Mechanism of ACh-induced asynchronous calcium waves and tonic contraction in porcine tracheal muscle bundle

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Dai, Jiazhen M., Kuo-Hsing Kuo, Joyce M. Leo, Cornelis van Breemen, and Cheng-Han Lee. Mechanism of ACh-induced asynchronous calcium waves and tonic contraction in porcine tracheal muscle bundle. Am J Physiol Lung Cell Mol Physiol 290: L459–L469, 2006.—Stimulation of the tracheal muscle bundle by acetylcholine (ACh) results in the generation of asynchronous repetitive Ca2+ waves (ACW) in intact tracheal smooth muscle (TSM) cells. We showed previously that ACW underlie cholinergic excitation-contraction coupling in porcine TSM and that Ca2+ entry through the L-type voltage-gated Ca2+ channel (VGCC) contributes partially to maintenance of the ACW. However, the mechanism of the ACW remains undefined. In this study, we pharmacologically characterized the mechanism of ACh-induced ACW in the intact porcine tracheal muscle bundle. We found that inhibition of receptor-operated channels/store-operated channels (ROC/SOC) by SKF-96365 completely abolished the nifedipine-insensitive component of ACh-mediated ACW and tonic contraction. Blockade of Na+/Ca2+ exchange with KB-R7943 or 2,4'-dichlorobenzamil or removal of extracellular Na+ resulted in nearly complete inhibition of the nifedipine-insensitive component of ACh-mediated ACW and tonic contraction. Inhibition of the sarco(endo)plasmic reticulum (SR)-mediated Ca2+-ATPase by cyclopiazonic acid abolished the ongoing ACW. Application of 2-aminoethoxydiphenyl borate (2-APB) or xestospongin C to inhibit the inositol 1,4,5-trisphosphate-sensitive sarcoplasmic reticulum (SR) Ca2+ release channels produced no effect on ACh-mediated ACW and tonic contraction. However, pretreatment with caffeine or ryanodine inhibited ACh-induced ACW. Furthermore, application of procaine or tetracaine prevented the generation and abolished the ongoing ACh-mediated ACW and tonic contraction. Collectively, these results indicate that the ACh-stimulated ACW in porcine TSM are produced by repetitive cycles of Ca2+ release from SR through 2-APB- and xestospongin C-insensitive Ca2+ release channels, and plasmalemmal Ca2+ entry involving reverse-mode Na+/Ca2+ exchange, ROC/SOC, and L-type VGCC is required to refill the SR via SERCA to support the ongoing ACW.

confocal calcium imaging; excitation-contraction coupling; intact airway smooth muscle

ACTIVATION OF THE MUSCARINIC RECEPTOR by acetylcholine (ACh) or other cholinergic agonists is known to result in tonic contraction of airway smooth muscle. Recent findings by Bergner and Sanderson (4) and by our laboratory (19) have revealed that smooth muscle cells of the intact tracheal and bronchial muscle bundle respond to cholinergic stimulation with repetitive Ca2+ waves that are not synchronized between neighboring cells. Functionally, these asynchronous Ca2+ waves (ACW) were found to be responsible for the development of force in the intact smooth muscle cells (TSMC) (19). In the case of ACh stimulation, the regulation of force generation by the TSMC is achieved initially by differential recruitment of cells to initiate ACW and subsequently by the enhancement of the frequency of the ACW and the elevation of interspike intracellular Ca2+ concentration ([(Ca2+)]i) once the cells are recruited (19). Mechanistically, we found that the blockade of the L-type voltage-gated Ca2+ channel (VGCC) with high-dose nifedipine resulted in only partial reduction in frequency of the ACW and partial inhibition of the tonic contraction induced by ACh (19). However, the detailed mechanism for the generation of ACh-induced ACW and tonic contraction has not been elucidated in the smooth muscle cells of the intact tracheal muscle bundle.

In enzymatically isolated porcine TSMC, ACh-induced repetitive Ca2+ waves have been described, and their mechanism has been extensively studied. It was found that these agonist-induced repetitive Ca2+ waves are produced by sarcoplasmic reticulum (SR)-mediated Ca2+ release (9, 18, 23, 36, 37). More specifically, Ca2+ release through the inositol-1,4,5-trisphosphate-sensitive SR Ca2+ release channels (IP3R) is important in initiating the Ca2+ waves, whereas Ca2+ release through the ryanodine-sensitive SR Ca2+ release channels (RyR) is responsible for the recurrent wave generation (9, 18, 23). The repetitive Ca2+ waves in the isolated TSMC can be abolished with 100 nM nifedipine (36), indicating that Ca2+ entry through the L-type VGCC is the main pathway utilized for refilling the SR Ca2+ store and the maintenance of the repetitive cycles of SR Ca2+ release in these cells. However, our initial investigation into the mechanism of the ACW of the intact porcine TSMC revealed a crucial difference from the isolated cell preparation. Blockade of the L-type VGCC with high-dose nifedipine attenuated the frequency of the ACW observed in TSMC of the intact tissue but did not abolish the ongoing ACW or the tonic contraction stimulated with ACh.

