Ca\(^{2+}\) Sparks Act as Potent Regulators of Excitation-Contraction Coupling in Airway Smooth Muscle

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Ca\(^{2+}\) sparks are short lived and localized Ca\(^{2+}\) transients resulting from the opening of ryanodine receptors in sarcoplasmic reticulum. These events relax certain types of smooth muscle by activating big conductance Ca\(^{2+}\)-activated K\(^{+}\) channels to produce spontaneous transient outward currents (STOCs) and the resultant closure of voltage-dependent Ca\(^{2+}\) channels. But in many smooth muscles from a variety of organs, Ca\(^{2+}\) sparks can additionally activate Ca\(^{2+}\)-activated Cl\(^{-}\) channels to generate spontaneous transient inward current (STICs). To date, the physiological roles of Ca\(^{2+}\) sparks in this latter group of smooth muscle remain elusive. Here, we show that in airway smooth muscle, Ca\(^{2+}\) sparks under physiological conditions, activating STOCs and STICs, induce biphasic membrane potential transients (BiMPTs), leading to membrane potential oscillations. Paradoxically, BiMPTs stabilize the membrane potential by clamping it within a negative range and prevent the generation of action potentials. Moreover, blocking either Ca\(^{2+}\) sparks or hyperpolarization components of BiMPTs activates voltage-dependent Ca\(^{2+}\) channels, resulting in an increase in global [Ca\(^{2+}\)] and cell contraction. Therefore, Ca\(^{2+}\) sparks in smooth muscle presenting both STICs and STOCs act as a stabilizer of membrane potential, and altering the balance can profoundly alter the status of excitability and contractility. These results reveal a novel mechanism underpinning the control of excitability and contractility in smooth muscle.

Ca\(^{2+}\) sparks, which are highly localized, short lived Ca\(^{2+}\) transients due to the opening of ryanodine receptors (RyRs) in sarcoplasmic reticulum, play pivotal roles in a variety of cellular functions and may contribute to an array of diseases when compromised (1–3). In vascular, gastric, ureteral, and bladder smooth muscle, Ca\(^{2+}\) sparks activate nearby big conductance Ca\(^{2+}\)-activated K\(^{+}\) (BK) channels in the plasma membrane to generate spontaneous transient outward currents (STOCs) (4–7). STOCs hyperpolarize the membrane and turn off pre-activated voltage-dependent Ca\(^{2+}\) channels (VDCCs), leading to the relaxation of smooth muscle (4, 6). Knock-out of either the pore-forming BK \(\alpha\) subunit or the auxiliary BK \(\beta\) subunit results in elevated blood pressure or overactive bladder in mice (8–10). A weaker coupling between Ca\(^{2+}\) sparks and STOCs has also been linked to angiotensin II-induced hypertension (11) and diabetic retinopathy (12) in animal models.

In many smooth muscles from a variety of organs, however, Ca\(^{2+}\) sparks, in addition to activating STOCs, turn on Ca\(^{2+}\)-activated Cl\(^{-}\) (C\(_{\text{ic}}\)) channels to generate spontaneous transient inward currents (STICs) (7, 13–17). Because \(E_{\text{C}_{\text{ic}}}\) in smooth muscle is less negative than resting membrane potential (RMP), the activation of STICs by Ca\(^{2+}\) sparks is expected to depolarize the membrane, an opposite effect to that of STOCs. But the precise effect of Ca\(^{2+}\) sparks on membrane potential in this class of smooth muscle has not been experimentally determined nor has the physiological consequence of changes in membrane potentials caused by Ca\(^{2+}\) sparks. Therefore, a major unsolved question is the physiological function of Ca\(^{2+}\) sparks in smooth muscle cells that possess both STOCs and STICs.

