ORIGINAL RESEARCH

Arachidonate-Regulated Ca^{2+} Influx in Human Airway Smooth Muscle

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Abstract

Plasma membrane Ca^{2+} influx, especially store-operated Ca^{2+} entry triggered by sarcoplasmic reticulum (SR) Ca^{2+} release, is a key component of intracellular calcium concentration ($[Ca^{2+}]_i$) regulation in airway smooth muscle (ASM). Agonist-induced Ca^{2+} oscillations in ASM that involve both influx and SR mechanisms have been previously demonstrated. In nonexcitable cells, $[Ca^{2+}]_i$ oscillations involve Ca^{2+} influx via arachidonic acid (AA)–stimulated channels, which show similarities to store-operated Ca^{2+} entry, although their molecular identity remains undetermined. Little is known about AA-regulated Ca^{2+} channels or their regulation in ASM. In enzymatically dissociated human ASM cells loaded with the Ca^{2+} indicator, fura-2, AA (1–10 μM) triggered $[Ca^{2+}]_i$ oscillations that were inhibited by removal of extracellular Ca^{2+}. Other fatty acids, such as the diacylglycerol analog, 1-oleoyl-2-acetyl-SN-glycerol, oleic acid, and palmitic acid (10 μM each), failed to elicit similar $[Ca^{2+}]_i$ responses. Preincubation with LaCl$_3$ (1 μM or 1 mM) inhibited AA-induced oscillations. Inhibition of receptor-operated channels (SKF96,365 [10 μM]), lipoxygenase (zileuton [10 μM]), or cyclooxygenase (indomethacin [10 μM]) did not affect oscillation parameters. Inhibition of SR Ca^{2+} release (ryanodine [10 μM] or inositol 1,4,5-trisphosphate receptor inhibitor, xestospongin C [1 μM]) decreased $[Ca^{2+}]_i$ oscillation frequency and amplitude. Small interfering RNA against caveolin-1, stromal interaction molecule 1, or Orai3 (20 nM each) reduced the frequency and amplitude of AA-induced $[Ca^{2+}]_i$ oscillations. In ASM cells derived from individuals with asthma, AA increased oscillation amplitude, but not frequency. These results are highly suggestive of a novel AA-mediated Ca^{2+}–regulatory mechanism in human ASM, reminiscent of agonist-induced oscillations. Given the role of AA in ASM intracellular signaling, especially with inflammation, AA-regulated Ca^{2+} channels could potentially contribute to increased $[Ca^{2+}]_i$ in diseases such asthma.

Keywords: bronchial smooth muscle; calcium; influx; arachidonic acid; sarcoplasmic reticulum

Clinical Relevance

Arachidonic acid (AA) and its downstream metabolites are important in inflammatory conditions, such as asthma. Here, we present novel data on the expression and properties of a Ca^{2+} influx pathway regulated by AA in human airway smooth muscle. We believe that the basic science described here is highly relevant to understanding the mechanisms by which agonists and inflammation can enhance Ca^{2+} in airways and contribute to increased airway contractility.

In human airway smooth muscle (ASM), changes in intracellular calcium concentration ($[Ca^{2+}]_i$) are mediated by both Ca^{2+} influx/efflux and Ca^{2+} release/reuptake from the sarcoplasmic reticulum (SR) (1–4). We and others have previously shown a variety of Ca^{2+} influx mechanisms in ASM, including voltage-gated and receptor-operated channels (5–8). In addition, an important influx mechanism that occurs upon SR depletion (e.g., after agonist stimulation) is store-operated Ca^{2+} entry (SOCE), which aids SR Ca^{2+} refilling. We and others have demonstrated the presence of SOCE in ASM (9–11). However, one caveat in regard to SOCE is that physiologically relevant agonist concentrations do not necessarily deplete SR Ca^{2+} stores, raising the question of whether SOCE normally contributes to Ca^{2+} influx during agonist-induced airway contractions. We previously demonstrated in porcine ASM that physiologic concentrations of agonists result in the generation of repetitive and stable Ca^{2+} oscillations characterized by a rapid and transient release of submaximal Ca^{2+} from

the SR, with concomitant rapid refilling of such intracellular Ca^{2+} stores via influx (5, 12). Such oscillations would prevent full depletion of SR Ca^{2+} stores, and thus mechanisms that mediate SOCE *per se* may not be as important in agonist-induced $[Ca^{2+}]_i$ oscillations. Accordingly, the question is whether a noncapacitative influx pathway exists that may regulate Ca^{2+} oscillatory signaling in ASM.

Data in nonexcitable cells show the presence of a novel noncapacitative Ca^{2+} influx mechanism evoked by arachidonic acid (AA) stimulation (arachidonate-regulated Ca^{2+} [ARC] channels), that is independent of SR store depletion (13–16). Although initially thought to reflect AA effects on a noncapacitative influx pathway, the role of ARC channels is now considered much more diverse, with evidence that these channels are also involved in capacitative Ca^{2+} entry (17, 18). Evidence in nonexcitable cells for this connection comes from data that stromal interaction molecule (STIM) and Orai proteins, which are key regulators of SOCE, can also regulate ARC channels (18). However, what appears to distinguish SOCE from ARC channels is the role of a plasma membrane STIM1 in the regulation of ARC channels (18, 19) in contrast to endoplasmic reticulum STIM1 in SOCE. Furthermore, the ARC channel pore is thought to be formed by assembly of three plasma membrane proteins, Orai1 and two Orai3 subunits (18). There is now substantial evidence that plasma membrane Orai1, and STIM1 at the level of the SR, are mediators of SOCE in ASM (10, 11, 20–25). However, there is currently no information on the role of ARC channels in ASM, their molecular identity, or the mechanisms that may regulate such channels. Accordingly, we hypothesized that ARC channels exist in ASM, and the goal of the present study was to examine their regulation in human ASM cells.

