Ionic mechanisms and Ca$^{2+}$ regulation in airway smooth muscle contraction: do the data contradict dogma?

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Janssen, Luke J. Ionic mechanisms and Ca$^{2+}$ regulation in airway smooth muscle contraction: do the data contradict dogma? Am J Physiol Lung Cell Mol Physiol 282: L1161–L1178, 2002; 10.1152/ajplung.00452.2001.—In general, excitation-contraction coupling in muscle is dependent on membrane depolarization and hyperpolarization to regulate the opening of voltage-dependent Ca$^{2+}$/H11001 channels and, thereby, influence intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]). Thus Ca$^{2+}$ channel blockers and K$^{+}$ channel openers are important tools in the arsenals against hypertension, stroke, and myocardial infarction, etc. Airway smooth muscle (ASM) also exhibits robust Ca$^{2+}$, K$^{+}$, and Cl$^{-}$ currents, and there are elaborate signaling pathways that regulate them. It is easy, then, to presume that these also play a central role in contraction/relaxation of ASM. However, several lines of evidence speak to the contrary. Also, too many researchers in the ASM field view the sarcoplasmic reticulum as being centrally located and displacing its contents uniformly throughout the cell, and they have focused almost exclusively on the initial single [Ca$^{2+}$] spike evoked by excitatory agonists. Several recent studies have revealed complex spatial and temporal heterogeneity in [Ca$^{2+}$], the significance of which is only just beginning to be appreciated. In this review, we will compare what is known about ion channels in ASM with what is believed to be their roles in ASM physiology. Also, we will examine some novel ionic mechanisms in the context of Ca$^{2+}$ handling and excitation-contraction coupling in ASM.

excitation-contraction coupling; ion channels; membrane potential

AIRWAY HYPERREACTIVITY and variable airflow obstruction are key features of asthma. Indeed, one might say these are its most clinically relevant features. For this reason, it is essential to have a good understanding of the mechanisms underlying excitation-contraction (EC) coupling in airway smooth muscle (ASM). A great deal of research is being focused on the electrophysiology of ASM, given the importance of ion channels in EC coupling in other muscle types. The goal of this review is to stimulate a reevaluation of the existing literature on the electrophysiology of ASM as it pertains to EC coupling, with a view to redirect those research efforts.

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TRADITIONAL VIEWS

EC coupling in nonairway muscles: adequate models for ASM? In striated muscles as well as vascular and gastrointestinal smooth muscle, EC coupling is largely dependent on membrane depolarization, although for very different reasons.

In cardiac muscle, Na⁺ channel opening depolarizes the membrane, resulting in Ca²⁺ entry via voltage-dependent ("L-type") Ca²⁺ channels, producing a transient elevation of [Ca²⁺], immediately under the plasmalemma (17). This initial rise in [Ca²⁺], does not trigger contraction directly; instead, it activates ryanodine receptors on the sarcoplasmic reticulum (SR), causing a massive discharge of Ca²⁺ from the internal store, resulting in contraction (17). Voltage-dependent Ca²⁺ influx also contributes to a number of other cellular events, including refilling of the SR, activation of plasmalemmal ion channels, and modulation of various enzyme activities, etc.

In vascular and gastrointestinal smooth muscle, on the other hand, voltage-dependent Ca²⁺ influx through L-type channels is sufficient for contraction (17). Moreover, many electrophysiological studies of these tissues reveal a "window current" spanning the physiologically relevant range of membrane potentials, i.e., a range of potentials more positive than the threshold for activation of the Ca²⁺ channels but over which voltage-dependent inactivation is not complete, giving rise to a persistent Ca²⁺ influx. Thus small hyperpolarizations lead to decreased activation of the channels and a drop in [Ca²⁺], while small depolarizations increase Ca²⁺ channel activation and elevate [Ca²⁺].

Despite the differences in the mechanisms underlying EC coupling in these tissues, the central role played by dihydropyridine-sensitive Ca²⁺ channels in both cases provides the rationale for the use of Ca²⁺ channel blockers and K⁺ channel agonists in controlling cardiac and smooth muscle contractions in hypertension, stroke, myocardial infarction, and gastrointestinal motility disorders, etc. More importantly, clinical studies attest to the efficacy of these tools for these purposes (59, 66, 187).

Agonist-mediated EC coupling in ASM. Excitation of ASM is similar in many respects to that of vascular or gastrointestinal smooth muscles. First, it is accompanied by membrane depolarization (60) mediated primarily by activation of Cl⁻ and nonselective cation currents as well as suppression of K⁺ currents (106, 117, 119, 120, 236). Patch-clamp studies have documented the large voltage-dependent Ca²⁺ currents activated by membrane depolarization (68, 140) (Fig. 1). Finally, these Ca²⁺ currents are sufficient to produce contraction, as manifest in the robust dihydropyridine-sensitive contractions evoked by potassium chloride (108, 122) or K⁺ channel blockers such as tetraethylammonium (TEA), 4-aminopyridine, or charybotoxin (45), although these contractions are generally only a fraction of the size of those evoked by physiological agonists such as carbachol, histamine, and endothelin, etc.

Given these similarities with other muscle types, it is understandable that many treat Ca²⁺, K⁺, and Cl⁻ channels as central players in EC coupling in ASM. However, a large body of data speaks to the contrary.