This finding suggests that, unlike the enzymatically isolated TSMC, Ca2+ influx through the L-type VGCC is not obligatory for maintaining the ACW of the TSMC of the intact tissue. Such a significant difference in the phenotypic characteristics between freshly dissociated cells and the intact tissue may be the result of the disruption of crucial intercellular communication or may reflect damage of important surface proteins on the TSMC due to nonspecific enzymatic digestion (16). Because of the observed discrepancy between the isolated TSMC and the
TSMC of the intact tissue, the data deduced from studies that examined either the cultured or the enzymatically isolated TSMC may not accurately depict the physiology of the intact airway smooth muscle. Therefore, the detailed mechanism for the generation of ACW in the intact tracheal muscle bundle requires further investigation.

In this study, we examine the mechanism for the generation of ACh-induced ACW in the TSMC of the intact porcine tracheal muscle bundle. The focus of this study was to identify the Ca\textsuperscript{2+}/H\textsubscript{11001}\textsuperscript{+} transport molecules involved in the generation and maintenance of ACh-mediated ACW.

**MATERIALS AND METHODS**

**Tissue preparations.** Porcine trachea obtained from a local abattoir was placed in physiological saline solution (PSS) at 4°C. Tracheal smooth muscle strips (~6 × 1.5 × 0.3 mm in dimension) free of epithelium and connective tissue were isolated from the trachea. Each tracheal muscle strip contains multiple muscle bundles (19). The tracheal muscle strips were subsequently attached at both ends to aluminum foil clips designed for mounting onto the custom-built setup.

**Cell permeabilization.** Tracheal muscle strips were permeabilized using 10 μM digitonin in an intracellular substitution solution (see Solutions and chemicals) for 10 min. The experiments were performed in intracellular substitution solution for permeabilized tracheal muscle strips. Successful permeabilization of tracheal muscle bundle was verified by the observation of force generation following either the application of inositol 1,4,5-trisphosphate (IP\textsubscript{3}) or increased extracellular [Ca\textsuperscript{2+}].

Fig. 1. Effect of removal of extracellular Ca\textsuperscript{2+} on acetylcholine (ACh)-induced asynchronous Ca\textsuperscript{2+} waves (ACW). Application of 3 μM ACh elicited ACW in porcine tracheal smooth muscle cells (TSMC) placed in normal physiological saline solution (PSS) with 1.5 mM extracellular Ca\textsuperscript{2+}. Replacement of the normal PSS with 0-Ca\textsuperscript{2+} PSS resulted in the cessation of the ACW. Experimental intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) trace is representative of results in 30 cells from 5 different animals.

Fig. 2. Effect of 10 μM nifedipine and 50 μM SKF-96365 on ACh-induced ACW and tonic contraction. A: L-type voltage-gated Ca\textsuperscript{2+} channel (VGCC) blockade by nifedipine did not abolish ACh-induced ACW but reduced the frequency of the ACW. Additional application of SK-F96365 abolished ACh-induced ACW completely. Experimental [Ca\textsuperscript{2+}]\textsubscript{i} trace is representative of results in 30 cells from 4 different animals. B: application of nifedipine partially reduced the ACh-induced contraction, whereas SKF-96365 nearly abolished the remaining contraction. Experimental tissue contraction trace is representative of results from 5 different animals. C: time-control traces of ACh-mediated ACW at 30 s and 45 min poststimulation.

Fig. 3. Effect of 10 μM 2′,4′-dichlorobenzamil (2′,4′-DCB), 20 μM KB-R7943, and 0-Na\textsuperscript{+} PSS on the nifedipine-resistant component of ACh-induced ACW and tonic contraction. A: application of 10 μM nifedipine reduced the frequency of ACh-induced ACW, whereas additional application of 2′,4′-DCB resulted in inhibition of the nifedipine-resistant component of the ACh-induced ACW and tonic contraction. Experimental [Ca\textsuperscript{2+}]\textsubscript{i} trace is representative of results in 45 cells from 7 different animals, and tissue contraction trace is representative of results from 8 different animals. B: the nifedipine-insensitive portion of the ACh-induced ACW and tonic contraction were inhibited by KB-R7943. Experimental [Ca\textsuperscript{2+}]\textsubscript{i} trace is representative of results in 45 cells from 7 different animals, and tissue contraction trace is representative of results from 8 different animals. C: replacement of the bathing solution with 0-Na\textsuperscript{+} PSS resulted in cessation of ACh-induced ACW and significant inhibition of the tonic contraction. Experimental [Ca\textsuperscript{2+}]\textsubscript{i} trace is representative of results in 40 cells in 5 different animals, and tissue contraction trace is representative of results from 5 different animals.
Isometric force measurement. The porcine tracheal muscle strips were attached to an isometric force transducer and equilibrated in PSS at 37°C for 1 h. During this time, the resting tension was maintained at 0.3 g. Exchange of bathing solutions was accomplished by simultaneously draining and refilling the tissue bath. The muscle strips were stimulated twice with 80 mM K⁺ PSS for 5 min each time. The experiment protocols were applied after complete relaxation of the tissue from the second dose of 80 mM K⁺ stimulation. Chart v3.4.5
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software (ADInstruments) was employed for data acquisition and analysis.