In this study, we used airway smooth muscle (ASM), a prototypical smooth muscle exhibiting STOCs and STICs (7, 14, 18), to explore the physiological function of Ca\(^{2+}\) sparks by directly measuring Ca\(^{2+}\) sparks, membrane potential, global intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{i}}\)), and contractile state. We demonstrated that Ca\(^{2+}\) sparks under physiological conditions induce biphasic membrane potential transients (BiMPTs), leading to membrane potential oscillations. BiMPTs clamp membrane potential within a negative range and prevent the generation of action potentials, thus serving as a potent mechanism to maintain a low excitability of the cells. Strikingly, the reagents that block Ca\(^{2+}\) sparks or upset the balance of BiMPTs can depolarize the membrane and activate VDCCs, resulting in an increase in global [Ca\(^{2+}\)]. Therefore, Ca\(^{2+}\) sparks and BiMPTs function as powerful regulators of membrane excitability and contractility in smooth muscle.

**EXPERIMENTAL PROCEDURES**

**Cell Isolation**—Male Swiss Webster mice (4–6 weeks) were euthanized with intraperitoneal injection of a lethal dose of sodium pentobarbital (50 mg kg\(^{-1}\)). After each animal was unresponsive to applied stimulus, the trachea was quickly removed and placed in pre-chilled dissociation solution consisting of (in mM) the following: 136 NaCl, 5.36 KCl, 0.44...
The difference between maximum potential and on-set potential. SM is expressed in terms of the number of BiMPTs (open triangles) phase; 2) the endogenous fixed Ca\(^{2+}\)/H\(_{9262}\) sparks and their evoked membrane potential transients. A, images of approximately one-third of a cell display the spatiotemporal evolution of a single Ca\(^{2+}\) spark. The cell was current-clamped without injecting current. The reversal potentials for Cl\(^{-}\) and K\(^{+}\) were set at −15 and −80 mV, respectively, in accordance with the concentration gradient of these two ions in smooth muscle under physiological conditions (28). The images were acquired at a rate of 100 Hz with an exposure time of 3 ms. Cytosolic Ca\(^{2+}\) was measured using fluo-3 (50 \(\mu\)M), which was introduced into the cell in the K\(^{+}\) form through the patch pipette. Changes in Ca\(^{2+}\) concentration in the images are expressed as \(\Delta F/F_0\) (%) and displayed on a pseudocolor scale calibrated at the right of images. Letters above the images correspond to the letters in the top panel of B and indicate the time at which the images were obtained. B, change in fluorescence (panel i) at the epicenter pixel of the spark shown in A, and its SM (panel ii), \(I_{Ca\text{spark}}\) (panel iii), i.e. Ca\(^{2+}\) current flowing from the intracellular Ca\(^{2+}\) store into the cytosol during the spark, and the corresponding change in membrane potential (MP) (panel iv). Note the following: 1) the membrane potential transient is biphasic with a hyperpolarization phase followed by a depolarization phase, so it is designated as BiMPT; 2) the endogenous fixed Ca\(^{2+}\) buffer (i.e. 81 \(\mu\)M with a K\(_0\) of 0.66 \(\mu\)M) as estimated in the same type of cells by Bao et al. (13) was taken into account in this and following calculations of signal mass and \(I_{Ca\text{spark}}\), C, panel i, no or weak correlation exists between Ca\(^{2+}\) SM and BiMPT amplitude (red open circles, \(r = 0.0198\) and \(p = 0.8804\) for SM versus hyperpolarization phase; blue open triangles, \(r = 0.3410\) and \(p = 0.007\) for SM and depolarization phase, and between SM and on-set potential of BiMPTs (black open squares, \(r = 0.1647\) and \(p = 0.2085\)). The amplitude of hyperpolarization phase equals the difference between on-set potential and minimum potential, and the amplitude of depolarization phase the difference between maximum potential and on-set potential. SM is expressed in terms of the number of Ca\(^{2+}\) ions liberated during Ca\(^{2+}\) sparks. \(n = 60\) for both panels i and ii. Panel ii, lack of correlation between \(I_{Ca\text{spark}}\) and BiMPT amplitude (red open circles, \(r = -0.0686\) and \(p = 0.602\) for \(I_{Ca\text{spark}}\) versus hyperpolarization phase; blue open triangles, \(r = 0.234\) and \(p = 0.0719\) for \(I_{Ca\text{spark}}\) versus depolarization phase), and between \(I_{Ca\text{spark}}\) and on-set potential of BiMPTs (black open squares, \(r = -0.0239\) and \(p = 0.8563\)).