CHALLENGING THE DOGMA

Do agonist-evoked contractions in ASM require voltage-dependent Ca²⁺ influx? Many groups have characterized the voltage-dependent Ca²⁺ currents in ASM (usually of the trachealis) and found these to be almost exclusively L-type in nature (77, 81, 84, 107, 140, 152, 164). It is particularly important to bear in mind a number of biophysical properties of these currents. 1) All electrophysiological studies find the threshold potential for these currents to be in excess of −40 mV, and almost all of them show little or no Ca²⁺ current until membrane voltages rise above −20 mV (Fig. 1); peak activation occurs at +10 to +20 mV. 2) These currents can develop substantial voltage- and Ca²⁺-dependent inactivation, and they are also suppressed by various second messenger signaling pathways (233, 242, 246). 3) L-type channels are selectively and potently blocked by dihydropyridines (Fig. 2), with an IC₅₀ in the nanomolar range (158).

Given these properties of the Ca²⁺ channels, several observations call into question the view that voltage-dependent Ca²⁺ channels play a central role in ASM contraction.
First, many have shown that agonist-evoked contraction of ASM is seemingly unaffected under conditions in which voltage-dependent Ca\textsuperscript{2+}/H\textsubscript{11001} influx is prevented by Ca\textsuperscript{2+}/H\textsubscript{11001} channel blockers (25, 26, 48, 61) (Fig. 2) by “clamping” the membrane potential to very negative values far below the threshold for Ca\textsuperscript{2+} channel activation (118) (Fig. 3) or even by removal of external Ca\textsuperscript{2+} (48, 61). Likewise, although Cl\textsuperscript{−} channels are primarily responsible for the membrane depolarization (106, 117, 119, 120, 147, 148, 237, 238), agents such as niflumic acid, which are able to completely block the Cl\textsuperscript{−} currents in ASM, have essentially no effect on resting membrane potential (113) or agonist-evoked contractions (Fig. 2). The only studies that do describe an inhibitory effect of dihydropyridines on mechanical responses in ASM were carried out under very non-physiological conditions (complete depletion of the internal Ca\textsuperscript{2+} pool) (8, 24–26, 114, 123, 197, 224) or used supramaximally effective concentrations of dihydropyridines. For example, in the case of nifedipine, most groups tend to use 10\textsuperscript{−6} M, and may even use 10\textsuperscript{−5} M (231), even though the IC\textsubscript{50} value reported for this agent is in the nanomolar range (158); submicromolar concentrations are sufficient to completely block the Ca\textsuperscript{2+} channels in ASM (77, 84, 243) and to suppress contractions in vascular or gastrointestinal smooth muscle (13, 38, 57, 70, 145). Thus nonspecific effects of the dihydropyridines (191) need to be kept in mind when interpreting such data.

Second, the range of membrane potentials typically seen in ASM at rest and during excitation ranges from −70 to −30 mV (1, 27, 40, 46, 60, 85, 95, 96, 99–102, 109, 111, 112, 125, 130, 132, 136, 155, 188, 215, 220), which is well below the range of potentials required for Ca\textsuperscript{2+} channel activation (−30 to +20 mV) (68, 84, 107, 140, 152, 164, 242, 243, 247) (Figs. 1 and 4). More to the point, the voltages required to only marginally activate voltage-dependent Ca\textsuperscript{2+} channels (−40 to −30 mV) are attained only with concentrations of agonist that evoke nearly complete contraction, and Ca\textsuperscript{2+} currents are maximal at membrane potentials never seen during agonist stimulation (+10 to +20 mV; see Figs. 1 and 4). Simultaneous electrophysiological and fura 2 fluorimetric recordings in equine ASM have shown that the Ca\textsuperscript{2+} currents evoked by voltage step commands to potentials in the physiologically relevant range produce elevations in [Ca\textsuperscript{2+}]\textsubscript{i} of <50 nM (68), which pales in comparison with bronchoconstrictor-evoked Ca\textsuperscript{2+} responses (both the peak and plateau

Fig. 2. Contractions in airway smooth muscle (ASM) are seemingly unaffected by blockers of voltage-dependent Ca\textsuperscript{2+} channels or of Cl\textsuperscript{−} channels. Despite the ability of niflumic acid and nifedipine to abolish Cl\textsuperscript{−} and Ca\textsuperscript{2+} currents in ASM (A and B, respectively) [adapted from Janssen and Sims (117) and Janssen (107)], these agents have no substantial effect on agonist-evoked contractions (C).

Fig. 3. Substantial contraction can still be evoked at very negative membrane potentials. Vertical axis indicates cell length in an ASM cell held continuously under voltage clamp at −60 mV. Acetylcholine (10\textsuperscript{−4} M) was applied at −3-min intervals (>). Numerous contractions (to almost 50% of resting length) can still be evoked at this potential, which is far below threshold for opening of voltage-dependent Ca\textsuperscript{2+} channels. [From Janssen and Sims (118).]
Fig. 4. Membrane voltages and Ca\(^{2+}\) currents. Estimates of membrane potentials at rest (●) and during stimulation with various excitatory agents (■), obtained using intracellular microelectrode techniques. Numerals in parentheses indicate reference citation from which data were extracted. Cch, carbachol; EFS, electric field stimulation; 4-AP, 4-aminopyridine; TEA, tetraethylammonium; 5-HT, 5-hydroxytryptamine. Also shown are voltages for threshold and peak (○ and ●, respectively) activation of voltage-dependent Ca\(^{2+}\) currents, as determined using patch-clamp electrophysiological techniques. The voltages at which those currents are half inactivated are also indicated by the solid vertical bar. Shaded box indicates the range of potentials at which membrane oscillations (slow waves) are typically seen in ASM. The purpose of this figure is to highlight the lack of overlap between the physiologically relevant range of potentials and those potentials required for significant voltage-dependent Ca\(^{2+}\) influx. TSM, tracheal smooth muscle; BSM, bronchial smooth muscle; LTC, leukotriene C\(_4\).
values typically measure several hundreds of nanomolars (249–251).