Confocal imaging. The details of the confocal Ca$^{2+}$ imaging method have been described previously by investigators in our labor

atory (19). Briefly, the clipped muscle strips were loaded with fluo-4 AM (5 μM, with 5 μM Pluronic F-127) for 120 min at 25°C and then left to equilibrate for 10 min in normal PSS. They were then isomet-

rically mounted onto the custom-made stiff force transducer setup (20). Briefly, the clipped muscle strips were loaded with fluo-4 AM for determining relative changes in [Ca$^{2+}$]. The changes in [Ca$^{2+}$] were measured using an inverted Leica TCS SP2 AOBLS laser scanning confocal microscope with an air ×10 (numerical aperture 0.3) lens. The tissue was illuminated using the 488-nm line of a argon-krypton laser, and a high-gain photomultiplier tube collected the emission at wavelengths between 505 and 550 nm. The acquisition rate was 3 frame/s. The measured changes in fluo-4 fluorescence level are proportional to the relative changes in [Ca$^{2+}$]. All parameters (laser intensity, gain) were maintained during the experiment.

Data analysis. All confocal image analysis was performed with ImageProPlus software using customized routines written in Visual Basic. Analysis of frequency of the ACW was performed using a three-pixel-wide line along the longitudinal axis of a single cell. The resulting x-t plot revealed the point of origin as well as the progression of the apparent “Ca$^{2+}$ wave.” The frequency of the ACW was determined by counting the number of waves occurring during a period of 50 s. The amplitude of the ACW reflects the difference between the peak fluorescence of individual Ca$^{2+}$ spikes in the ACW and the prestimulation baseline level. The fluorescence level derived in each region is linearly proportional to the [Ca$^{2+}$], in that region in such a fashion that any fluctuation in [Ca$^{2+}$] would be proportionally reflected in the changes in fluorescence.

All summarized data are presented as means ± SE. For numerical analysis, all data were analyzed with Excel or SigmaPlot software using the appropriate statistical tests. Paired Student’s $t$-test was used for comparisons. A value of $P < 0.05$ was considered significant. The $n$ values indicated for contraction experiments represent the number of animals studied, and the $n$ values indicated for the Ca$^{2+}$ studies represent the number of TSMC studied from the specified numbers of animals. For each study protocol, only one muscle strip from each animal was used, and the number of tissues indicated therefore reflects the number of animals. For the analysis of the Ca$^{2+}$ signals, when the data obtained from individual TSMC were pooled together from different animals, ANOVA was performed and variance is reported when significant. In our current study, no significant variance was found.

Solutions and chemicals. Normal PSS containing (in mM) 140 NaCl, 5 KCl, 1.5 CaCl$_2$, 1 MgCl$_2$, 10 glucose, and 5 HEPES (pH 7.4 at 37°C) was used for all of the studies. High-K$^+$ (80 mM extracell-

ular K$^+$) PSS was identical in composition to normal PSS with the exception of (in mM) 65 NaCl and 80 KCl. Zero-Ca$^{2+}$ PSS and zero-Na$^+$ PSS were prepared in the same way as normal PSS, but CaCl$_2$ was replaced with 1 mM EGTA and NaCl was replaced with equimolar N-methyl-D-glucamine. The intracellular substitution solution used contained (in mM) 130 KCl, 10 NaCl, 5 K$_2$HP0$_4$, 5.6 glucose, 1 MgSO$_4$, 5 Tris-succinate, 1 ATP, 75 μM EGTA, and 20 HEPES (pH 7.0 at 37°C). Fluo-4 AM, Pluronic F-127, and 2′,4′-dichlorobenzamil (2′,4′-DCB) were purchased from Molecular Probes and were dissolved in dimethyl sulfoxide (DMSO). Stocks of ACh, caffeine, digitonin, and IP$_3$ (Sigma) were prepared in normal PSS, and stocks of 2-aminoethoxydiphenyl borate (2-APB), xestospongin C, nifedipine, cyclopiazonic acid (CPA), KB-R7943, SKF-96365, procaine, and tetracaine (Sigma) were prepared in 100% ethanol.