Materials and Methods

Isolation of Human ASM Cells
In lung resections of patients undergoing surgery for focal tumors (excluding infectious pathologies, bullae, or widely disseminated cancers), normal third- to sixth-generation human bronchi distant from the tumor were identified and dissected. Patient histories were used to identify subjects with asthma versus those

without asthma. Procedures were approved by the Mayo Clinic Institutional Review Board (minimal risk). ASM cells were enzymatically dissociated using papain/collagenase, and maintained in culture at 37°C (5% CO_2, 95% air; less than fifth passage subculture; 4, 24). Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10% FBS was used until approximately 75% confluence, with 48-hour serum deprivation before experimentation. Verification of ASM phenotype was performed as previously described (4), using Westerns or immunocytochemistry for presence of smooth muscle actin and myosin, but absence of fibroblast surface protein, and robust $[Ca^{2+}]_i$ responses to acetylcholine (ACh).

Western Blot Analysis
Standard techniques involved SDS-PAGE, transfer to polyvinylidene fluoride membranes, blocking with 5% milk, overnight incubation at 4°C with 1 µg/ml primary antibody, exposure to horseradish peroxidase–conjugated secondary antibodies, and signal detection by enhanced chemiluminescence.

Knockdown by Small Interfering RNA
Cells were transfected using 20 nM STIM1 small interfering RNA (siRNA; GCCUAUAUCCAGAACCGUtt) (Ambion, Inc., Lafayette, CO), or caveolin-1 siRNA (CUAAACACCUCAACGAUGAUU) or Orai3 siRNA (GCACCUCUUUGCACUCAUG) (Thermo Fisher Scientific, Pittsburgh, PA) using 1 µl/ml lipofectamine in DMEM F/12 lacking FBS. After 6 hours, fresh growth medium was added and cells maintained overnight. Transfection complexes were then aspirated and fresh DMEM/F12 containing 10% FBS added for an additional 24 hours. Serum deprivation was then maintained for 48 hours, followed by experimentation. Western blots verified specific protein knockdown.

$[Ca^{2+}]_i$ Imaging
Techniques for real-time $[Ca^{2+}]_i$ fluorescence imaging of human ASM cells have been previously described (4, 24). Fura-2/AM (Invitrogen, Carlsbad, CA) –loaded cells were visualized using a Metafluor-based imaging system on a Nikon microscope (Nikon, Melville, NY). Software regions of interest encompassing substantial intracellular areas were

delineated for individual cells (multiple cells per visual field), and fluorescence changes calibrated to nM Ca^{2+} using established techniques. Estimated "noise" levels were roughly 10 nM, and a valid $[Ca^{2+}]_i$ oscillation was considered at greater than 40-nM increase above baseline (measured immediately before initiation of an oscillation). Initial observations suggested temporal summation of oscillations immediately upon AA exposure. For consistency, oscillations were analyzed after 2 minutes of AA exposure, and all valid oscillations considered as individual events. Oscillation frequency was calculated as number of valid events over a defined time period (typically 20 min).

Materials
Chemicals were from Sigma-Aldrich (St. Louis, MO) unless mentioned otherwise. Tissue culture reagents (DMEM F/12, FBS) were from Invitrogen, zileuton from R&D Systems Inc. (Minneapolis, MN), xestospongin C (XeC) and ryanodine from Millipore Inc. (Billerica, MA), caveolin-1 and secondary antibodies from Santa Cruz Biotechnology, Inc. (Dallas, TX), STIM1 antibody from Novus (Littleton, CO), and Orai3 antibody from Abcam (Cambridge, MA).

Statistical Analysis
From ASM cells of five different patients (those with asthma or those without), $[Ca^{2+}]_i$ experiments were performed using at least 20 cells per patient with three repeats per protocol. Statistically significant ($P < 0.05$) effects of drugs and/or siRNA were evaluated using Student's t test or two-way ANOVA, as appropriate, with Bonferroni's corrections for repeated measures, and Tukey's test for *post hoc* analyses. Values are expressed as means ($\pm$SE).

Results

$[Ca^{2+}]_i$ Responses of Human ASM Cells to AA
In untreated cells (from subjects without asthma), baseline $[Ca^{2+}]_i$ levels before any AA exposure ranged between 80 and 120 nM, comparable to our previous reports (5). No spontaneous $[Ca^{2+}]_i$ oscillations were observed at baseline. Exposure to AA (0.1–10 µM) in the presence of 2 mM extracellular Ca^{2+} resulted in $[Ca^{2+}]_i$ oscillations (Figure 1). Interestingly, there
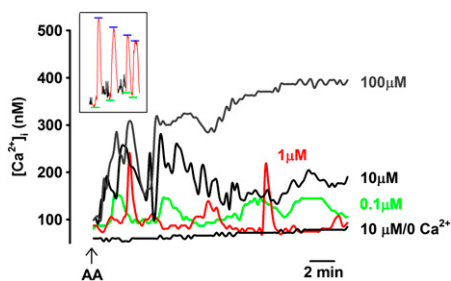
Figure 1. Arachidonic acid (AA) induces intracellular calcium concentration ($[Ca^{2+}]_i$) oscillations in human airway smooth muscle (ASM). In the presence of 2 mM extracellular Ca^{2+}, exposure of human ASM cells to different concentrations of AA (0.1–100 μM) rapidly resulted in $[Ca^{2+}]_i$ oscillations. Removal of extracellular Ca^{2+} eliminated these AA-induced $[Ca^{2+}]_i$ responses. These data suggest the presence of arachidonate-regulated Ca^{2+} (ARC) channels in human ASM cells, which modulate Ca^{2+} influx. *Inset* shows approach to evaluation of baseline Ca^{2+} (*green*) and peak Ca^{2+} (*blue*) for individual oscillations. *See* Table 1 for summary and statistics.

was no systematic relationship between AA concentration and oscillation features (Table 1; *see inset* of Figure 1 for evaluation of baseline and peak of valid oscillations). For example, 0.1 and 1 μM AA induced slow $[Ca^{2+}]_i$ oscillations of comparable amplitude and frequency (Figure 1), whereas 10 μM AA showed oscillations at a significantly higher frequency of 0.015 (± 0.005) Hz (which subsided as $[Ca^{2+}]_i$ concentrations rose to a higher level; Figure 1; $P < 0.05$ compared with lower AA concentrations). Exposure to 10 nM AA did not result in $[Ca^{2+}]_i$ oscillations, and inconsistently elevated the baseline $[Ca^{2+}]_i$ levels in roughly 10% of the cells (data not shown; in these cells, average baseline at 20 min after 10 nM AA was

175 ± 24 nM). On the other hand, exposure to 100 μM AA resulted in an initial $[Ca^{2+}]_i$ response similar to that produced by 10 μM AA, but rapidly progressed to a sustained plateau absent of any oscillations.