Third, the \( \text{Ca}^{2+} \) currents and changes in \( [\text{Ca}^{2+}]_i \) described above are overestimations of those one should expect to see in vivo. Because they were evoked under experimental conditions in which voltage-dependent inactivation had not developed to any substantial degree, i.e., the depolarizing steps used to evoke these currents are generally less than a few hundred milliseconds in duration and are each separated by several seconds to minimize inactivation. This inactivation is half-maximal (meaning the currents are halved in size) whenever membrane potential approaches \(-30 \text{ mV}\) for more than a few seconds (77, 84, 107, 140, 164) and is nearly complete as membrane potential approaches 0 mV. Thus during slow wave activity, when the membrane potential is slowly oscillating between \(-40 \text{ and } -20 \text{ mV}\) for many minutes or even hours, substantial inactivation of these currents will have occurred. One group has described a small persistent \( \text{Ca}^{2+} \) inactivation of these currents will have occurred.

Fourth, whenever the tissues are stimulated by bronchoconstrictors such as cholinergic agonists, voltage-dependent \( \text{Ca}^{2+} \) currents are further suppressed via phosphorylation of the channels by protein kinase C (246) and/or through \( \text{Ca}^{2+} \)-induced inactivation of the \( \text{Ca}^{2+} \) channels (233). Thus the small window current that might exist would be wiped out by many bronchoconstrictors. Surprisingly, \( \beta \)-agonists have been shown to augment L-type \( \text{Ca}^{2+} \) currents in ASM (242) even though they are powerful relaxants.

Finally, and perhaps most importantly, clinical studies have found \( \text{Ca}^{2+} \) channel blockers to be ineffective as therapeutic agents in asthma (14, 65, 76, 86, 162, 189, 202).

This begs the question: Why does ASM exhibit such large \( \text{Ca}^{2+} \) and \( \text{Cl}^- \) currents? We will propose several answers to this question in this review.

Do \( \text{K}^+ \) channels play a major role in agonist-evoked relaxations? In general, \( \text{K}^+ \) channels are divided into four major classes: \( \text{Ca}^{2+} \)-dependent (\( \text{K}_{\text{Ca}} \)), voltage dependent (\( \text{K}_V \)), ATP dependent (\( \text{K}_{\text{ATP}} \)), and inward rectifier (\( \text{K}_{\text{IR}} \)) (142, 169, 187), of which there is substantial direct evidence in ASM for two of these classes and very limited evidence for the other two classes.

McCann and Welsh (157) were the first to directly record \( \text{K}_{\text{Ca}} \) in ASM, and there have since been innumerable studies that add to this evidence. These channels are generally of the large conductance subtype because they are highly sensitive to \( \text{K}^+ \) channel blockers such as TEA, charybdotoxin, and iberiotoxin, with very little effect of the small conductance blocker apamin (28, 141, 165, 207, 208). Also, direct patch-clamp recordings show these channels to have unitary conductances of several hundred picoamperes (207, 208, 216). One of these studies (216) further found that these channels can undergo random conformational changes that lead to subconductance states of 17, 33, 41, 52, 63, and 72% of the full conductance. These currents can appear to be very “noisy” with chaotic oscillations and spikes, in part due to marked spatial/temporal changes in \( [\text{Ca}^{2+}]_i \) and the large unitary conductance of these channels.

\( \text{K}_V \) currents in ASM have also been studied in detail at the whole cell (69, 141) and single channel (28) levels. Activation of these channels ensues after somewhat of a delay (28) (thus they are also referred to as “delayed rectifier” currents) and occurs much more “smoothly” than \( \text{K}_{\text{Ca}} \) given their smaller unitary conductance (10–15 pS) and insensitivity to \( [\text{Ca}^{2+}]_i \), relative to \( \text{K}_{\text{Ca}} \). The channels also exhibit voltage-dependent inactivation, which is roughly half-maximal at the resting membrane potential. \( \text{K}_V \) channels are effectively blocked by 4-aminopyridine (1–5 mM) or dantrolin (1–100 nM) but not by TEA, charybdotoxin, or glybenclamide unless unreasonably high concentrations of these agents are used (169).

Although there are many studies providing indirect evidence for \( \text{K}_{\text{ATP}} \) in ASM (in that relaxations are evoked by \( \text{K}_{\text{ATP}} \) agonists such as chromakalim) and these mechanical responses are antagonized by \( \text{K}_{\text{ATP}} \) blockers such as glybenclamide (21, 22, 36, 42, 43, 97, 131, 169, 178), direct electrophysiological evidence for these channels is essentially nonexistent. Many groups that have characterized \( \text{K}^+ \) currents in ASM in detail directly using patch-clamp techniques have not reported a glybenclamide-sensitive component. Rather than an action on some channel per se, some evidence suggests that \( \text{K}_{\text{ATP}} \) agonists act instead by suppressing phosphodiesterase activity (178, 203).

Dozens of studies of ASM cells from the larger airways have failed to identify any inward rectifier \( \text{K}^+ \) currents. However, one recent study (208) of cells obtained from small human bronchioles (outer diameter 0.3–1.0 mm) has done so. The physiological relevance of this possible regional heterogeneity is unclear.