RESULTS

Dependence of ACW on plasmalemmal Ca$^{2+}$ influx. There are two potential sources of Ca$^{2+}$ that can contribute to the generation of ACh-mediated ACW: Ca$^{2+}$ release from intracellular store and Ca$^{2+}$ influx from the extracellular space. To determine the significance of plasmalemmal Ca$^{2+}$ entry in ACh-mediated ACW, we studied the effect of extracellular Ca$^{2+}$ removal on the ACh-induced ACW. As shown in Fig. 1, ACh-mediated ACW were completely abolished in zero-Ca$^{2+}$ PSS within 5 min of treatment ($n$ = 30 cells from 5 animals). This finding indicates that Ca$^{2+}$ entry from the extracellular space is required for maintaining ACh-induced ACW.

To further define the Ca$^{2+}$ entry pathways that are responsible for maintaining the ACW, we used SKF-96365, an inhibitor of receptor-operated channels (ROC) and store-operated channels (SOC), together with nifedipine, a selective inhibitor of L-type VGCC. Results from a previous study in our laboratory (19) showed that high-dose nifedipine (10 μM) reduced the frequency of ACh-mediated ACW by 29% and partially inhibited ACh-mediated tonic contraction by 32.8%. It is important to note that at 10 μM, nifedipine was able to completely abolish the contraction induced by 80 mM K$^+$-PSS in the tracheal muscle bundle. These findings indicate that Ca$^{2+}$ influx via L-type VGCC plays only a minor role in the

![Fig. 4. Effect of 10 μM nifedipine and 50 μM SKF-96365 on the initiation of ACh-induced ACW. Application of ACh elicited transient repetitive Ca$^{2+}$ waves in tissues pretreated with nifedipine (L-type VGCC blocker) and SKF-96365 [receptor-operated channels/store-operated channels (ROC/SOC) blocker] in contrast to the sustained repetitive Ca$^{2+}$ waves in the control tissue. Experimental [Ca$^{2+}$], trace is representative of results in 20 cells from 4 different animals.](http://ajplung.physiology.org/)

![Fig. 5. Effect of 10 μM cyclopiazonic acid (CPA) on ACh-induced ACW. Application of CPA to ACh-stimulated porcine TSMC completely abolished the ongoing ACW. Experimental [Ca$^{2+}$], trace is representative of results in 30 cells from 4 different animals.](http://ajplung.physiology.org/)
maintenance of ACh-induced ACW and tonic contraction in the porcine TSMC. Therefore, an additional pathway(s) for Ca\(^{2+}\) entry must be responsible for supporting the nifedipine-resistant component of the ACh-mediated ACW and tonic contraction. In the isolated airway smooth muscle cells, both SOC and ROC have been implicated in Ca\(^{2+}\) signaling (3, 24, 28, 29). As shown in Fig. 2, 50 \(\mu M\) SKF-96365 abolished the nifedipine-resistant ACW within 2 min of its application (\(n = 30\) cells from 4 animals). A corresponding time control trace of the ACh-mediated ACW in Fig. 2C shows the persistent generation of ACW up to 45 min after ACh stimulation, and the frequency of the ACW at 30 s and 45 min poststimulation was 0.36 ± 0.02 and 0.33 ± 0.02 Hz, respectively (\(P = 0.168, n = 40\) cells from 5 animals). In parallel contraction studies (Fig. 2), a combination of 10 \(\mu M\) nifedipine and 50 \(\mu M\) SKF-96365 abolished 93.7 ± 1% of ACh-induced tonic contraction compared with the 32.8 ± 2.9% reduction in ACh-induced tonic contraction seen with nifedipine alone (\(P < 0.0001, n = 5\) animals). SKF-96365 was used after the addition of nifedipine but not in the reverse sequence, because it is known to inhibit the L-type VGCC as well. To examine the possibility that the high-dose nifedipine may produce nonspecific inhibition beyond the blockade of the L-type VGCC, we used 100 \(nM\) nifedipine, and it produced a degree of inhibition (33.0 ± 3.3%, \(P = 0.0096, n = 3\) animals) of ACh-induced tonic contraction similar to that produced by 10 \(\mu M\) nifedipine. These findings suggest that Ca\(^{2+}\) entry via the ROC/SOC and the L-type VGCC are important in maintaining ACh-induced ACW as well as tonic contraction in the tracheal muscle bundle.