These interesting data raised the issue of what AA concentration to use in examining the potential role of ARC channels in ASM. Based on our observations, a lack of difference between 1 and 10 μM AA, previous studies in other cell types using greater than 1 μM AA (15, 16, 26), and indications that endogenous, active AA levels are in the tens of micromolar range (27), further studies were conducted using 10 μM AA.

Characterization of ARC Channels in Human ASM

In contrast to the consistent effect of 10 μM AA on ASM cells reflected by $[Ca^{2+}]_i$ oscillations, exposure to other fatty acids, such as the monounsaturated oleic acid, the saturated palmitic acid, and the diacylglycerol analog, 1-oleoyl-2-acetyl-SN-glycerol (OAG) (10 μM each), had minimal effects on $[Ca^{2+}]_i$ levels with occasional, extremely slow oscillations induced by OAG and oleic acid, but no effects of palmitic acid (Figure 2, Table 2). Oscillations induced by OAG or oleic acid were significantly smaller and slower compared with those by 10 μM AA (Table 2; $P < 0.05$). With palmitic acid and OAG, baseline Ca^{2+} levels at 20 minutes were not significantly increased compared with that before exposure to these fatty acids (110 ± 19 nM versus 125 ± 25 nM, respectively). Exposure to oleic acid resulted in a slow increase in baseline to 180 (± 39) nM.

In ASM cells perfused with zero extracellular Ca^{2+}, 10 μM AA–induced

oscillations were completely abolished (Figure 1, Table 2), suggesting an influx-mediated mechanism. This was further confirmed in β-escin–permeabilized ASM cells (28) in calcium potential (pCa) 7.0, 6.3, or 6.0 solutions, where 10 μM AA did not induce any oscillations (*also see below*). To investigate whether the AA-induced increase in $[Ca^{2+}]_i$ concentration is due to receptor-operated Ca^{2+} influx, $[Ca^{2+}]_i$ responses to AA were repeated in the presence of 10 μM SKF96365 (Figure 3A). However, 10 μM AA–induced increases in baseline, average oscillation amplitude, or frequency were unaffected by inhibition of receptor-operated influx (Figure 3, Table 2).

Although the above protocols did not specifically involve depletion of intracellular Ca^{2+} stores, it was important to determine whether La^{3+}-sensitive influx pathways (reflecting SOCE at lower concentrations [9]) were influenced by AA. Experiments on AA exposure were performed in the presence of 1 μM or 1 mM La^{3+}. Oscillation frequency was maintained in the presence of 1 μM La^{3+}, but amplitude is decreased, whereas 1 mM La^{3+} completely abolished oscillations as well as any change in baseline with AA (Figure 3B, Table 2; $P < 0.05$). Conversely, clamping membrane potential using 60 mM KCl increased baseline Ca^{2+} to 230 (± 49) nM, but did not induce any oscillations or substantially influence AA-induced $[Ca^{2+}]_i$ oscillations (Table 2), suggesting that voltage-gated influx channels were not contributory in this phenomenon.

STIM1 is an important regulatory mechanism in SOCE (22, 23, 29). We previously demonstrated that STIM1 in human ASM is mostly intracellular (e.g., at SR) and, to a lesser extent, within the plasma membrane (23). However, other investigators (17, 18) demonstrated that, in nonexcitable cells, STIM1 is located at the plasma membrane. To further investigate whether STIM1 plays a role in AA effects, AA-induced $[Ca^{2+}]_i$ responses were evaluated in ASM cells transfected with STIM1 siRNA (Figure 3C). STIM1 siRNA significantly decreased the frequency and amplitude AA-induced $[Ca^{2+}]_i$ oscillations, but did not eliminate them. Baseline $[Ca^{2+}]_i$ levels were not significantly influenced by STIM1 siRNA (102 ± 31 nM versus 121 ± 20 nM in lipofectamine controls). In this regard, we previously demonstrated the importance of caveolae and its constituent protein, caveolin-1, in

Table 1. Summary of Intracellular Calcium Concentration Responses of Human Airway Smooth Muscle Cells to Arachidonic Acid

	Oscillation Amplitude (nM Ca²⁺)	Oscillation Frequency (Hz)
Baseline	No oscillations	—
0.1 μM AA	101+14	0.003 ± 0.0004
1 μM AA	118 ± 20	0.002 ± 0.0004
10 μM AA	150 ± 20*	0.027 ± 0.007*
100 μM AA	No oscillation, 350 ± 48 peak*	—

Definition of abbreviation: AA, arachidonic acid
Values are means ± SE.
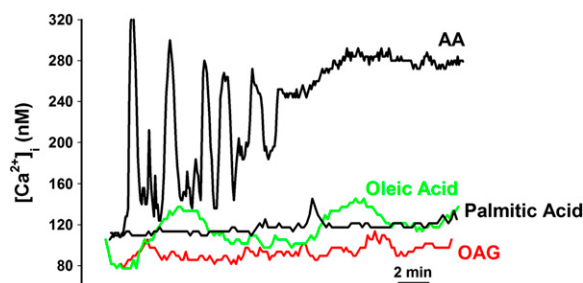*Significant difference from lower AA concentration ($P < 0.05$).

Figure 2. Effect of other fatty acids on $[Ca^{2+}]_i$ responses in ASM cells. In contrast to the consistent AA-induced $[Ca^{2+}]_i$ oscillations, other fatty acids, such as the lipid diacylglycerol analog, 1-oleoyl-2-acetyl-SN-glycerol, the saturated fatty acid, palmitic acid, or the monounsaturated oleic acid did not elicit any $[Ca^{2+}]_i$ oscillations. Indeed, only oleic acid induced any significant increase in $[Ca^{2+}]_i$ in human ASM cells. These data suggest that the observed $[Ca^{2+}]_i$ responses are specific to AA. See Table 2 for summary and statistics.