General dogma has it that relaxants act by opening \( \text{K}^+ \) channels and hyperpolarizing the membrane. However, again, several lines of evidence speak to the contrary.

For example, bronchodilators such as \( \beta \)-agonists and nitric oxide can still evoke substantial or even complete relaxation in the presence of \( \text{K}^+ \) channel blockers (6, 7, 45, 102, 116, 127, 128, 165, 219) (Fig. 5). Although a rightward shift in the concentration-response relationship for the bronchodilator is sometimes seen (127, 128, 163), this should be interpreted carefully. Such a parallel shift is a hallmark of competitive inhibition, yet the \( \text{K}^+ \) channel blocker and bronchodilator agonist are not competing at a common receptor, and one should not expect that stimulating the receptor more aggressively (by using higher concentrations of agonist) would displace the blocker from the channel and thus unmask the relaxation. Instead, it may be that the \( \text{K}^+ \) channel blockers are depolarizing nerve endings in the tissues, causing them to release excitatory agonists (97, 132) that then antagonize the bronchodilator response. Such functional antagonism can be
overcome by using higher concentrations of bronchodilator agonist (64, 110, 185). Tetrodotoxin is not a guarantee against this because it only prevents depolarization caused by Na\textsuperscript{+} channel activation but not that caused by suppression of outward K\textsuperscript{+} currents in the nerve endings. Instead, agents such as \textomega-conotoxin should be used to prevent the subsequent Ca\textsuperscript{2+} influx and neurotransmitter release.

Also, bronchodilators can often cause relaxations when voltage-dependent Ca\textsuperscript{2+} influx has already been abolished beforehand using Ca\textsuperscript{2+} channel blockers (22, 102, 130, 218). Others have found that relaxations are not evoked by artificially imposed hyperpolarizing current (39, 48); again, bronchoconstrictors can still evoke substantial contraction during voltage clamp at resting membrane potentials (117, 118) (Fig. 3).

Clearly, then, membrane hyperpolarization alone is neither necessary nor sufficient for relaxation in ASM. Consistent with this, K\textsuperscript{+} channel openers have been found to be ineffective as a therapy for asthma (41, 62, 135, 204). Thus a better understanding of agonist-evoked relaxation demands a new emphasis on mechanisms other than K\textsuperscript{+} channel activation. Many (perhaps all) bronchodilators that activate K\textsuperscript{+} channels are also known to exert other effects on ASM, including decreased Ca\textsuperscript{2+} sensitivity of the contractile apparatus (126, 174, 209, 210, 212), inhibition of D-myoinositol 1,4,5-trisphosphate (IP\textsubscript{3}) binding to its receptor on the SR (196), suppression of IP\textsubscript{3}-induced Ca\textsuperscript{2+} release (133, 241), and enhancement of Ca\textsuperscript{2+} uptake/extrusion (133).

EC coupling in ASM owes much more to voltage-independent mechanisms. In contrast to the questionable significance of membrane voltage-regulated Ca\textsuperscript{2+} influx, a number of voltage-independent mechanisms are primarily responsible for contraction in ASM.

The most widely recognized involves the release of Ca\textsuperscript{2+} sequestered within the SR. Cholinergic agonists, histamine, endothelin, leukotrienes, and thromboxane A\textsubscript{2} activate phospholipase C, which, in turn, generates the second messengers diacylglycerol and IP\textsubscript{3} (32–34). The latter of these two messengers activates Ca\textsuperscript{2+}-permeable ion channels on the membrane of the SR, releasing its store of Ca\textsuperscript{2+} and, thereby, triggering contraction (212). The SR also expresses another group

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**Fig. 5.** Substantial relaxation can still be evoked during blockade of K\textsuperscript{+} channels. Relaxations were evoked by the NO donor soluble \textomega-ethylmaleimide-sensitive factor attachment protein (SNAP) in ASM tissues pretreated with the K\textsuperscript{+} channel blockers charybotoxin (ChTx; 0.1 \textmu M), TEA (30 mM), or 4-AP (1 mM). These data suggest that K\textsuperscript{+} channels are not absolutely necessary for NO-evoked relaxation. [From Janssen et al. (116).]
of Ca\(^{2+}\)-permeable ion channels that are activated by Ca\(^{2+}\) itself, caffeine, ryanodine, or by cyclic ADP ribose (71, 201, 209, 212). The physiological role of these “ryanodine receptors” is still debated (see Bronchodilators: is K\(^{+}\) channel activation a causal event or an epiphenomenon?). Refilling of the SR involves primarily the sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA). However, the existence of at least one other novel refilling pathway has been suggested (25, 26, 118, 186) as summarized in Voltage-dependent Ca\(^{2+}\) channels and SR refilling.

More recently, a great deal of attention has been focused on agonist-induced changes in the sensitivity of the contractile apparatus to [Ca\(^{2+}\)]. One such mechanism is that gaining a great deal of momentum both in the vascular and ASM fields involves activation of the monomeric G protein Rho, which in turn translocates to the membrane and activates Rho kinase (31, 98, 124, 253). The latter phosphorylates and thereby inactivates MLCP, leading to a net accumulation of phosphorylated myosin light chains, and thus contraction (211). Others are investigating mechanisms such as extracellular regulated kinase (ERK)-mediated phosphorylation of caldesmon and calponin (72, 75), integrin-mediated tyrosine phosphorylation of focal adhesion kinase, paxillin, and talin (73, 159, 160, 177, 221, 223, 240), and protein kinase C activation (2, 29, 93). Endothelin-stimulated activation of ERK was recently shown to be dependent on Ca\(^{2+}\) influx (232), indicating some “cross talk” between Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent pathways.