In addition to the conventional plasmalemmal Ca\(^{2+}\)-permeable channels, the Na\(^{+}/Ca^{2+}\) exchanger (NCX) operating in the reverse mode can be an important pathway for Ca\(^{2+}\) entry into smooth muscle cells (1, 6). The role of NCX in airway smooth muscle is controversial. Although some studies have reported little contribution by NCX (13, 17), others have implicated NCX in the Ca\(^{2+}\) and contractile regulation of airway smooth muscle (8, 11, 12, 30, 35, 38). To examine whether the reverse-mode Na\(^{+}/Ca^{2+}\) exchange is involved in supporting the nifedipine-resistant ACW in the porcine TSMC, we used an inhibitor of both the forward- and the reverse-mode Na\(^{+}/Ca^{2+}\) exchange, 2',4'-DCB, and a selective reverse-mode inhibitor of NCX, KB-R7943 (6, 21, 43, 45, 48). As shown in Fig. 3, the application of 10 \(\mu M\) 2',4'-DCB abolished nifedipine-resistant ACW induced by ACh (\(n = 45\) cells from 7 animals) and inhibited nifedipine-resistant tonic contraction by 91.0 ± 1.4% (\(P < 0.001, n = 8\) animals). Similarly, the application of 20 \(\mu M\) KB-R7943 abolished nifedipine-resistant ACW induced by ACh (\(n = 45\) cells from 7 animals) and inhibited the corresponding tonic contraction by 86.9 ± 4.3% (\(P < 0.0001, n = 8\) animals). In addition to the use of the above-mentioned pharmacological inhibitors, we also examined the effect of removal of extracellular Na\(^{+}\) with the use of zero-Na\(^{+}\) PSS abolished ACh-mediated ACW within 10 min (\(n = 40\) cells from 5 animals). In a parallel contraction study (Fig. 3C), the removal of extracellular Na\(^{+}\) reduced ACh-mediated tonic contraction by 72.7 ± 2.2% (\(P < 0.001, n = 5\) animals). These data uniformly indicate that reverse-mode Na\(^{+}/Ca^{2+}\) exchange is involved in maintaining ACh-induced ACW and tonic contraction. The sensitivity of the nifedipine-resistant ACW and tonic contraction to SKF-96365, 2',4'-DCB, and KB-R7943 suggest that the ROC/SOC are likely operating in series with the NCX, as proposed previously (1, 21).
Dependence of ACW on SR Ca\(^{2+}\) release. As described previously (19), ACh induces ACW in the TSMC of the intact porcine tracheal muscle bundle. The wavelike nature of the Ca\(^{2+}\) signal implies that SR Ca\(^{2+}\) release is most likely responsible for raising the \([\text{Ca}^{2+}]_{i}\), because the Ca\(^{2+}\) signal produced by extracellular Ca\(^{2+}\) entry would typically result in a spatially more uniform elevation of \([\text{Ca}^{2+}]_{i}\) (39). To examine the role of plasmalemmal Ca\(^{2+}\) entry in the generation of the ACW, we pretreated the tracheal muscle bundle with 10 μM nifedipine and 50 μM SKF-96365 for 5 min before ACh stimulation to block all the Ca\(^{2+}\) entry pathways that were found to be important in supporting the ACW (Fig. 2). As indicated in Fig. 4, blockade of the L-type VGCC and ROC/SOC did not prevent the induction of ACW, because ACh was able to induce Ca\(^{2+}\) waves initially (n = 20 cells from 4 animals). However, the ACW did not persist as they did in the absence of nifedipine and SKF-96365 (Fig. 4). More interestingly, caffeine (12.5 mM) induced no significant rise in \([\text{Ca}^{2+}]_{i}\), because the peak \([\text{Ca}^{2+}]_{i}\), levels before and immediately after the addition of caffeine were 100.9 ± 0.8 and 100.6 ± 1.0% of baseline level pre-ACh stimulation, respectively (P = 0.862, n = 20 cells from 4 animals), indicating that the SR Ca\(^{2+}\) stores had been emptied. It is important to note that the pretreatment of the muscle bundle with only nifedipine and SKF-96365 at the same concentration did not affect the amplitude of the caffeine (12.5 mM)-induced Ca\(^{2+}\) transient compared with the control amplitude before the addition of nifedipine and SKF-96365 (P = 0.896, n = 28 cells from 4 animals). This finding suggests that the ACW are the result of SR Ca\(^{2+}\) release and that the SR Ca\(^{2+}\) release can be initiated in the absence of extracellular Ca\(^{2+}\) entry, thereby excluding plasmalemmal Ca\(^{2+}\) entry-induced SR Ca\(^{2+}\) release by the process of Ca\(^{2+}\)-induced Ca\(^{2+}\) release as the primary activation mechanism. Furthermore, if SR Ca\(^{2+}\) release is responsible for the generation of the ACW, blockade of the sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) should completely inhibit the ACW, because the SR Ca\(^{2+}\) store can no longer be replenished. As shown in Fig. 5, the application of CPA (10 μM), a selective inhibitor of SERCA, resulted in a brief broadening followed by complete abolition of the ACh-mediated ACW within 10 s, leaving behind a small but significant elevation in baseline \([\text{Ca}^{2+}]_{i}\) (P < 0.0001, n = 30 cells from 4 animals) that corresponds to 26 ± 2% of the peak \([\text{Ca}^{2+}]_{i}\) of the ACW. In a parallel contraction study, the application of 10 μM CPA produced a 83.4 ± 2.8% inhibition of the tonic contraction induced by ACh (P < 0.001, n = 4 animals). These findings collectively indicate that ACW are produced by repetitive cycles of SR Ca\(^{2+}\) release followed by Ca\(^{2+}\) reuptake and that Ca\(^{2+}\) entry through the L-type VGCC, ROC/SOC, and NCX pathway is
necessary to ensure the continual proper refilling of the SR Ca\textsuperscript{2+} store to sustain the ongoing ACW.