$[Ca^{2+}]_i$ regulation in human ASM (4), further showing that plasma membrane STIM1 is within caveolae (23). Based on the above data showing an effect of STIM1 on AA-induced oscillations, to investigate the role of caveolae, we performed experiments in caveolin-1 siRNA–transfected ASM cells. Compared with lipofectamine control, caveolin-1 siRNA significantly blunted both amplitude and frequency of AA-induced $[Ca^{2+}]_i$ oscillations (Figure 3D, Table 2; $P < 0.05$). Baseline $[Ca^{2+}]_i$ levels were decreased by caveolin-1 siRNA, but not

significantly (76 ± 21 nM versus 111 ± 22 nM in lipofectamine controls).

Although the identity of ARC channels is still under investigation, previous work in nonexcitable cells (30, 31) suggests plasma membrane Orai3. In human ASM cells transfected with Orai3 siRNA, AA-induced $[Ca^{2+}]_i$ oscillation frequency was significantly reduced (Figure 3E, Table 2; $P < 0.05$). However, there was no significant effect on oscillation amplitude, or baseline Ca^{2+} levels.

For STIM1, caveolin-1, and Orai3 siRNA experiments, nonsense siRNAs did not

substantially influence AA-induced $[Ca^{2+}]_i$ oscillations, nor did lipofectamine alone (vehicle control). Western analysis confirmed substantial knockdown of these three proteins by the specific siRNAs, with lack of effect of the nonsense siRNAs (Figure 3F).

AA can serve as a substrate for the lipoxygenase (LOX), cyclo-oxygenase (COX), and CYP450 pathways, which produce metabolites, such as leukotrienes, prostaglandins, epoxyeicosatrienoic acid, and hydroxyeicosatetraenoic acid. These metabolites can induce inflammation and alter Ca^{2+} influx (32). Additional experiments were performed in the presence of the LOX inhibitor, zileuton (10 μM), and the COX inhibitor, indomethacin (10 μM; Figure 4). Cells were incubated with inhibitor 15 minutes before AA. Neither inhibitor showed a significant effect on AA-induced $[Ca^{2+}]_i$ oscillations (Figure 4, Table 2; $P < 0.05$). Based on these data showing lack of effect of LOX or COX inhibition on AA-induced oscillations, we did not explore the potential role of AA metabolites further.

We previously demonstrated that agonist-induced $[Ca^{2+}]_i$ oscillations represent repetitive SR Ca^{2+} release via ryanodine receptor (RyR) channels or inositol 1,4,5-trisphosphate (IP_3) receptor channels (5, 12). To determine whether AA-induced $[Ca^{2+}]_i$ oscillations also represented such repetitive Ca^{2+} release from intracellular stores, we repeated experiments in the presence of high, blocking concentrations of the RyR modulator, ryanodine (10 μM) or the IP_3 receptor blocker, XeC (1 μM; Figure 5). In the presence of either ryanodine or XeC, AA-induced $[Ca^{2+}]_i$ oscillations were abolished, and only minimally elevated $[Ca^{2+}]_i$ levels were observed (Figure 5), demonstrating that AA effects do involve SR Ca^{2+} release, but likely due to indirect effects on the SR resulting from Ca^{2+} influx. This was supported by the lack of $[Ca^{2+}]_i$ oscillations in the absence of extracellular Ca^{2+} (Figure 1) as well as in β-escin–permeabilized ASM cells in pCa 7.0 (corresponding to baseline Ca^{2+} of ~ 100 nM; Figure 5). A similar lack of effect of AA was observed in permeabilized ASM cells in pCa 6.3 (~ 500 nM; data not shown).

AA Effects in ASM Cells from Subjects with Asthma
In general, baseline $[Ca^{2+}]_i$ levels before AA exposure were not higher in ASM cells

Table 2. Characterization of Arachidonic Acid–Regulated Ca^{2+} Channels in Human Airway Smooth Muscle Cells

	Oscillation Amplitude (nM Ca^{2+})	Oscillation Frequency (Hz)
2 mM Ca^{2+}, AA	178 ± 19	0.018 ± 0.007
Zero Ca^{2+}, AA	No oscillations	—
β-escin permeabilized, pCa 7.0 AA	No oscillations	—
10 μM OAG	33 ± 9*	0.009 ± 0.003*
10 μM Oleic acid	42 ± 7*	0.002 ± 0.007*
10 μM Palmitic acid	No oscillations	—
10 μM SKF96,365 + AA	182 ± 12	0.021 ± 0.009
1 μM $LaCl_3$ + AA	110 ± 17*	0.024 ± 0.003
1 mM $LaCl_3$ + AA	33 ± 9*	0.002 ± 0.006*
60mM KCl +AA	132 ± 33	0.021+0.002
Lipofectamine + AA	155 ± 15*	0.022 ± 0.007
Nonsense siRNA + AA	138+40	0.022+0.002
STIM1 siRNA + AA	91 ± 9†	0.010 ± 0.001†
Caveolin-1 siRNA + AA	101 ± 17†	0.005 ± 0.005†
Orai3 siRNA + AA	117 ± 22	0.002 ± 0.001†
Zileuton + AA	130 ± 10	0.014 ± 0.001
Indomethacin + AA	122 ± 15	0.016 ± 0.001
Asthmatic ASM, AA	301 ± 90*	0.013+0.001

Definition of abbreviations: AA, arachidonic acid; ASM, airway smooth muscle; OAG, 1-oleoyl-2-acetyl-SN-glycerol; pCa, calcium potential; siRNA, small interfering RNA; STIM, stromal interaction molecule