The superficial buffer barrier is another interesting topic. The superficial buffer barrier model is shown in Fig. 6. The saroplastic reticulum (SR) divides the cytosol into two spatially and functionally distinct compartments: the subplasmalemmal space and the deep cytosol. Bottom left: excitatory agonists release Ca\(^{2+}\) into the deep cytosol to trigger contraction, as well as into the subplasmalemmal space to activate Ca\(^{2+}\)-dependent Cl\(^{-}\) channels. Bottom right: relaxants, on the other hand, promote Ca\(^{2+}\) uptake and Ca\(^{2+}\) extrusion but also trigger Ca\(^{2+}\) release via ryanodine receptors. The latter effect allows for unloading of the SR, increasing its buffering capacity, without triggering contraction. It may also lead to activation of Ca\(^{2+}\)-dependent K\(^{+}\) channels and membrane hyperpolarization.
Ca\(^{2+}\) release preferentially into the peripheral space (and, thereby, activate ion channels), and the contractile apparatus only becomes activated when this release is so massive that it "spills over" into the deep space. It can also explain how subnanomolar concentrations of acetylcholine can evoke substantial ionic current but without any change in tension (124). That is, the ionic currents indicate that Ca\(^{2+}\) is in fact being released (because they are totally dependent on that process) (108, 117, 118, 121, 143, 238) although this release cannot be discerned using fluorimetric techniques that measure the average change in \([\text{Ca}^{2+}]_i\) throughout the entire cell (124, 198, 200, 249). However, the anticipated mechanical response is quelled by the barrier function of the SR. Thus it is essential to use more refined Ca\(^{2+}\) imaging techniques, ones that can resolve subcellular regions, for further studies of Ca\(^{2+}\) handling in ASM.

Another change in our understanding of Ca\(^{2+}\) handling was stimulated by advances made in other cell types using highly sophisticated Ca\(^{2+}\) imaging equipment. In the ASM field, many focus their attention on the magnitude of the solitary, brief, spikelike Ca\(^{2+}\) transient evoked by a high concentration of agonist and relate that to much more persistent cellular events, such as contraction. In other words, this Ca\(^{2+}\) transient is being interpreted as a persistent elevation of \([\text{Ca}^{2+}]_i\), throughout the entire cell. Instead, the sustained changes in \([\text{Ca}^{2+}]_i\), evoked by lower concentrations of agonist might be more physiologically relevant. It is now known that agonists can evoke recurring changes in \([\text{Ca}^{2+}]_i\), ("oscillations"), which propagate throughout the cell ("waves") (225), and are further refined or shaped as they progress through the cytosol (139). These oscillations convey information within their amplitudes (peak height as well as their mean or temporally averaged amplitude) as well as in their frequency, and this information may be decoded by Ca\(^{2+}\)/calmodulin-dependent kinase (19, 20, 30, 35, 50, 52, 55, 184), MLCK (50), the SR Ca\(^{2+}\) pump (50), calpain (229), adenyl cyclase (44), or mitochondria (79). For example, gene expression of several proinflammatory cytokines is differentially regulated by Ca\(^{2+}\) oscillations in a frequency- and amplitude-dependent fashion (54, 146). Ca\(^{2+}\) oscillations have been reported in ASM from the human (56), rat (193, 194, 227), guinea pig (117, 200), pig (171, 180–183), and dog (117), but their underlying mechanism and their physiological relevance are still poorly understood.

Finally, a great deal of work has been done in the cardiac and vascular smooth muscle fields looking at Ca\(^{2+}\) sparks: small and transient elevations of \([\text{Ca}^{2+}]_i\), produced by localized bursts of Ca\(^{2+}\) from a handful of ryanodine receptors (23, 82, 103, 104, 168). These are proposed to be the triggers for spontaneous transient ion currents, which, in turn, are believed to modulate mechanical activity (168, 254). They also represent the fundamental event underlying Ca\(^{2+}\) oscillations and relaxations in vascular smooth muscle (168). There have been limited studies of sparks in ASM (172, 173, 199, 254), but their physiological role(s) is unclear. Although they may lead to activation of K\(^+\) and Cl\(^-\) channels (254), electromechanical coupling is of limited importance in ASM. Instead, their primary function may be to discharge the SR contents, without evoking contraction, toward the plasmalemmal Ca\(^{2+}\) pump to increase the buffering capacity of the SR.

Thus Ca\(^{2+}\) signals are organized into complex temporal and spatial patterns that are lost using techniques and models that focus solely on the spikelike elevation averaged across the entire ASM cell. Many previous reports of Ca\(^{2+}\) responses in ASM were severely limited, because whole cell photometry was used: this approach averages the changes in \([\text{Ca}^{2+}]_i\) across the entire cell. None have yet compared bronchoconstrictor-induced changes in the deep cytosol vs. the subplasmalemmal space. Also, the sampling rate of most of these studies (on the order of 1 Hz) was much too slow to adequately resolve events with time courses on the millisecond scale, such as Ca\(^{2+}\) oscillations and Ca\(^{2+}\) sparks (199). Finally, the few studies able to resolve subcellular regions generally used only a maximally effective concentration of excitatory agonist: we have previously obtained evidence (using patch-clamp recordings) that concentrations of acetylcholine that were subthreshold for mechanical or fluorimetric responses nonetheless evoked substantial membrane currents (124), suggesting important differences between global and subplasmalemmal measurements of \([\text{Ca}^{2+}]_i\). The concentration of agonist used is also important with respect to the likelihood of observing Ca\(^{2+}\) oscillations, since models of these phenomena indicate a critical dependence on variables such as \([\text{IP}_3]\) and basal \([\text{Ca}^{2+}]_i\) (199, 225).