Given that SR Ca\textsuperscript{2+} release produces the ACW, we proceeded to identify the type(s) of SR Ca\textsuperscript{2+} release channels involved in the generation of ACh-induced ACW. As mentioned earlier, in isolated TSMC, SR Ca\textsuperscript{2+} release through the IP\textsubscript{3}R is required for the initiation of ACh-induced repetitive Ca\textsuperscript{2+} waves (18). To determine whether SR Ca\textsuperscript{2+} release through the IP\textsubscript{3}R is responsible for producing ACh-induced ACW in the intact porcine tracheal muscle bundle, we used 2-APB and xestospongin C, both well-established cell-permeable inhibitors of IP\textsubscript{3}R that have been found to block IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} release in a variety of tissues, including the airway smooth muscle as well as non-smooth muscle cell types (2, 14, 25, 26, 27, 32, 33, 34, 46). As shown in Fig. 6, after pretreatment of the tissue with 75 \(\mu\)M 2-APB for 30 min, ACh (3 \(\mu\)M) was still able to induce ACW and tonic contraction in a manner similar to that observed in the absence of 2-APB in the same tissue. Furthermore, the addition of 2-APB to ongoing ACh-mediated ACW and tonic contraction produced no measurable effect on the ACW and the tonic contraction (Fig. 6).

It is important to note that the 30-min pretreatment of the tissue with the same batch of 2-APB at 75 \(\mu\)M was able to inhibit phenylephrine (5 \(\mu\)M)-induced tonic contraction of the porcine aorta by 94.4 \pm 5.4\% \((P < 0.0001, n = 4\) animals). More importantly, in a separate experiment in which we permeabilized the tracheal muscle bundle with 10 \(\mu\)M digitonin (31, 40, 44, 47), the addition of IP\textsubscript{3}R (10 \(\mu\)M) produced a transient contraction that was completely prevented with 2-APB (75 \(\mu\)M) pretreatment \((n = 4\) animals) (Fig. 6E). In contrast, as shown in Fig. 6F, 2-APB pretreatment did not prevent or attenuate caffeine-induced contraction \((n = 4\) animals). These positive control studies show that 75 \(\mu\)M 2-APB is able to effectively inhibit porcine airway smooth muscle IP\textsubscript{3}R and porcine vascular smooth muscle IP\textsubscript{3}R, because the vascular smooth muscle in the aorta is known to exhibit ACW, and the phenylephrine-induced ACW in the vascular muscle depend on SR Ca\textsuperscript{2+} release through the IP\textsubscript{3}R (22). Similar to the results with 2-APB, pretreatment of the tissue for 45 min with 10 \(\mu\)M xestospongin C produced no significant effect on ACh-induced ACW and tonic contraction in the intact porcine muscle bundle, and the addition of 10 \(\mu\)M xestospongin C to tissues already stimulated with ACh produced no measurable effect on the ongoing ACW and tonic contraction (Fig. 7). The lack of effect seen with these two structurally distinct inhibitors of the IP\textsubscript{3}R suggests that Ca\textsuperscript{2+} release via the IP\textsubscript{3}R is not required for the generation or maintenance of ACh-induced ACW and tonic contraction.

