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

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). STICs are produced by directly measuring Ca\(^{2+}\) sparks in smooth muscle cells that possess both STOCs and STICs. Thus, they 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 can activate nearby big conductance Ca\(^{2+}\)-activated K\(^{+}\) (BK) channels 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 \(\beta 1\) 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\(_{I}\)) channels to generate spontaneous transient inward currents (STICs) (7, 13–17). Because \(E_{C_{I}}\) 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\(^{2+}\)]\(_{i}\)), 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. 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 underlying 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)\(^2\) 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)

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2 The abbreviations used are: RyR, ryanodine receptor; STOC, spontaneous transient outward current; STIC, spontaneous transient inward current; BiMPT, biphasic membrane potential transient; RMP, resting membrane potential; VDCC, voltage-dependent Ca\(^{2+}\) channel; \(I_{Ca,sparks}\), Ca\(^{2+}\) current via RyRs underlying Ca\(^{2+}\) spark; ASM, airway smooth muscle; SM, signal mass.
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**FIGURE 1. Relationship between Ca\(^{2+}\) 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\(_{\text{Ca(spark)}}\) (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\(_{d}\) 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\(_{\text{Ca(spark)}}\); 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\(_{\text{Ca(spark)}}\) and BiMPT amplitude (red open circles, \(r = -0.0686\) and \(p = 0.602\) for I\(_{\text{Ca(spark)}}\) versus hyperpolarization phase; blue open triangles, \(r = 0.234\) and \(p = 0.0719\) for I\(_{\text{Ca(spark)}}\) versus depolarization phase), and between I\(_{\text{Ca(spark)}}\) and on-set potential of BiMPTs (black open squares, \(r = -0.0239\) and \(p = 0.8563\)).

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 0.02 mM EDTA at room temperature for 30 min. The tissue was then incubated at 32 \(^\circ\)C for another ~6 min with 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 \(^\circ\)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, Na oil, giving a pixel size of 333 nm at the specimen. The 488 nm line of an argon ion laser provided fluorescence excitation, with a shutter to control exposure duration, and emission of the Ca\(^{2+}\) indicator was monitored at wavelengths of >500 nm. Subsequent image processing and analysis were performed off line using a custom-designed software package, running on either a Silicon Graphics or Linux/PC workstation. Signal mass of Ca\(^{2+}\) sparks was estimated according to the methodology published previously (19). The measured endogenous Ca\(^{2+}\) buffers from the same type of the cells were used to correct for the signal mass (13).

**Measurement of Global [Ca\(^{2+}\)]\(_i\)—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 \(\times 40\), 1.3-NA) with a specially designed excitation path and photomultiplier tube (Thorn EMI type 9954A, Thorn EMI, Rock-

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RESULTS

Ca\textsuperscript{2+} Sparks Induce BiMPTs via Activating BK Channels and Cl\textsubscript{Ca} Channels under Physiological Conditions—Ca\textsuperscript{2+} sparks activate only STICs at $E_{K}$, only STOCs at $E_{C}$, and spontaneous transient outward and inward currents at potentials between $E_{K}$ and $E_{C}$ 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\textsuperscript{2+} sparks under physiological conditions, Ca\textsuperscript{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\textsuperscript{2+} transients were detected (Fig. 1A), and they were Ca\textsuperscript{2+} sparks because no transient was observed in the presence of 100 μM ryanodine. Estimated with signal mass (SM) methodology (19), the signal mass and peak current underlying these Ca\textsuperscript{2+} sparks ($I_{Ca(spark)}$) were 244,794 ± 24,818 Ca\textsuperscript{2+} ions and 3.56 ± 0.32 pA ($n = 60$), respectively. The amplitudes of SM and $I_{Ca(spark)}$ were independent of the onset membrane potential ($V_{on}$) as determined by the events activated by Ca\textsuperscript{2+} sparks (see below). Collectively, these results suggest that Ca\textsuperscript{2+} underlying Ca\textsuperscript{2+} sparks in phys-
BiMPTs Set the Range of Membrane Potential under Physiological Conditions—Ca^{2+} sparks continually activated BiMPTs, resulting in the oscillation of membrane potentials with varying amplitude (Fig. 2). Given the variation between the coupling of Ca^{2+} sparks and BiMPTs (Fig. 1C), all-points histograms of the recorded membrane potentials were used to quantify the relationship between BiMPT and RMP. Because the histogram could not fit well with a Gaussian function, the mode of the histogram was designated as RMP (Fig. 2A, panel i). The average amplitude of the hyperpolarization phase in BiMPTs was −20.1 ± 1.4 mV and that of the depolarization was 10.1 ± 1.3 mV (n = 60). These values are in line with those obtained in much longer recordings under perforated whole-cell configurations (see Fig. 2). Thus, a single Ca^{2+} spark can change membrane potential by ~30 mV under physiological conditions.