Values are means ± SE. All AA experiments were at 10-μM concentration.
*Significant difference from 10 μM AA ($P < 0.05$).
†Significant difference from 10 μM AA effects on lipofectamine-transfected cells ($P < 0.05$).
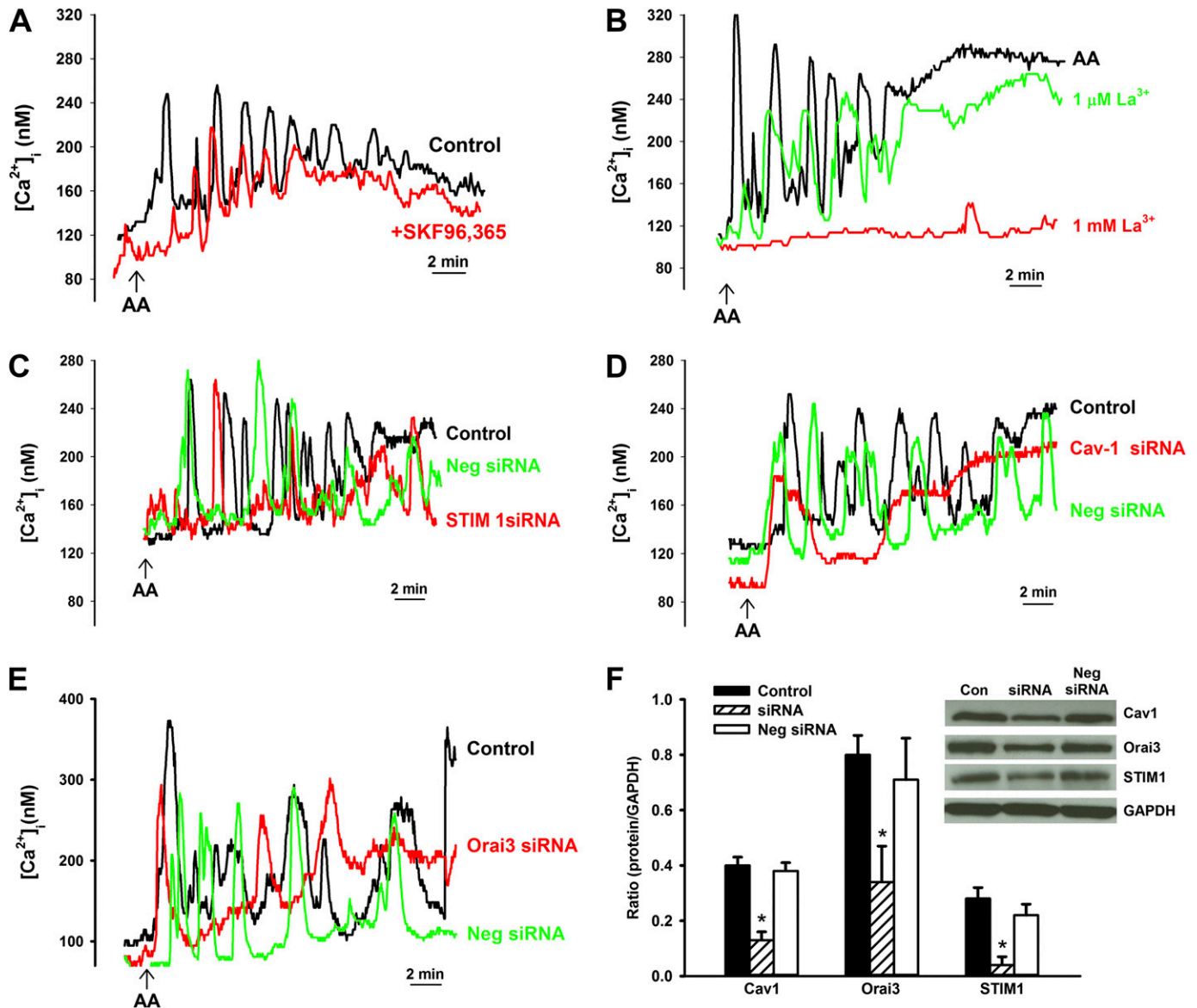
Figure 3. Mechanisms of AA-induced Ca^{2+} influx. (*A*) Role of receptor-operated Ca^{2+} entry in AA-induced Ca^{2+} influx. In the presence of the receptor-operated channel inhibitor, SKF96365 (10 μM), AA-induced $[Ca^{2+}]_i$ responses were not significantly altered, suggesting that the observed Ca^{2+} influx was not via this mechanism. (*B*) Role of store-operated Ca^{2+} entry (SOCE) in AA-induced Ca^{2+} influx. La^{3+} (1 μM; which inhibits SOCE) slightly decreased the amplitude of AA-induced $[Ca^{2+}]_i$ oscillations, but did not affect oscillation frequency. In contrast, 1 mM La^{3+} completely eliminated $[Ca^{2+}]_i$ responses, suggesting that the observed influx is unlikely to be SOCE, especially because sarcoplasmic reticulum (SR) stores were not depleted during these experiments. (*C*) Role of stromal interaction molecule (STIM) 1 in AA-induced $[Ca^{2+}]_i$ responses. In the presence of STIM1 small interfering RNA (siRNA), AA-induced elevations in $[Ca^{2+}]_i$ levels were reduced and $[Ca^{2+}]_i$ oscillations decreased compared with lipofectamine (vehicle) control, whereas negative siRNA was without effect. These data suggest that STIM1 modulates AA-induced $[Ca^{2+}]_i$ responses. (*D*) Role of caveolae/caveolin-1 in AA-induced $[Ca^{2+}]_i$ responses. Suppression of caveolin-1 using siRNA eliminated AA-induced $[Ca^{2+}]_i$ oscillations, whereas lipofectamine or negative siRNA were without effect. These data suggest that caveolin-1 modulates AA-induced Ca^{2+} influx in human ASM. (*E*) Role of Orai3 on AA-induced $[Ca^{2+}]_i$ responses. Orai3 siRNA reduced the frequency of AA-induced $[Ca^{2+}]_i$ oscillations and slightly altered the amplitudes of $[Ca^{2+}]_i$ compared with lipofectamine or negative siRNA. These data suggest a role for Orai 3 in modulating AA-induced $[Ca^{2+}]_i$ responses. (*F*) Western analysis confirmed siRNA knockdown of STIM1, caveolin-1, and Orai3, and lack of effect of negative siRNA. *See* Table 2 for summary and statistics of Ca^{2+}. *Significant siRNA effect ($P < 0.05$). Cav1, caveolin-1; Con, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Neg, negative.