In addition to the IP\textsubscript{3}R, another type of SR Ca\textsuperscript{2+} release channel that is known to be functionally important in the TSMC is the RyR. As shown in Fig. 8, pretreatment of the tracheal muscle bundle for <2 min with either 12.5 mM caffeine or 25 \(\mu\)M ryanodine to empty Ca\textsuperscript{2+} from the RyR-sensitive SR Ca\textsuperscript{2+} store completely prevented the generation of ACh-induced ACW. These findings suggest that SR Ca\textsuperscript{2+} release from the RyR-dependent SR store is responsible for the generation of ACW but nonetheless do not prove the involvement of RyR in the Ca\textsuperscript{2+} release. We therefore employed procaine and tetracaine, both known membrane-permeable inhibitors of RyR-mediated SR Ca\textsuperscript{2+} release, to block the RyR channels (7, 10, 15). As demonstrated in Fig. 9, pretreatment of tracheal muscle bundle with 2 mM procaine for 30 min completely prevented ACh-induced ACW and tonic contraction. When 2 mM procaine was applied to ACh-stimulated tracheal muscle bundles exhibiting ongoing ACW and tonic contraction, it immediately abolished ongoing ACW and reduced the ongoing tonic contraction to 5.3 \pm 0.7\% of the original level \((P < 0.0001, n = 6\) animals). Similarly, as shown in Fig. 10, pretreatment of the tracheal muscle bundle with 100 \(\mu\)M tetracaine for 30 min completely prevented ACh-induced ACW and tonic contraction as well. The application of 100 \(\mu\)M tetracaine to ACh-stimulated tissues resulted in the immediate cessation of the ongoing ACW and near-complete inhibition of the ongoing tonic contraction to 13.3 \pm 5.3\% of the original level \((P = 0.0038, n = 5\) animals). These findings together suggest that SR Ca\textsuperscript{2+} release via the RyR channel is responsible for both the initiation and maintenance of ACh-mediated ACW and tonic contraction in the porcine tracheal smooth muscle.
DISCUSSION

The aim of this study was to examine the mechanism of the ACW in the porcine tracheal smooth muscle. Our findings show that extracellular Ca\(^{2+}\) entry is not directly responsible for the generation of the Ca\(^{2+}\) waves (Fig. 4) and that the ACW are produced by recurring cycles of SR Ca\(^{2+}\) release and SR Ca\(^{2+}\) reuptake (Fig. 5). We also characterized the type of SR Ca\(^{2+}\) release channels involved. As shown in Figs. 6 and 7, the application of IP\(_3\)R inhibitors 2-APB and xestospongin C produced no measurable effect on the initial generation and the maintenance of ACh-induced ACW and tonic contraction. It is unlikely that two structurally unrelated inhibitors of IP3R with well-demonstrated efficacy in various smooth muscle and non-smooth muscle cell types would be completely ineffective in inhibiting the IP\(_3\)R in the porcine tracheal smooth muscle (2, 14, 25–27, 32–34, 46). In addition, our control studies demonstrate that 2-APB is an effective inhibitor of porcine airway IP\(_3\)R, because 2-APB pretreatment completely inhibited IP\(_3\)-induced force transient in permeabilized tracheal muscle bundle. 2-APB also produced significant inhibition of phenylephrine-induced tonic contraction of the nonpermeabilized porcine aorta, a process that is driven by IP\(_3\)R-mediated ACW in the vascular smooth muscle (20). Furthermore, a similar concentration of xestospongin C has been shown to be effective in preventing ACh and ATP-induced ACW in the mouse bronchiolar smooth muscle cells (5, 34), suggesting that xestospongin C is able to inhibit IP\(_3\)R in the airway smooth muscle as well. The observed difference in the sensitivity to xestospongin C between ACh-induced ACW in the porcine tracheal smooth muscle and ACh-induced ACW in the mouse bronchial smooth muscle may reflect interspecies or inter-airway segment heterogeneity in the mechanism of ACW. The lack of effect with 2-APB and xestospongin C observed in our study therefore suggests that Ca\(^{2+}\) release through the IP\(_3\)R is not involved in the generation of the ACW in ACh-stimulated intact porcine tracheal smooth muscle. In contrast, pretreatment of the tracheal muscle bundle with caffeine or ryanodine prevented the generation of the ACW by ACh. More importantly, application of procaine or tetracaine prevented the generation of the ACW and produced immediate and complete inhibition of ongoing ACW induced by ACh. These findings show that ACh-induced ACW in the intact porcine smooth muscle are the result of repetitive waves of SR Ca\(^{2+}\) release through the RyR. Even though extracellular Ca\(^{2+}\) entry is not immediately required for the generation of the ACW, it is necessary to support ongoing ACW over time, as shown in Fig. 4. This is likely due to the fact that a proportion of the Ca\(^{2+}\) released by the SR to produce the Ca\(^{2+}\) waves is inevitably extruded to the extracellular space, possibly via the plasma membrane Ca\(^{2+}\)-ATPase. In the absence of sufficient extracellular Ca\(^{2+}\) entry, the SR is unable to continually replenish itself after repetitive waves of Ca\(^{2+}\) release, and the ACW cease after a period of time because the
SR Ca\(^{2+}\) store is depleted (Fig. 4). Therefore, to maintain the ongoing ACW, it is important to replenish the SR Ca\(^{2+}\) store with additional Ca\(^{2+}\) from the extracellular space. Our pharmacological characterizations have implicated the L-type VGCC, the NCX operating in the reverse mode, and the ROC/SOC-type channel. The role of the L-type VGCC is ascertained when both low-dose (100 nM) and high-dose (10 \(\mu\)M) nifedipine produce comparable partial inhibitory effects on ACh-induced ACW and tonic contraction (Fig. 2). The exact nature of the ROC/SOC-type channel is not known at this point. However, our findings have revealed a few characteristics of this ROC/SOC-type channel. First, it is sensitive to SKF-96365 but resistant to nifedipine, 2-APB, and xestospon- gin C. Second, this channel is likely a nonselective cation channel, given the involvement of the reverse-mode NCX in supporting the ACW. For the NCX to contribute to Ca\(^{2+}\) entry into the cell, it would need to operate in its reverse mode. To activate reverse-mode Na\(^{+}\)/Ca\(^{2+}\) exchange, ACh-stimulated TSMC must allow for an influx of Na\(^{+}\) into the cell, and such influx of Na\(^{+}\) in smooth muscle cells typically occurs through a nonselective cation channel (1). Na\(^{+}\) entry from the nonselective cation channel then accumulates in the subplasmalemmal space and drives the NCX into the reverse mode of operation. In this model, the nonselective cation ROC/SOC-type channel is coupled in series to the NCX operating in the reverse mode. This would explain our findings that the nifedipine-resistant ACW and tonic contraction are similarly sensitive to both SKF-96365, an inhibitor of the ROC/SOC, and 2,4'-DCB and KB-R7943, inhibitors of the NCX. It is important to note that although the phenomenon of reverse-mode Na\(^{+}\)/Ca\(^{2+}\) exchange coupled with the nonselective cation channel has never been reported previously in airway smooth muscle, it has been well described in vascular smooth muscle (1, 21). In both animal and human airway smooth muscle, it is known that NCX is present and is capable of operating in the reverse mode (8, 35, 38).