Measurement and Analysis of Ca\(^{2+}\) Sparks—Fluorescence images using fluo-3 as a calcium indicator were obtained using a custom-built wide field digital imaging system (7). Rapid imaging at 100 Hz (exposure, 3 ms) was made possible by equipping the system with a cooled high sensitivity, charge-coupled device camera developed in conjunction with the Lincoln Laboratory, Massachusetts Institute of Technology (Lexington, MA (7)). The camera was interfaced to a custom-made inverted microscope, and the cells were imaged using a \(\times60\) Nikon 1.4 KH\(_2\)PO\(_4\), 4.16 NaHCO\(_3\), 0.34 Na\(_2\)HPO\(_4\), 5 MgCl\(_2\), 20 Hepes, and 10 glucose (pH 7.1). The trachea was dissected free from the surface of connective tissues and incubated in the dissociation medium with 30 units/ml papain, 0.2 mM dithiothreitol, and 10 glucose (pH 7.1). The trachea was dissected free from the surface of connective tissues and incubated in the dissociation medium containing 3 units/ml collagenase 1A, 0.2 mg/ml Pronase E, 0.1 mg/ml DNase I, and 1 mg/ml bovine serum albumin. Finally, the tissue was agitated with a fire-polished wide bore glass pipette to release the cells. The isolated single cells were used on the day of isolation, and all the experiments were carried out at room temperature (22–25 °C).

Patch Clamp Recording and Analysis—Conventional or perforated whole-cell voltage clamp or current clamp recording was done with an Axopatch-1D amplifier or a HEKA EPC10 amplifier. The extracellular solution contained (in mm) the following: 130 NaCl, 5.5 KCl, 2.2 CaCl\(_2\), 1 MgCl\(_2\), and 10 Hepes, pH adjusted to 7.4 with NaOH. The pipette solution contained (in mm) the following: 75 KCl, 64 potassium aspartate, 1 MgCl\(_2\), 3 Na\(_2\)ATP, and 10 Hepes, pH adjusted to 7.3 with KOH. In conventional whole-cell clamp, 0.05 mm fluo-3 K\(_5\) was included in the patch pipette. For perforated patch technique, fluo-3 K\(_5\) was omitted, and 160 \(\mu\)g/ml amphotericin B was added in the patch pipette. Events were analyzed by the mini analysis program. To minimize the effect of leak conductance at the seal between the patch pipette and membrane, only cells with a seal resistance greater than >1 gigaohm were recorded and analyzed. The capacitance of these cells was 41.3 ± 1.5 picofarads (\(n = 27\)).

Because of oscillating nature of the membrane potential, all-point histograms were constructed using the segments of interest, and the mode values were taken as the RMP.

**Measurement of Global [Ca\(^{2+}\)]**—To monitor global cytosolic [Ca\(^{2+}\)]\(_i\), fura-2 fluorescence was measured using a custom-built multichannel microfluorimeter (20). Briefly, the system consisted of a Zeiss IM-35 inverted microscope (Nikon \(\times40\), 1.3NA) with a specially designed excitation path and photomultiplier tube (Thorn EMI type 9954A, Thorn EMI, Rockingham, MA), and a silicon photodiode array detector (PC 1280, PAR). The fura-2 solution contained 1% dimethyl sulfoxide and 0.05% pluronic F-127 in 1X Hank’s balanced salt solution (HBSS, in mm) 137 NaCl, 2.7 KCl, 10 Hepes, 6.7 glucose, 1M Mg\(^{2+}\), pH adjusted to 7.3 with KOH.
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**RESULTS**