NOVEL IONIC MECHANISMS

Voltage-dependent Ca\(^{2+}\) channels and SR refilling. Several studies show voltage-dependent Ca\(^{2+}\) channels in ASM to be important for refilling and maintenance of the SR (25, 26, 118, 149, 186). For example, we used agonist-evoked Cl\(^-\) currents to assess the filling state of the SR, and we found that we could completely deplete the SR using cyclopiazonic acid (SERCA inhibitor) and then refill the SR in a dihydropyridine-sensitive fashion using a series of depolarizing pulses (Fig. 7) (118). More surprisingly, though, this refill occurred in the maintained presence of cyclopiazonic acid (which was completely sufficient to functionally deplete the SR), suggesting that this refilling pathway did not involve SERCA. In other words, Ca\(^{2+}\) was crossing the plasmalemma and entering the SR without being pumped by the Ca\(^{2+}\)-ATPase.

These paradoxical findings can be explained using a recently proposed model that describes a physical interaction between the SR and plasmalemmal store-operated Ca\(^{2+}\) channels (18, 138, 176, 190, 191, 252). Briefly, agonist-induced depletion of the internal store triggers activation of tyrosine kinase(s), Ras, and reorganization of the cytoskeleton in such a way that it directly couples IP\(_3\) receptors on the SR with Ca\(^{2+}\) channels on the plasmalemma. Similarly, a functional
interaction between voltage-dependent Ca\textsuperscript{2+} channels and the nucleus has recently been described. Dolmetsch et al. (53) found that gene transcription could be stimulated by Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channels but not through N- or P/Q-type Ca\textsuperscript{2+} channels (which are also present but seem to contribute to other physiological responses in these cells). Their data show that calmodulin, which is tethered at the mouth of the L-type Ca\textsuperscript{2+} channels and is activated by Ca\textsuperscript{2+} influx through them, signals to the nucleus via a mitogen-activated protein kinase pathway (53).

Several observations made in ASM are consistent with a physical coupling between plasmalemmal Ca\textsuperscript{2+} channels and SR membranes. First, excitatory stimulation of ASM is accompanied by activation of tyrosine kinases (206, 228) and Ras/Rho (58, 80, 83, 226) as well as cytoskeletal rearrangement (78, 83, 226). Second, inhibition of tyrosine kinases compromises SR refilling (151). Third, ASM depleted of focal adhesion kinase (which regulates cytoskeleton stability) shows marked suppression of acetylcholine-evoked Ca\textsuperscript{2+} transients and contractions as well as changes in voltage-dependent Ca\textsuperscript{2+} channel function without any disruptive changes in the contractile apparatus per se (assessed by addition of Ca\textsuperscript{2+} to permeabilized strips) (222). Fourth, our observation that the SR can be refilled by voltage-dependent Ca\textsuperscript{2+} influx in the maintained presence of cyclopiazonic acid is difficult to explain otherwise, because there is no other Ca\textsuperscript{2+} pump on the SR other than SERCA.

At first glance, this novel model of SR refilling also suffers from the criticism raised earlier in this review that membrane potential rarely reaches the threshold for opening of the voltage-dependent Ca\textsuperscript{2+} channels. However, if there is a very close apposition or even physical coupling between the plasmalemmal and SR membranes, it is unclear what transmembrane potentials the plasmalemmal Ca\textsuperscript{2+} channels would experience. That is, the close proximity and physical interaction of a large polypeptide (the IP\textsubscript{3} receptor) and the SR membrane with the inner face of the Ca\textsuperscript{2+} channel, with the resultant changes in membrane surface charge and/or induction of conformational changes in the Ca\textsuperscript{2+} channel, could easily alter their voltage characteristics, allowing them to open at physiologically relevant potentials (167). Also, access of Ca\textsuperscript{2+} and of protein kinase C to the inner face of the Ca\textsuperscript{2+} channels might be hindered during this interaction, thereby preventing Ca\textsuperscript{2+} channel inactivation.

**Bronchodilators: is K\textsuperscript{+} channel activation a causal event or an epiphenomenon?** The traditional view has been that bronchodilators act by decreasing [Ca\textsuperscript{2+}], throughout the cell. Surprisingly, however, we and others have described elevations in [Ca\textsuperscript{2+}], in response to relaxant agents (63, 115, 116, 245). Yamaguchi et al. (245) resolved the effects of isoproterenol on [Ca\textsuperscript{2+}] in greater detail and showed that it increases [Ca\textsuperscript{2+}] in the peripheral regions of the ASM cells and decreases it in their more central regions. One interpretation of these findings is that bronchodilators act by triggering Ca\textsuperscript{2+} release from the SR, completely contrary to current dogma, but not in the same fashion as bronchoconstrictors. That is, rather than elevating [Ca\textsuperscript{2+}], globally throughout the cell (via IP\textsubscript{3}-induced Ca\textsuperscript{2+} release), the data suggest that ryanodine receptors are involved and direct Ca\textsuperscript{2+} into the subplasmalemmal space where it is extruded from the cell by the plasmalemmal Ca\textsuperscript{2+}-ATPase. In the process, Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channels may or may not be activated. Although extensive evidence has been given for such a mechanism in vascular smooth muscle (103, 105, 137, 168), this model has not been examined in ASM. However, there has been one report of K\textsuperscript{+} currents activated by Ca\textsuperscript{2+} sparks in ASM (although relaxants were not used in this study) (254), and the isoproterenol-induced elevation of [Ca\textsuperscript{2+}], was shown to involve ryanodine receptors (245). Isoproterenol (171), cAMP (171), and cGMP (37) suppress the frequency of cholinergic Ca\textsuperscript{2+} oscillations in ASM.