To determine the underlying channels for BiMPTs, cells were treated with the blockers of BK channels and Cl_{Ca} channels, respectively. Paxilline (1 μM), a BK channel blocker, abolished the hyperpolarization phases of BiMPTs (Fig. 2A, panel ii). Niflumic acid (100 μM), a Cl_{Ca} channel blocker, gradually abolished the depolarization phases of BiMPTs (Fig. 2A, panel ii). (Changes in the RMP in these experiments will be discussed below.) Therefore, Ca^{2+} sparks induce BiMPTs by activating BK channels and Cl_{Ca} channels in ASM from mouse.

BiMPTs Set the Range of Membrane Potential under Physiological Conditions—Ca^{2+} sparks correlated temporally with BiMPTs with a hyperpolarization followed by a depolarization (Fig. 1A and B). (At times, Ca^{2+} sparks correlated only with either hyperpolarization transients or depolarization transients, as indicated by the events with zero value in Fig. 1C.) The V_{on} of BiMPTs was −46.3 ± 0.9 mV (n = 60); and the average amplitude of the hyperpolarization phase in BiMPTs was −20.1 ± 1.4 mV and that of the depolarization was 10.1 ± 1.3 mV (n = 60). These values are in line with those obtained in much longer recordings under perforated whole-cell configurations (see Fig. 2). Thus, a single Ca^{2+} spark can change membrane potential by ~30 mV under physiological conditions.

Scatter plots of the parameters of Ca^{2+} sparks and BiMPTs in Fig. 1C show weak correlations between SM and the hyperpolarization phase or depolarization phase of BiMPTs, and between I_{Ca(spark)} and both phases of BiMPTs. These data indicate a great variation between the coupling of Ca^{2+} sparks and their target channels, as suggested in the voltage clamp studies (13, 23).

By blocking the hyperpolarization phase of BiMPTs, paxilline 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 paxilline, 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 depolarized (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), as 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 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.

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FIGURE 3. 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.
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**A**

![Activation of VDCCs and Cl$_{Ca}$ channels underlies paxilline- and ryanodine-induced depolarization.](image)

**B**

![Membrane potential close to RMP](image)

**C**

![1 μM Nif + 100 μM Ry](image)

**D**

![1 μM Nif + 1 μM Pax](image)

**FIGURE 4.** Activation of VDCCs and Cl$_{Ca}$ channels underlies paxilline- and ryanodine-induced depolarization. A, niflumic acid (NA, 100 μM) plus ryanodine (Ry, 100 μM) 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 μM) and paxilline (Pax, 1 μM) abolished BiMPTs without changing RMP (** p > 0.05, n = 4). C, nifedipine (Nif, 1 μM) plus ryanodine (100 μM) blocked BiMPTs without changing RMP (** p > 0.05, n = 4). D, nifedipine (1 μM) and paxilline (1 μM) suppressed the hyperpolarizing component of BiMPTs without causing a sustained depolarization (p > 0.05, n = 5).