Ca\(^{2+}\) Sparks Induce BiMPTs via Activating BK Channels and \(Cl_{ca}\) Channels under Physiological Conditions—Ca\(^{2+}\) sparks activate only STICs at \(E_{K}\), only STOCs at \(E_{Cl}\), and spontaneous transient outward and inward currents at potentials between \(E_{K}\) and \(E_{Cl}\) in ASM from mouse as they do in ASM from other species, and in the smooth muscle from other tissues (supplemental Fig. S1) (7, 14, 15, 17). To explore the role of Ca\(^{2+}\) sparks under physiological conditions, Ca\(^{2+}\) sparks and membrane potential were simultaneously measured with a combination of high speed fluorescence imaging and conventional whole-cell current clamp technology. At rest (i.e. without injection of current), spontaneous local Ca\(^{2+}\) transients were detected (Fig. 1A), and they were Ca\(^{2+}\) sparks because no transient was observed in the presence of 100 \(\mu M\) ryanodine. Estimated with signal mass (SM) methodology (19), the signal mass and peak Ca\(^{2+}\) current underlying these Ca\(^{2+}\) sparks (\(I_{Ca\text{(spark)}}\)) were 244,794 ± 24,818 Ca\(^{2+}\) ions and 3.56 ± 0.32 pA \((n = 60)\), respectively. The amplitudes of SM and \(I_{Ca\text{(spark)}}\) were independent of the onset membrane potential \((V_{on})\) as determined by the events activated by Ca\(^{2+}\) sparks (see below). Collectively, these results suggest that Ca\(^{2+}\) underlying Ca\(^{2+}\) sparks in phys-
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BiMPTs prevent action potentials evoked by depolarizing currents. A, depolarizing current (400 pA for 5 ms) failed to elicit action potential. B, same depolarizing current as in A triggered an action potential when BiMPTs were blocked by ryanodine (Ry, 100 μM). To prevent the inactivation of VDCCs by the sustained depolarization caused by ryanodine (Fig. 2), −50 pA was applied to reset RMP to approximately −60 mV. C, nifedipine (Nif, 1 μM) blocked the evoked action potential in the presence of ryanodine, indicating that this potential is mediated by L-type VDCCs. Insets depict expanded views of the recordings marked by dotted boxes. Four cells gave similar responses.

By blocking the hyperpolarization phase of BiMPTs, paixilline shifted RMP to a more depolarized level (Fig. 2A, panel i, RMP: −44 ± 1.8 mV in control versus −22 ± 2.4 mV in the presence of paixilline, n = 5, p < 0.001 with paired t test). Along with inhibiting the depolarization phase of BiMPTs, niflumic acid (100 μM) caused the membrane to become more hyperpolarized (Fig. 2A, panel ii, RMP: −44 ± 3.7 mV in control versus −56 ± 3.6 mV in the presence of niflumic acid, n = 5, p < 0.01 with paired t test). Because niflumic acid does not activate BK channels in airway smooth muscle (14), it does in vascular smooth muscle (24), the hyperpolarization by niflumic acid is most likely mediated by its inhibition of Cl\(_{Ca}\) channels. Therefore, both BK channels and Cl\(_{Ca}\) channels are critical to membrane potential, and their balanced activation is required to maintain RMP at physiological conditions.