from subjects with moderate asthma (defined by forced expiratory volume in 1 second/forced vital capacity, daily use of short-acting bronchodilator). However, exposure of ASM cells from subjects with asthma to 10 μM AA resulted in higher oscillation amplitude compared with cells from subjects without asthma, but there was no difference in oscillation frequency (Figure 6, Table 2). Removal of extracellular Ca^{2+} prevented $[Ca^{2+}]_i$ oscillations as with nonasthmatic cells.
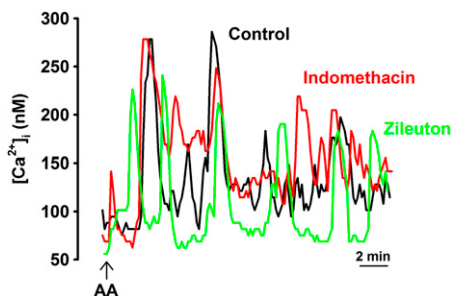
Figure 4. Role of lipoxygenase (LOX) and cyclo-oxygenase (COX) pathways in AA-mediated $[Ca^{2+}]_i$ responses. AA can serve as a substrate for the LOX and COX pathways that produce metabolites (leukotrienes, prostaglandins, and prostacyclins), which can mediate inflammation and alter Ca^{2+} influx. Inhibitors of LOX (zileuton) and COX (indomethacin) did not significantly influence 10 μM AA–induced $[Ca^{2+}]_i$ oscillations, suggesting that ARC channels are specifically modulated by AA, and not by products downstream to AA. *See* Table 2 for summary and statistics.

Discussion

Ca^{2+} influx through a variety of mechanisms is a key aspect of $[Ca^{2+}]_i$ regulation in ASM. In this regard, capacitative Ca^{2+} entry in response to intracellular store depletion (i.e., SOCE) is an important mechanism in ASM (9–11, 25). However, there is increasing evidence in nonexcitable cells for an additional noncapacitative Ca^{2+} influx mechanism regulated by AA (13, 33). What is less clear is the molecular identity of such ARC channels, with data in other cell types suggesting a role for Orai proteins that are also involved in SOCE. The data in the current study strongly suggest the existence of ARC channels in human ASM, and such channels involve Ca^{2+} influx and are mediated via Orai3 proteins. However, other modulatory mechanisms, such as STIM1 and caveolin-1, also appear to be important. Although AA generation is known to occur in human ASM in response to agonist stimulation and in inflammation, our novel results suggest that such AA, by itself, may act through a plasma membrane caveolar mechanism, perhaps Orai3 with a role for plasma membrane STIM1, to induce $[Ca^{2+}]_i$ oscillations (Figure 7), akin to those induced by agonists, such as ACh (5). Such AA-induced oscillations appear to involve intracellular Ca^{2+} release as well, and may thus also partly result from store-operated mechanisms (but not receptor-operated influx). Furthermore, AA-induced $[Ca^{2+}]_i$ responses appear to be enhanced in asthmatic ASM. Overall, these data indicate that ARC channels may be an important Ca^{2+}-regulatory mechanism in human ASM, and may contribute to modulation of $[Ca^{2+}]_i$ during agonist stimulation or with inflammation.

Although ARC channels have been demonstrated in a variety of nonexcitable cells (16, 34), their existence and role in ASM has not been studied. In terms of smooth muscle, a previous study reported AA-sensitive Ca^{2+} influx pathways in A7R5 vascular smooth muscle cells (35). In the current study, we demonstrate that concentrations of AA in the micromolar range induce $[Ca^{2+}]_i$ responses, including oscillations. This effect is completely abolished upon removal of extracellular Ca^{2+}, demonstrating the importance of Ca^{2+} influx in responses of ASM cells to AA. An interesting observation was that there was no clear relationship between AA concentration and the amplitude or frequency of $[Ca^{2+}]_i$ oscillations. Furthermore, $[Ca^{2+}]_i$ responsiveness to AA in terms of oscillations seems to occur over a narrow concentration range (> 0.1 to < 100 μM), and involves just frequency modulation (Table 1). In this regard, it may be important to consider the appropriate concentrations of AA to examine. There is currently no information on levels in ASM *per se*. However, published literature (e.g., Ref. 27) suggests that the concentration of free AA in resting cells is "low." Esterified (presumably inactive) AA levels in platelets are in the 5-mM range, with plasma membrane concentrations being even higher. Thus, even small amounts of release of this AA would provide 50 μM. Free AA levels in tissues or cells has been reported to be anywhere from 0.5–100 μM, and cells are thought to respond in the 1–10 μM AA range. Therefore, our use of 10 μM AA is consistent with previous data.

Although the underlying reasons for the lack of a classical dose–response curve to AA are not entirely clear, oscillations only at higher AA concentrations may represent a threshold within ASM cells to prevent inappropriate $[Ca^{2+}]_i$ responses to AA. Accordingly, the higher $[Ca^{2+}]_i$ responses to AA in asthmatic ASM may reflect a change in this threshold, contributing to an overall increase in $[Ca^{2+}]_i$ that does occur in asthmatic ASM cells. Alternatively, because ARC channels are regulated by AA, but do not necessarily involve receptor-coupled mechanisms, perhaps a classical dose–response cannot be expected. Furthermore, it may be necessary to examine the precise relationships between AA-induced regulatory mechanisms in ASM, and the sensitivity of putative ARC channel elements, such as Orai3 or STIM1, to these mechanisms: topics for future studies.