It appears, then, that bronchodilators simultaneously trigger uptake of Ca\textsuperscript{2+} from the deep cytosol into the SR as well as release of SR Ca\textsuperscript{2+} into the subplasmalemmal space, followed by extrusion of Ca\textsuperscript{2+} from that peripheral space into the extracellular space (Fig. 6). Our proposal that relaxants stimulate Ca\textsuperscript{2+} release might be counterintuitive, but it should be expected. The cells must be able to discharge internally sequestered Ca\textsuperscript{2+}, but without triggering contraction, to increase/maintain the Ca\textsuperscript{2+}-buffering capacity of the
SR. Data presented in an earlier study (108) suggest that ryanodine receptors are involved and that this Ca$^{2+}$ release is preferentially directed into the subplasmalemmal space. The important point to be made in all of this is that K$^+$ channels appear to be more like bystanders than key players in the process of relaxation.

What role do plasmalemmal Cl$^-$ channels play in ASM physiology? There is substantial electrophysiological evidence for a population of Cl$^-$ channels activated during excitatory stimulation (81, 106, 117, 119, 120, 147, 148, 237, 238). These channels exhibit a small unitary conductance (far below 20 pS) (121), and their activation is Ca$^{2+}$ dependent (via G$q$/G$11$-stimulated release of internal Ca$^{2+}$) (238) but voltage independent (121). Moreover, these inactivate in a voltage-sensitive fashion (121) via phosphorylation by Ca$^{2+}$/calmodulin-dependent kinase II (237) (which is in turn triggered by the elevation of [Ca$^{2+}$], that activated the channels in the first place).

Given that excitatory stimulation is generally associated with such large Cl$^-$ currents that can depolarize the membrane and are tightly regulated by second messenger signaling events (121, 237, 241, 254), much as is the case in vascular smooth muscle, it is easy to conclude that the Cl$^-$ currents play a key role in contraction of ASM by depolarizing the membrane and thus triggering voltage-dependent Ca$^{2+}$ influx. However, the truth of the matter is that agonist-evoked contractions are not affected by Cl$^-$ channel blockers (Fig. 2). Also, given that the equilibrium potential for Cl$^-$ is approximately $-40$ to $-30$ mV (4, 5), activation of Cl$^-$ channels will indeed depolarize the membrane but will essentially clamp it at potentials barely sufficient for activation of voltage-dependent Ca$^{2+}$ channels. Again, it bears repeating that substantial contractions can still be evoked during voltage clamp at very negative potentials (118) (Fig. 3), indicating that depolarization is not necessary for contraction anyway. Why, then, are Cl$^-$ currents so prominent in ASM?

Recently, another type of Cl$^-$ channel has been isolated from ASM with properties diametrically opposite to those described above (195). That is, they have a large unitary conductance (several hundred picosiemens) and their activation is voltage dependent but Ca$^{2+}$ independent. All of these properties are similar to those of the Cl$^-$ channels present on the SR of skeletal and cardiac muscle and facilitate Ca$^{2+}$ flux by neutralizing charge buildup on the SR membranes (3).

This finding prompts us to propose an entirely novel and testable hypothesis: that agonists activate Cl$^-$ currents in the plasmalemma of the ASM cell to facilitate Ca$^{2+}$ release/uptake. That is, Ca$^{2+}$ efflux from the SR leads to a net negative charge on the inner face of the SR membrane that hinders Ca$^{2+}$ release (Fig. 8A) unless alleviated by compensatory fluxes of Cl$^-$ out of...
the SR (Fig. 8B) (134, 179). However, the accumulation of Cl⁻ outside the SR opposes further Cl⁻ efflux from the SR (and thus Ca²⁺ release; Fig. 8B). A sudden opening of Cl⁻ channels on the plasmalemma, with subsequent loss of Cl⁻ from the subplasmalemmal space, would instantaneously alter the equilibrium potential for Cl⁻ across the SR membrane, thereby boosting efflux of Cl⁻ (and Ca²⁺) from the SR (Fig. 8C). Consistent with this, we have noted anecdotally that agonist-evoked membrane currents and contractions were lost in cells studied using a low internal [Cl⁻] solution (20 mM) (117).

Voltage-independent Ca²⁺ influx pathways? Agonists activate a membrane conductance that is nonselective for various monovalent cations (117, 235, 239). It is unclear whether this conductance is Ca²⁺ permeable and, thereby, serves as a source of Ca²⁺ for contraction as is the case in vascular and gastrointestinal smooth muscle (51, 90, 94). Elevation of [Ca²⁺]i is necessary but not sufficient for activation of these nonselective cation channels; instead, they are activated by Gα/Gβγ (activated by M₂ muscarinic or H₁ histaminergic receptors). While the Cl⁻ current that is concurrently activated by these agonists decays within seconds, the nonselective cation channels remain open as long as the agonists are present, resulting in a persistent non-inactivating inward current.