Ca\(^{2+}\) Sparks and BiMPTs Prevent Evoked Action Potential—ASM does not fire action potentials under physiological conditions, but the underlying reasons remain incompletely understood (25). In light of the stabilizing effect of Ca\(^{2+}\) sparks on the membrane potential, we explored the hypothesis that Ca\(^{2+}\) sparks and BiMPTs are the signals that prevent ASM from generating action potential. In the presence of Ca\(^{2+}\) sparks and BiMPTs, short depolarizing currents with amplitude (10–1200

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\(^3\) R. ZhuGe, R. Bao, K. E. Fogarty, and L. M. Lifshitz, unpublished results.
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**Results in Membrane Depolarization**—The inhibitory and stabilizing nature of Ca\(^{2+}\) sparks and BiMPTs on membrane excitability predicts that reagents that alter these events could change membrane excitability. This prediction was confirmed in Fig. 2A where paxilline (1 \(\mu\)M), by blocking the hyperpolarization phase of BiMPTs, depolarized membrane excitability. 

**Tipping the Balance of BiMPTs**—Increasing the excitability of ASM.

\begin{align*}
\text{Activation of VDCCs and Cl}_{\text{Ca}} \text{ channels underlies paxilline- and ryanodine-induced depolarization.} \\
\text{A, niflumic acid (NA, 100 \(\mu\)M) plus ryanodine (Ry, 100 \(\mu\)M) blocked BiMPTs and hyperpolarized membrane. Insets in this panel and other three panels in the figure show the mean RMP before (open bars) and during (filled bars) treatments (**, \(p < 0.01\), control versus niflumic acid plus ryanodine, \(n = 4\)). B, niflumic acid (100 \(\mu\)M) and paxilline (Pax, 1 \(\mu\)M) abolished BiMPTs without changing RMP (\(p > 0.05\), \(n = 4\)). C, nifedipine (Nif, 1 \(\mu\)M) plus ryanodine (100 \(\mu\)M) blocked BiMPTs without changing RMP (\(p > 0.05\), \(n = 4\)). D, nifedipine (1 \(\mu\)M) and paxilline (1 \(\mu\)M) suppressed the hyperpolarizing component of BiMPTs without causing a sustained depolarization (\(p < 0.05\), \(n = 5\)).}
\end{align*}

\begin{align*}
\text{100 \(\mu\)M Ry} & \text{ (A)} \\
\text{1 \(\mu\)M Pax} & \text{ (A)} \\
\text{1 \(\mu\)M Nif + 100 \(\mu\)M Ry} & \text{ (A)} \\
\text{100 \(\mu\)M NA + 100 \(\mu\)M Ry} & \text{ (A)} \\
\text{100 nM 50 Sec} & \text{ (A)}
\end{align*}

\begin{align*}
\text{1 \(\mu\)M Nif + 100 \(\mu\)M Ry} & \text{ (B)} \\
\text{Nif (7)} & \text{ vs. Ry (6), Pi (6), NA (7), Nif + Ry (6), Nif + Pax (6), NA + Ry (7), NA + Pax (10) (**, \(p < 0.01\) with paired t test). These observations prompted us to determine the underlying channels for the induced depolarization.}
\end{align*}
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illine-induced sustained depolarization, respectively ($-45.3 \pm 2.8$ mV in the control versus $-51.5 \pm 2.1$ mV in the presence of niflumic acid and ryanodine, $n = 4, p < 0.01$; and $-45.5 \pm 1.7$ mV in the control versus $-46 \pm 1.3$ mV in the presence of niflumic acid and paxilline, $p > 0.05, n = 4$).

Because the depolarization caused by paxilline and ryanodine is more positive than the potential at which the L-type Ca\textsuperscript{2+} channel current was detected in these cells (supplemental Fig. S2), the effect of nifedipine on the depolarization induced by these two compounds was next assessed. Fig. 4, C and D, demonstrates that nifedipine (1 \textmu M) blocked the sustained depolarization caused by ryanodine (see Fig. 2A, panel ii) and paxilline (see Fig. 2A, panel i), respectively ($-47.8 \pm 2.9$ in the control versus $-47.3 \pm 3.2$ in the presence of nifedipine and ryanodine, $n = 5, p > 0.05$; and $-47.5 \pm 1.7$ in the control and $-45.0 \pm 2.3$ mV in the presence of nifedipine and paxilline, $n = 4, p > 0.05$). Contrary to the effect on ryanodine, nifedipine did not affect the depolarization components induced by paxilline. These results indicate that blocking either Ca\textsuperscript{2+} sparks or the hyperpolarization phase of BiMPTs can trigger a positive feedback loop between Cl\textsubscript{Ca} channels and VDCCs, resulting in a depolarization close to $E_{Cl}$.