**FIGURE 5.** Ryanodine and paxilline activate Cl$_{Ca}$ channels and VDCCs, resulting in an increase in global [Ca$^{2+}$]. The cells were loaded with fura-2 AM (1 μM), and the global [Ca$^{2+}$]$_i$ was determined with a microfluorometer (20). A, representative traces showing that 100 μM ryanodine (i, panel i) and 1 μM paxilline (panel ii) increased global [Ca$^{2+}$], whereas 1 μM nifedipine (Nif, panel iii) and 100 μM niflumic acid (NA, panel iv) blocked the effect of ryanodine. B, average responses in global [Ca$^{2+}$]$_i$ upon stimulation with reagents indicated below the bars. The [Ca$^{2+}$]$_i$ during the pretreatment in each experiment was set as control, and the differences between treatments (+) and controls (−) were compared with paired t test. Values in parentheses are the number of cells used in each treatment. **, p < 0.01.

pA) and duration (2–20 ms) did not induce an action potential. Fig. 3A shows a typical response to a pulse of 400 pA for 5 ms. When delivered either at the valley or the peak of BiMPTs, this pulse only caused a passive potential transient. Interestingly, in the presence of ryanodine at the concentration sufficient to block Ca$^{2+}$ sparks and BiMPTs, the same stimulation generated an action potential after the passive potential transient (Fig. 3B). (The membrane potential was reset to around −60 mV after ryanodine but prior to stimulation, because ryanodine caused sustained depolarization, which in turn could inactivate L-type VDCCs (Fig. 2A, panel iii) (26). Nifedipine (1 μM) blocked the action potential induced by the depolarizing current, confirming that the action potential is mediated by the opening of L-type VDCCs. In conjunction with results in Fig. 2, it is reasonable to suggest that at rest Ca$^{2+}$ sparks and the resulting BiMPTs act as a major inhibitory mechanism of membrane excitability in ASM.

**Tipping the Balance of BiMPTs**

**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 μM), by blocking the hyperpolarization phase of BiMPTs, depolarized the membrane to a level approaching $E_{Cl}$. Strikingly, ryanodine by blocking BiMPTs also depolarized the membrane close to $E_{Cl}$ (Fig. 2A, panel iii; RMP: −48 ± 2.3 mV in the control versus −27 ± 4.5 mV in the presence of ryanodine, n = 5, p < 0.001 with paired t test). These observations prompted us to determine the underlying channels for the induced depolarization. Because both reagents depolarize membrane potential close to $E_{Cl}$, the involvement of Cl$_{Ca}$ channels was first examined. Fig. 4, A and B, shows that niflumic acid (100 μM) prevented both ryanodine- and pax-
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illine-induced sustained depolarization, respectively (−45.3 ± 2.8 mV in the control versus −51.5 ± 2.1 mV in the presence of niflumic acid and ryanodine, \(n = 4, p < 0.01\); and −45.5 ± 1.7 mV in the control versus −46 ± 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\(^{2+}\) 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 \(\mu M\)) blocked the sustained depolarization caused by ryanodine (see Fig. 2A, panel ii) and paxilline (see Fig. 2A, panel i), respectively (−47.8 ± 2.9 in the control versus −47.3 ± 3.2 in the presence of nifedipine and ryanodine, \(n = 5, p > 0.05\); and −47.5 ± 1.7 in the control and −45.0 ± 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\(^{2+}\) sparks or the hyperpolarization phase of BiMPTs can trigger a positive feedback loop between Cl\(_{Ca}\) channels and VDCCs, resulting in a depolarization close to \(E_{Cl}\).

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

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

Because global [Ca\(^{2+}\)] 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\(^{2+}\) sparks/BiMPTs and contractility. Cells used in this study are relaxed and spindle-shaped, with a length of 166 ± 5 \(\mu M\) (\(n = 57\)). Fig. 6A demonstrates that ryanodine (100 \(\mu M\)) initiated a contraction within 5 s of application and short-ened the cell by 39% within 90 s of treatment. On average, ryanodine contracted the cells by 20.4 ± 3.7% (\(p < 0.01, n = 10\)). Paxilline (1 \(\mu M\)) caused a similar effect on cell shortening as ryanodine. On average, paxilline contracted the cells by 15.5 ± 2.2% (\(p < 0.01, n = 9\)). Both niflumic acid (100 \(\mu M\)) and nifedipine (1 \(\mu M\)) prevented ryanodine- and paxilline-induced contraction (Fig. 6B).