Mignen and colleagues (36) demonstrated in HEK293, COS, and HeLa cells the existence and high selectivity of ARC channels for Ca^{2+} as well as AA itself. In comparison to AA, other monounsaturated and saturated fatty acids had no effect. In the current study using human ASM, we demonstrated that, in comparison to AA, the diacylglycerol analog, OAG, oleic acid, and palmitic acid had minimal effects on $[Ca^{2+}]_i$, and only occasionally induced $[Ca^{2+}]_i$ oscillations.
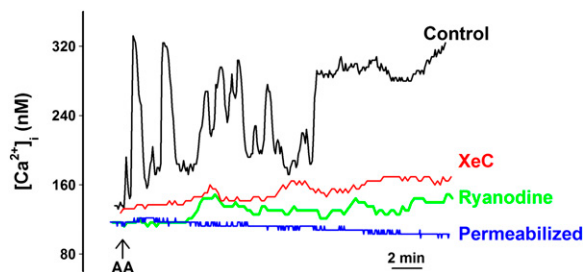

Figure 5. Role of SR Ca^{2+} release in AA-induced Ca^{2+} oscillations. The presence of the cell-permeant inositol 1,4,5-trisphosphate (IP_3) receptor blocker, xestospongin C (XeC; 1 μM) or blocking concentrations of ryanodine (10 μM) eliminated all $[Ca^{2+}]_i$ oscillations, with only slight increases in $[Ca^{2+}]_i$ levels. Separately, in β-escin–permeabilized cells in a pCa of 7.0 (corresponding to 100 nM Ca^{2+} as reflected by baseline Ca^{2+}), AA was without effect on $[Ca^{2+}]_i$, indicating that AA-induced $[Ca^{2+}]_i$ responses only indirectly modulate SR Ca^{2+} release. *See* Table 2 for summary and statistics.
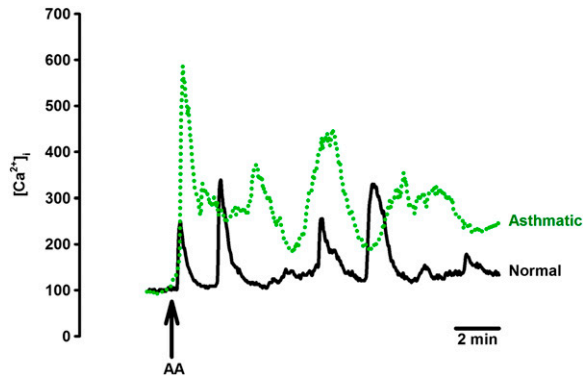
Figure 6. Effect of AA on $[Ca^{2+}]_i$ in asthmatic ASM. In isolated ASM cells from patients with moderate asthma, 10 μM AA induced $[Ca^{2+}]_i$ oscillations of higher amplitude compared with nonasthmatic ASM cells. However, oscillation frequency was not different. See Table 2 for summary and statistics.

Furthermore, inhibition of LOX and COX pathways downstream to AA were without effect. These novel data are highly suggestive of an AA-specific influx pathway in ASM similar to that in nonexcitable cells.

Intracellular Ca^{2+} regulation in ASM involves interplay between plasma membrane Ca^{2+} influx/efflux and SR Ca^{2+} release/reuptake (1–4). Accordingly, overall $[Ca^{2+}]_i$ regulation may be dependent on both physical proximity of regulatory elements as well as changes in Ca^{2+} gradients and fluxes induced by these regulatory elements. In this regard, our

Figure 7. Proposed model of ARC channels in human ASM. Regardless of the source (agonist- or cytokine-induced production), AA activates plasma membrane ARC channels that are within caveolae. Such activation is facilitated by plasma membrane STIM1, and involves repeated SR Ca^{2+} release. IP$_3$R, IP$_3$ receptor; RyR, ryanodine receptor; SERCA, sarco/endoplasmic reticulum calcium ATPase.

previous data, showing that agonists, such as ACh, induce repetitive $[Ca^{2+}]_i$ oscillations representing cyclical SR Ca^{2+} release/reuptake with a supportive role for Ca^{2+} influx, are significant. Here, in addition to voltage-operated and receptor-operated Ca^{2+} influx, we and others have demonstrated the importance of SOCE in ASM (9–11). What is less clear is whether SOCE per se is important in agonist-induced $[Ca^{2+}]_i$ oscillations, because store depletion may or may not occur, especially at lower agonist concentration. In this regard, data from nonexcitable cells show that, in contrast to SOCE, which relies on high agonist concentrations and almost complete SR Ca^{2+} depletion, ARC channel activity depends on low agonist concentrations (16, 34). Accordingly, the relevance of ARC channels in ASM cells may lie in providing a non-SOCE Ca^{2+} influx pathway after submaximal agonist stimulation typically associated with $[Ca^{2+}]_i$ oscillations (37, 38). Here, the higher $[Ca^{2+}]_i$ responses of asthmatic ASM cells to AA may contribute to the higher $[Ca^{2+}]_i$ responses to bronchoconstrictor agonists in such cells.

Although it is clear that ARC channels involve Ca^{2+} influx (demonstrated by the abolition of responses to AA in zero extracellular Ca^{2+}, by high levels of LaCl$_3$, and the lack of responses to AA in β-escin–permeabilized cells), given the lack of specific pharmacological inhibitors, and only limited data suggesting actual molecular identities of these channels, it is difficult to state with complete confidence that the AA-sensitive $[Ca^{2+}]_i$ responses observed in ASM cells in this study truly present ARC channels per se. However,