**Tipping the Balance of BiMPTs Leads to an Increase in Global [Ca\textsuperscript{2+}], and Cell Shortening**—The activation of VDCCs by depolarization induced by ryanodine and paxilline suggests Ca\textsuperscript{2+} sparks and BiMPTs could regulate global [Ca\textsuperscript{2+}]. Yet, as is evident in Fig. 1, a single Ca\textsuperscript{2+} spark raised Ca\textsuperscript{2+} locally but exerted no effect on the global [Ca\textsuperscript{2+}]. Therefore, we examined the accumulated effect of Ca\textsuperscript{2+} sparks and BiMPTs on [Ca\textsuperscript{2+}], by monitoring global [Ca\textsuperscript{2+}], dynamics using fura-2, a ratio-metric indicator that is well suited for quantifying [Ca\textsuperscript{2+}]. Although these cells exhibit spontaneous Ca\textsuperscript{2+} sparks at rest, the global [Ca\textsuperscript{2+}] in the majority of cells (59 of 61) was stable with a mean value of $137 \pm 12 \text{nM}$, and nifedipine (1 \textmu M) caused no change in resting [Ca\textsuperscript{2+}], (Fig. 5), indicating that L-type Ca\textsuperscript{2+} channels do not contribute significantly to set the resting [Ca\textsuperscript{2+}], in these cells.

We next examined whether changes in Ca\textsuperscript{2+} sparks and BiMPTs alter global [Ca\textsuperscript{2+}], by treating cells with ryanodine and paxilline. At 100 \textmu M, ryanodine increased the [Ca\textsuperscript{2+}], from $136 \pm 18$ to $210 \pm 23 \text{nm}$ ($p < 0.01, n = 7$), and paxilline (1 \textmu M) elevated it to $213 \pm 25 \text{nm}$ from $131 \pm 19 \text{nm}$ ($p < 0.01, n = 11$) (Fig. 5). Removal of both agents restored global [Ca\textsuperscript{2+}], to its normal resting values. Nifedipine (1 \textmu M) blocked the increase in [Ca\textsuperscript{2+}], caused by either ryanodine or paxilline (Fig. 5B). Niflumic acid itself exerted no effect on [Ca\textsuperscript{2+}], but it blocked the increase in Ca\textsuperscript{2+} induced by either paxilline or ryanodine (Fig. 5B). Put together, the results in Fig. 5 indicate that Ca\textsuperscript{2+} sparks and resulting BiMPTs contribute to maintain resting [Ca\textsuperscript{2+}], and interrupting the balance of BK channels and Cl\textsubscript{Ca} channels can lead to a rise in global [Ca\textsuperscript{2+}],.

Because global [Ca\textsuperscript{2+}], is a key determinant of the contractile status of smooth muscle (27), we measured cell shortening at the single cell level in an attempt to establish a direct link between Ca\textsuperscript{2+} sparks/BiMPTs and contractility. Cells used in this study are relaxed and spindle-shaped, with a length of $166 \pm 5 \text{ mm} (n = 57)$. Fig. 6A demonstrates that ryanodine (100 \textmu M) initiated a contraction within 5 s of application and shortened the cell by 39% within 90 s of treatment. On average, ryanodine contracted the cells by $20.4 \pm 3.7\% (p < 0.01, n = 10)$. Paxilline (1 \textmu M) caused a similar effect on cell shortening as ryanodine. On average, paxilline contracted the cells by $15.5 \pm 2.2\% (p < 0.01, n = 9)$. Both niflumic acid (100 \textmu M) and nifedipine (1 \textmu M) prevented ryanodine- and paxilline-induced contraction (Fig. 6B).