**IONIC MECHANISMS IN ASM PATHOPHYSIOLOGY**

Inflammation plays a central role in asthma and airway hyperreactivity, and it is becoming increasingly clear that cytokines and inflammatory mediators exert a variety of effects on various aspects of ASM function. Several researchers have sought to examine whether ionic mechanisms are altered in asthma or in animal models of airway hyperresponsiveness. For example, it might be possible that membrane potentials are higher or Ca²⁺ currents greater in tissues/cells from asthmatics or hyperresponsive animals, as can happen for vascular smooth muscle cells and hypertension (150, 217). However, immunological stimulation of excised guinea pig tracheal tissues causes first a small and transient membrane depolarization, followed by a marked and prolonged membrane hyperpolarization (213, 215). In excised tissues exposed to allergen in vivo, membrane potentials were slightly more hyperpolarized (<5 mV) when the guinea pigs had been acutely sensitized to allergen but markedly depolarized (by >10 mV) when the animals had been chronically exposed to allergen (156, 214). While canine ASM is normally very polarized (resting potentials of approximately −60 mV) and does not show spontaneous phasic electrical activity, ASM from dogs with “aspirin-induced asthma” exhibited marked membrane depolarization and slow wave activity (101). This might be related to the suppression of delayed rectifier K⁺ current (more specifically, enhanced inactivation of the channels), which is reported to occur in allergen-sensitized canine bronchial smooth muscle (234). Despite all these observed changes in electrophysiological activity in vitro, their significance to airway physiology/pathophysiology is unclear given that electromechanical coupling is relatively unimportant in ASM and that Ca²⁺ channel blockers and K⁺ channel openers are generally ineffective in the treatment of asthma in the clinical setting (14, 41, 62, 65, 76, 86, 135, 162, 189, 202, 204).

On the other hand, asthma and airway hyperresponsiveness might be associated with changes in Ca²⁺ handling. Perhaps basal levels of [Ca²⁺]i are higher or Ca²⁺ release is greater. Several proinflammatory cytokines such as interleukin-1β, tumor necrosis factor-α, interferon-γ, platelet-derived growth factor, and eosinophil major basic protein markedly augment excitatory agonist-evoked Ca²⁺ transients (9, 10, 12, 244, 248), phosphoinositide turnover (10, 248), and contractions (11, 154, 175, 244). In some cases, these effects involve mitogen-activated protein kinase cascades (ras, raf, MEK, Rho, Rho kinase) and induction of gene expression, protein synthesis, and proliferation (10, 88, 89, 226).

Oxidizing pollutants such as ozone and acrolein induce airway hyperreactivity. Although these act in part through inflammatory cells, they can also alter EC coupling and other cellular events in isolated ASM cells or tissues (15, 16, 91, 92, 153, 192). This direct action on the ASM per se seems to involve changes in inositol phosphate metabolism and Ca²⁺ handling, with induction or augmentation of Ca²⁺ oscillations (91, 92, 192, 194).

**FUTURE DIRECTIONS**

On the basis of the arguments laid out in this review, we propose the following recommendations in future studies of ASM physiology.

*Ionic mechanisms.* It seems that too many groups are studying ionic mechanisms in ASM in the same fashion as they would a vascular or gastrointestinal smooth muscle preparation. There has been far too much emphasis on voltage-dependent mechanisms, to an extent that is not warranted by clinical studies of Ca²⁺ channel blockers or K⁺ channel agonists. As outlined above, ASM is distinct from skeletal, cardiac, and vascular smooth muscle in many respects pertaining to EC coupling, and a great deal remains to be learned about its unique physiology and pathophysiology. There needs to be greater consideration of other roles for the ion channels that do not depend on, or relate to, electromechanical coupling.

*Ca²⁺ handling.* The hypothesis that Ca²⁺ may play an important causal role in airway hyperreactivity and asthma was proposed more than two decades ago (161, 230) but still has not been explored in sufficient detail. We believe this has resulted in an overly simplistic model of Ca²⁺ handling in ASM and an imbalanced understanding of electromechanical coupling mechanisms, as outlined above. A breakdown in the superficial buffer barrier and/or changes in Ca²⁺ sparks/Ca²⁺ oscillations may be more relevant to airway hyperreactivity than increases in basal [Ca²⁺]i, or peak magnitudes of agonist-evoked Ca²⁺ transients.

AJP-Lung Cell Mol Physiol • VOL 282 • JUNE 2002 • www.ajplung.org
EC coupling. A great deal more attention needs to be focused on recently discovered EC coupling mechanisms in ASM, particularly Rho/Rho-activated kinase-mediated regulation of MLCP as well as pathways directed at thin filaments. Moreover, most studies use only a maximally effective concentration of agonist in their studies. The full range of agonist concentrations should be examined, from subthreshold to maximally effective concentrations, since there is now evidence that different EC coupling mechanisms may contribute to differing degrees depending on the level of excitation (124, 194). Also, experimental strategies and tools need to be wielded with a greater degree of sophistication. For example, we should be increasingly wary of the use of micromolar concentrations of dihydropyridine blockers, or of contractions as an index of \([Ca^{2+}]_i\), or of focusing almost exclusively on the temporally and spatially averaged spikelike elevations of \([Ca^{2+}]_i\) evoked by a maximally effective concentration of agonist. Finally, the vast majority of studies of airway function are done using tracheal tissues/cells. A greater use of smaller airways is advocated because evidence is accumulating for marked regional differences across the airway tree (47, 124, 208).

In summary, a more imaginative approach to the study of EC coupling in ASM, one that does not lean so heavily on electromechanical mechanisms, could lead to major advances in our understanding of the unique physiology (and pathophysiology) of ASM.

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