**DISCUSSION**

In this study we provide direct evidence that Ca\(^{2+}\) 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\(^{2+}\) sparks or the hyperpolarization components of BiMPTs turns this inhibitory mode into an excitable mode by activating Cl\(_{Ca}\) channels and VDCCs in a positive feedback manner, which in turn depolarizes the membrane, raises global [Ca\(^{2+}\)], and induces contraction.

It has long been recognized that Ca\(^{2+}\) sparks activate Cl\(_{Ca}\) channels and BK channels in a voltage-dependent manner in ASM (7). In smooth muscle cells, reversal potentials for Cl\(^−\) 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 [Ca\(^{2+}\)], 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 [Ca\(^{2+}\)]. 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 [Ca\(^{2+}\)]. 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 ~500 channels (assuming a \(P_{o}\) of 0.4 (supplemental Fig. S2) and a unitary conductance of 3 picosiemens (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 [Ca\(^{2+}\)]\(_{i}\) 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 at low

\[^{4}\text{L. M. Lifshitz, J. D. Carmichael, K. E. Fogarty, and R. ZhuGe, unpublished results.}\]
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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 picaxilline, 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 \(E_{Ca}\), 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 excitatory 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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REFERENCES

Supplemental materials

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Fig. S1. Ca\textsuperscript{2+} sparks activated STICs, STOICs, and STOCs at different membrane potentials. The cells were voltage-clamped at the potential as indicated in the panels. At -80 mV, i.e., $E_k$, the cell displays only STICs, at -15 mV, i.e., $E_{Cl}$, only STOCs, and at -50 mV STOICs. The same patterns were observed in 5 other cells.

Fig. S2. Characterization and molecular basis of voltage-dependent Ca\textsuperscript{2+} channels in murine ASM. (A) Depolarization from -80 mV to 0 mV activated $I_{Ca}$, 500 nM FPL increased the current while 500 nM nifedipine blocked the current. Ca\textsuperscript{2+} was the charge carrier. (B) I-V plots from the same cell in A under three conditions. (C) Transcripts of Ca\textsubscript{1.1} channels as detected by RT-PCR with specific primers (Ca\textsubscript{1.1}: 5'-CAGCAGAGGAGGAACGCTGGGAGAG-3' (f), 5'-ATCGGTCTTTTCAGTTTGTCCAC-3' (r), access number, NM_001081023, expected product, 442bp; Ca\textsubscript{1.2}: 5'-GAGACGACAGCTGTAGGAGGAGGACGTGGGAG-3' (f), 5'-ACAAAGGTAGAGAGGTGCGGT-3' (r), access number, NM_009781, expected product, 305bp; Ca\textsubscript{1.3}: 5'-TCCTACCCACCACATCCCACC-3' (f), 5'-AGTCAGACTAGCTGGCCGAAA-3' (r), access number, NM_028981, expected product, 391bp.) (D) Cellular distribution of Ca\textsubscript{1.1} and Ca\textsubscript{1.2} revealed by immunostaining. Nuclei shown in blue were stained with Hoechst 33342. Note that no specific antibody for Ca\textsubscript{1.3} is available so its expression could not be evaluated with immunostaining. Since this channel has a more negative threshold than observed (A and B), their contribution to $I_{Ca}$ can be ruled out in these cells.
Fig. S1. ZhuGe et al.
Fig. S2  ZhuGe et al

A

B

C

D

I_{Ca, pA}

50 pA

FPL

Control

Nifedipine

50 ms

1000

500

100

bps

Ca_{v, 1.1}  Ca_{v, 1.2}  Ca_{v, 1.3}

Ca_{v, 1.1}

Ca_{v, 1.2}
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