**DISCUSSION**

In this study we provide direct evidence that Ca\textsuperscript{2+} sparks and BiMPTs play critical roles in determining the status of membrane excitability and contractility in ASM. At rest, they maintain the membrane potential within a negative range and prevent the cells from generating action potentials triggered by external stimuli, thus keeping the cells at a low level of excitability. Blocking Ca\textsuperscript{2+} sparks or the hyperpolarization components of BiMPTs turns this inhibitory mode into an excitable mode by activating Cl\textsubscript{Ca} channels and VDCCs in a positive feedback manner, which in turn depolarizes the membrane, raises global [Ca\textsuperscript{2+}], and induces contraction.

It has long been recognized that Ca\textsuperscript{2+} sparks activate Cl\textsubscript{Ca} channels and BK channels in a voltage-dependent manner in ASM (7). In smooth muscle cells, reversal potentials for Cl\textsuperscript{–} and
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K$^+$ are around $-15$ and $-80$ mV, respectively (28). Therefore, under physiological conditions, Ca$^{2+}$ sparks are expected to cause biphasic membrane potential transients and, moreover, due to the depolarization component of the transients, to activate L-type Ca$^{2+}$ VDCCs, leading to an increase in global $\left[\text{Ca}^{2+}\right]_i$, or even to the generation of an action potential. Evidence from this study confirms the first prediction but does not validate the second one. We found that BiMPTs dwell most of the time below the potential at which the activity of L-type VDCCs was detected. This is in line with findings that the L-type Ca$^{2+}$ channel blocker nifedipine neither alters RMP amplitudes and membrane potential oscillations nor does it decrease resting $\left[\text{Ca}^{2+}\right]_i$. The ineffectiveness of nifedipine further indicates that Ca$^{2+}$ spark-induced depolarizations that transiently reach the potential where L-type VDCCs can be activated either do not activate these channels at all or activate them to such a minimal extent that Ca$^{2+}$ influx by them is not sufficient to alter global $\left[\text{Ca}^{2+}\right]_i$. The latter could happen if the entering Ca$^{2+}$ is buffered by endogenous Ca$^{2+}$ buffers (13) or is compensated by Ca$^{2+}$ extrusion mechanisms in the cells (29).

Not only do Ca$^{2+}$ sparks not activate L-type VDCCs at a detectable level, but also they suppress the generation of VDCC-mediated action potentials by external stimuli. As shown in this study, in the presence of Ca$^{2+}$ sparks and BiMPTs, ASM cells do not produce this form of action potential in response to depolarizing currents. Strikingly, when Ca$^{2+}$ sparks and their resulting BiMPTs were abolished, these cells generate action potentials upon stimulation with the same strength. The underlying mechanisms for this effect are to be determined. A likely possibility is that the currents resulting from the opening of BK channels and Cl$_{\text{Ca}}$ channels are much greater than that of L-type VDCCs, so the membrane potential is dominated by the activities of BK channels and Cl$_{\text{Ca}}$ channels. Both BK channels and Cl$_{\text{Ca}}$ channels in an ASM cell are in the range of 10,000–20,000, which could give rise to peak currents on the order of 2–5 nA (13, 30, 31). But the peak current for L-type VDCCs is around 50 pA, which could be accounted for by the opening of $\sim$500 channels (assuming a $P_o$ of 0.4 (supplemental Fig. S2) and a unitary conductance of 3 pico siemens (26)). When membrane is depolarized to the levels more negative than $E_{\text{Cl}}$, BK channels would be the dominant force to opposing the depolarizing effect of L-type VDCCs; when the membrane potential becomes less negative than $E_{\text{Cl}}$, both BK current and Cl$_{\text{Ca}}$ current act against the depolarization caused by L-type VDCCs, thus making the cells much harder to be depolarized. Because of the overwhelming effects of BK and Cl$_{\text{Ca}}$ channels on the membrane potential, Ca$^{2+}$ sparks serve as powerful safeguard devices to prevent hyper-excitability in ASM.