Our study of the mechanism of ACW in ACh-stimulated TSMC of the intact tissue has revealed some crucial differences from the enzymatically dissociated porcine TSMC. First, in isolated porcine TSMC challenged with ACh, it was shown previously that Ca\(^{2+}\) release through IP\(_3\)-R is important in initiating the repetitive Ca\(^{2+}\) waves (18). However, in the porcine TSMC of the intact tissue, two structurally unrelated inhibitors of IP\(_3\)-R did not affect either the initial generation or the maintenance of ACh-induced ACW and tonic contraction. Second, Prakash et al. (36) showed that the repetitive Ca\(^{2+}\) waves could be abolished by 100 nM nifedipine, which suggested that Ca\(^{2+}\) entry through the L-type VGCC is the main pathway utilized for refilling the SR Ca\(^{2+}\) store and maintenance of the repetitive cycles of SR Ca\(^{2+}\) release. Nonetheless, our previous study showed that the inhibition of the L-type VGCC by high-dose nifedipine attenuated the frequency of the ACW but did not abolish the ongoing ACW or the tonic contraction stimulated with ACh (19). This finding indicates that Ca\(^{2+}\) entry through the L-type VGCC is not obligatory for maintaining the ACW in intact tracheal smooth muscle, and as shown in this report, Ca\(^{2+}\) entry via the ROC/SOC and the NCX operating in the reverse mode are able to sustain the ACW in the absence of Ca\(^{2+}\) entry through the L-type VGCC. Third, results from earlier single-cell studies also showed that NCX plays only a minor role in the regulation of Ca\(^{2+}\) signaling in the tracheal smooth muscle (17). However, the significant inhibitory effects produced by KB-R7943 and 2,4'-DCB, as well as extracellular Na\(^{+}\) removal, on ACh-induced ACW and tonic contraction indicate that the NCX plays an important role in agonist-induced Ca\(^{2+}\) signaling in the porcine TSMC. These discrepancies in the characteristics between single-cell preparations and intact tissue show that enzymatically isolated TSMC are phenotypically different from the intact TSMC of intact tissue. It would appear that the process of enzymatic dissociation may have altered the phenotype of these TSMC, possibly as a result of the nonspecific proteolysis and the disruption of intercellular communication as suggested previously (16).

In summary, the data presented in this article show that multiple Ca\(^{2+}\) translocating proteins are involved in the generation of the ACW observed in ACh-stimulated smooth muscle cells of the intact porcine tracheal muscle bundle. The ACW appear to be produced by repetitive cycles of RyR-
mediated SR Ca\(^{2+}\) release followed by SERCA-mediated SR Ca\(^{2+}\) reuptake. Because a proportion of the released Ca\(^{2+}\) is lost to the extracellular space, extracellular Ca\(^{2+}\) entry involving the L-type VGCC, ROC/SOC, and reverse-mode Na\(^{+}/\)Ca\(^{2+}\) exchange is important to supply additional Ca\(^{2+}\) to refill the SR Ca\(^{2+}\) and to sustain the repetitive Ca\(^{2+}\) waves. In the future, more detailed characterization of the molecules important in ACh-mediated excitation-contraction coupling of the airway smooth muscle may help to identify a novel therapeutic target for the treatment of airway diseases characterized by excessive muscle contraction.

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