data included in our study are generally consistent with previous work in nonexcitable cells, and indirectly suggest that ARC channels do exist in ASM. For example, because ARC channel activity can depend on agonist concentration, receptor-operated Ca^{2+} channels need to be eliminated as potential confounders. Our data showing lack of effect of the receptor-operated Ca^{2+} channel inhibitor, SKF96365 (consistent with data in nonexcitable cells [34]), would indeed eliminate this possibility. Another potential confounder is channels that mediate SOCE that occurs in response to SR Ca^{2+} depletion, thus serving as an important mechanism for SR refilling (9, 39–42). In nonexcitable cells, it has been shown that Ca^{2+} influx switches from ARC channels at low agonist concentrations to SOCE at higher agonist concentrations (16). Previous studies in ASM demonstrate SOCE is triggered by SR Ca^{2+} release via both IP$_3$ receptor and RyR channels, and is modulated by agonists (9, 43). However, the sensing or activation mechanisms for SOCE, initially thought to involve transient receptor potential channels, including in ASM (4, 43), have been shown to involve STIM1 at the level of the SR and the plasma membrane Orai1 (44, 45). In this regard, our data (and those in nonexcitable cells) show that AA-mediated $[Ca^{2+}]_i$ responses do not represent STIM1/Orai1–mediated SOCE. However, it is also important to note that both STIM and Orai are highly relevant to ARC channels. The Orai family of proteins (Orai1–3) is thought to be the "pore-forming" unit in the plasma membrane that interacts with the C terminus of STIM1 to initiate SOCE or to activate ARC channels (46). Shuttleworth (18) and Thompson and Shuttleworth (19) demonstrated in nonexcitable cells a role for plasma membrane STIM1 in ARC channels. This is in contrast to SOCE, where SR STIM1 appears to be important. We previously found that SR STIM1 is involved in ASM SOCE (23), but found only small amounts of plasma membrane STIM1. Our finding that AA-induced $[Ca^{2+}]_i$ responses are substantially blunted in the presence of STIM1 siRNA now suggests a role for the latter. In addition, the units required for the assembly of SOCE channels in comparison to ARC channels are different (18). Whereas Orai1 is particularly important in SOCE (including in ASM), the ARC channel pore is thought to be formed by assembly of three Orai1
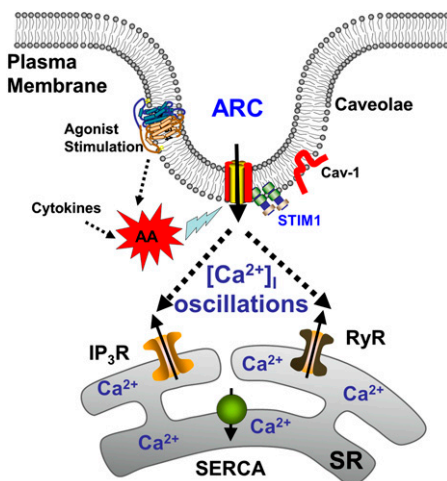
and two Orai3 subunits (18). Our observation that knockdown of Orai3 via siRNA results in a 50% decrease in AA-induced $[Ca^{2+}]_i$ oscillations is entirely consistent with this scenario in human ASM. Thus, our data overall underline the existence of ARC channels in human ASM.

A novel aspect of our study is the finding that plasma membrane caveolae are important for ARC channels in human ASM. Caveolae are specialized forms of lipid rafts found in most cells types. Previous studies by our group and others (4, 47, 48) suggest that caveolae contain a variety of proteins that are important for $[Ca^{2+}]_i$ signaling in ASM, including STIM1 and Orai proteins. Our data in this study, showing that caveolin-1 substantially blunts AA responses, suggests that the mechanisms that involve ARC channel activity, likely STIM1, Orai1, and Orai3, are located within caveolae. Thus, caveolae may facilitate interactions between plasma membrane STIM1 and Orai3, and thus be an important modulator of AA-induced Ca^{2+} influx.

An interesting aspect of AA-induced $[Ca^{2+}]_i$ oscillations is their inhibition by blocking of SR Ca^{2+} release channels via ryanodine and XeC. Although ARC channels are clearly an influx mechanism, such influx appears to trigger SR Ca^{2+} release, perhaps by the well known Ca^{2+}-induced Ca^{2+} release mechanism. What is less clear is whether any intermediate signaling pathways are involved. Furthermore, it is not clear whether AA or its metabolites separately affect IP_3 channel or RyR to alter their opening probability or sensitivity to $[Ca^{2+}]_i$. Although AA

effects on SR Ca^{2+} release channels are possible, the lack of AA-induced $[Ca^{2+}]_i$ oscillations in β-escin–permeabilized cells would suggest otherwise.

In addition to effects on mechanisms, such as Orai3, which may be part of the ARC channel pore, AA itself is known to activate a number of channels, particularly a range of K^+ channels (49), with both membrane depolarization and hyperpolarization being possible depending on whether AA or its metabolites are involved. Although we did not specifically examine the role of K^+ channels in this study, the observation that clamping membrane potential with 60 mM KCl did not produce spontaneous $[Ca^{2+}]_i$ oscillations or influence oscillations induced by AA would suggest that even if K^+ channels were affected by AA, membrane potential itself may not influence ARC channel regulation. With regard to AA metabolites, the downstream CYP450, COX, and LOX pathways can produce a number of metabolites that may potentially mediate AA effects on $[Ca^{2+}]_i$. However, our observations that inhibition of COX or LOX did not substantially influence AA-induced oscillations would suggest that metabolites of at least these two enzymes are likely not involved. Lack of enhanced AA effects in the presence of inhibitors of COX or LOX would further suggest that diversion of AA to the CYP450 pathway and resultant metabolites also may not play a role.

The relevance of AA-induced $[Ca^{2+}]_i$ responses and ARC channels in ASM lies in the potential role of this regulatory mechanism in inflammatory airway diseases, such as asthma, where AA-

mediated pathways are thought to be important (50). Accordingly, the limited but interesting data that asthmatic ASM cells have a higher amplitude of $[Ca^{2+}]_i$ responses to AA raise the question of whether ARC channels are potentially contributory to increased $[Ca^{2+}]_i$ in asthmatic ASM. Although the mechanisms for such up-regulation were beyond the scope of this study, enhanced Orai3 expression (or activation) may be involved, and would be consistent with previous findings that related mechanisms, such as STIM1, Orai1, and even caveolin-1, are up-regulated in human ASM after inflammation (11, 24, 25). Furthermore, inflammation can result in enhanced expression of SR Ca^{2+} proteins and functionality, and AA-induced influx could produce greater SR Ca^{2+} responses, leading to greater contraction.

In conclusion, our study demonstrates the existence of ARC channels in human ASM similar to that in nonexcitable cells. The relevance of ARC channels in ASM lies in its potential contribution to Ca^{2+} influx during agonist stimulation, when SR store depletion may not occur, thus allowing a non-SOCE pathway for Ca^{2+} influx to replenish stores. Given the generation of AA after agonist stimulation and inflammation, ARC channels may be particularly important in contributing to the elevated $[Ca^{2+}]_i$ levels observed in inflamed/asthmatic ASM, and thus to increased airway contractility. ■

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