The inhibitory and stabilizing nature of Ca$^{2+}$ sparks and BiMPTs suggest that tipping the balance between BK channels and Cl$_{\text{Ca}}$ channels can change the excitability and contractility in ASM. This study demonstrated two such mechanisms as follows: one is the blockage of BK channels by paxilline, and the other is the blockage of Ca$^{2+}$ sparks by ryanodine. Interestingly, although the first mechanism directly inhibits BK channels and the second indirectly blocks both BK channels and Cl$_{\text{Ca}}$ channels by stopping Ca$^{2+}$ sparks, both actions result in the activation of Cl$_{\text{Ca}}$ channels and L-type VDCCs in a positive feedback manner, leading to membrane depolarization, global $\left[\text{Ca}^{2+}\right]_i$ elevation, and contraction of the cells. These results suggest that BK channels and Cl$_{\text{Ca}}$ channels activated by Ca$^{2+}$ sparks exert a dominant influence on membrane potential in these cells. This is in line with the previous findings that BK channels form clusters near Ca$^{2+}$ spark sites, and almost all Cl$_{\text{Ca}}$ channels in the membrane appear to concentrate in the areas Ca$^{2+}$ sparks occur (13, 32). This is also supported by immunocytochemical studies revealing that BK channels localize in puncta in the surface membrane in several smooth muscle types, including ASM (33, 34).

We propose a model for the role of Ca$^{2+}$ sparks and BiMPTs in ASM as follows (Fig. 7). At rest, at a given moment the majority of Ca$^{2+}$ spark sites are quiet, although a few generate Ca$^{2+}$ sparks. BK channels and Cl$_{\text{Ca}}$ in the quiescent sites open low

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\(P_o\) at normal resting [Ca\(^{2+}\)], contributing to the RMP. Those in the active sites open at high \(P_o\) to generate BiMPTs, resulting in membrane potential oscillations. Because the RMP and membrane potential oscillations are below the activation potential of L-type Ca\(^{2+}\) channels for most of the time, VDCCs are not activated or activated at an undetectable level. When BK channels are blocked by paxilline, or the balanced activity of BK channels and Cl\(_{Ca}\) channels in Ca\(^{2+}\) activated or activated at an undetectable level. When BK channels are blocked by paxilline, or the balanced activity of BK channels and Cl\(_{Ca}\) channels in Ca\(^{2+}\) spark sites is disrupted by ryanodine, Ca\(^{2+}\) sparks continue to activate or preferentially activate Cl\(_{Ca}\) channels, leading to a stronger depolarization of the membrane. Such depolarization reaches the potential for the activation of VDCCs, resulting in their opening and Ca\(^{2+}\) influx. Ca\(^{2+}\) influx via VDCCs increases global [Ca\(^{2+}\)], which in turn activates more Cl\(_{Ca}\) channels, the membrane becomes more depolarized, and more VDCCs are consequently activated, until a new equilibrium potential, i.e. near \(E_{Cl}\), is reached. Finally, the increase in global [Ca\(^{2+}\)] causes cells to contract. It is likely that synchronizing activation of Ca\(^{2+}\) sparks, BiMPTs, and VDCCs in ASM could influence the contractility at the tissue and organ level under physiological conditions, a possibility that warrants further investigation.

In summary, our study reveals that in ASM Ca\(^{2+}\) sparks exert a bidirectional effect on membrane potential and can mediate both inhibitory and excitable responses. Therefore, Ca\(^{2+}\) sparks and their evoked currents serve as a powerful mechanism that allows ASM to adapt to diverse internal and external stimuli. A consequence of this mechanism is that any changes in the composition of the Ca\(^{2+}\) spark signaling complex could disrupt this plasticity, leading to an alteration in contractility with possible pathological consequences in ASM.

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