Physiological properties and functions of Ca\(^{2+}\) sparks in rat intrapulmonary arterial smooth muscle cells

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Remillard, Carmelle V., Wei-Min Zhang, Larissa A. Shimoda, and James S. K. Sham. Physiological properties and functions of Ca\(^{2+}\) sparks in rat intrapulmonary arterial smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 283: L433–L444, 2002. First published March 15, 2002; 10.1152/ajplung.00468.2001.—Ca\(^{2+}\) spark has been implicated as a pivotal feedback mechanism for regulating membrane potential and vasomotor tone in systemic arterial smooth muscle cells (SASMCs), but little is known about its properties in pulmonary arterial smooth muscle cells (PASMCs). Using confocal microscopy, we identified spontaneous Ca\(^{2+}\) sparks in rat intralobar PASMCs and characterized their spatiotemporal properties and physiological functions. Ca\(^{2+}\) sparks of PASMCs had a lower frequency and smaller amplitude than cardiac sparks. They were abolished by inhibition of ryanodine receptors but not by inhibition of inositol trisphosphate receptors and L-type Ca\(^{2+}\) channels. Enhanced Ca\(^{2+}\) influx by BAY K8644, K\(^{+}\), or high Ca\(^{2+}\) caused a significant increase in spark frequency. Functionally, enhancing Ca\(^{2+}\) sparks with caffeine (0.5 mM) caused membrane depolarization in PASMCs, in contrast to hyperpolarization in SASMCs. Norepinephrine and endothelin-1 both caused global elevations in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{cyt}\)), but only endothelin-1 increased spark frequency. These results suggest that Ca\(^{2+}\) sparks of PASMCs are similar to those of SASMCs, originate from ryanodine receptors, and are enhanced by Ca\(^{2+}\) influx. However, they play a different modulatory role on membrane potential and are under agonist-specific regulation independent of global [Ca\(^{2+}\)].

Muscle contraction is initiated by a global increase in cytosolic Ca\(^{2+}\) resulting from influx from extracellular compartments and release from intracellular stores. In cardiac and skeletal muscles, it has been demonstrated that the global Ca\(^{2+}\) transient activated during muscle contraction is generated by the summation of thousands of discrete local Ca\(^{2+}\) release events, or “Ca\(^{2+}\) sparks,” originating from ryanodine receptors (RyRs) on the sarcoplasmic reticulum (SR; see Refs. 9, 12, 36).

Similar Ca\(^{2+}\) sparks have been identified from various types of smooth muscle cells (2, 6, 16, 21, 23, 28, 35, 39–41, 72). Evidence from systemic vascular smooth muscle cells indicates that Ca\(^{2+}\) sparks cause local increases of Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{IC}\)) in subsarcolemmal spaces, activating nearby Ca\(^{2+}\)-activated K\(^{+}\) (K\(_{Ca}\)) channels, leading to membrane hyperpolarization, reduction of Ca\(^{2+}\) influx via L-type Ca\(^{2+}\) channels, and vasodilation (27, 29, 39, 40, 43). Therefore, they act as negative feedback modulators of membrane potential, rather than direct activators of myofilaments and the contractile apparatus (29, 40). On the other hand, Ca\(^{2+}\) sparks of tracheal smooth muscle cells have been shown to activate both K\(_{Ca}\) and Ca\(^{2+}\)-activated Cl\(^{−}\) (Cl\(_{Ca}\)) channels (72), depending on the membrane potential. Activation of Cl\(_{Ca}\) channels can cause membrane depolarization, increased Ca\(^{2+}\) influx, and cell contraction. Hence, the net physiological effects of Ca\(^{2+}\) sparks in these myocytes depends on the relative activities of these counteracting Ca\(^{2+}\)-activated channels and on the membrane potential at which the Ca\(^{2+}\) sparks are generated.

Despite the studies in systemic vascular smooth muscle cells, there is only minimal information on Ca\(^{2+}\) sparks in pulmonary arterial smooth muscle cells (PASMCs). A recent study on the heterogeneity of ryanodine- and inositol trisphosphate (IP\(_{3}\))-sensitive Ca\(^{2+}\) stores showed that Ca\(^{2+}\) sparks are present in canine PASMCs (30); another study reported that both Ca\(^{2+}\) sparks and spontaneous transient outward currents (STOCs) are present in fetal rabbit PASMCs (45). In the present study, we sought to provide a comprehensive characterization of the biophysical properties and physiological functions of Ca\(^{2+}\) sparks in intralobar PASMCs from adult rats. We have 1) quantified the spatiotemporal properties of spontaneous Ca\(^{2+}\) sparks in rat PASMCs, according to the criteria established in cardiac myocytes, 2) determined the nature of the intracellular Ca\(^{2+}\) store(s) from which they originate, 3) examined their modulation by enhanced Ca\(^{2+}\) influx, 4) determined their role in modulating resting membrane potential, and 5) investigated their regula-
tion by norepinephrine (NE) and endothelin-1 (ET-1). Our results indicate that, despite similarities in the basic properties of Ca\(^{2+}\) sparks in PASMCs and systemic vascular smooth muscle cells, the physiological functions are remarkably different. Inasmuch as the pulmonary circulation is often regulated differently from the systemic circulation (e.g., responses to hypoxia), the present study provides unique information on pulmonary Ca\(^{2+}\) sparks fundamental for future studies of local Ca\(^{2+}\) signaling in the pulmonary vasculature.

**METHODS**

Isolation and culture of PASMCs. PASMCs were enzymatically isolated and transiently cultured as previously described (58). Briefly, male Wistar rats (150–200 g) were injected with heparin and anesthetized with pentobarbital sodium (130 mg/kg ip). They were exsanguinated, and the lungs were removed and transferred to a petri dish filled with HEPES-buffered salt solution (HBSS) containing (in mM) 130 NaCl, 5 KCl, 1.2 MgCl\(_2\), 1.5 CaCl\(_2\), 10 HEPES, and 10 glucose, pH 7.4 (adjusted with NaOH). Second- and third-generation intrapulmonary arteries (~300–800 \(\mu\)m) were isolated and cleaned free of connective tissue. The endothelium was removed by gently rubbing the luminal surface with a cotton swab. Arteries were then allowed to recover for 30 min in cold (4°C) HBSS, followed by 20 min in reduced-Ca\(^{2+}\) (20 \(\mu\)M) HBSS at room temperature. The tissue was digested at 37°C for 20 min in 20 \(\mu\)M Ca\(^{2+}\)-free HBSS containing collagenase (type I, 1,750 U/ml), papain (9.5 U/ml), BSA (2 mg/ml), and dithiothreitol (1 mM), then removed and washed with Ca\(^{2+}\)-free HBSS to stop digestion. Single smooth muscle cells were gently dispersed by trituration with a small-bore pipette in Ca\(^{2+}\)-free HBSS at room temperature. The cell suspension was then placed on 25-mm glass coverslips and transiently (16–48 h) cultured in Ham’s F-12 medium (with L-glutamine) supplemented with 0.5% FCS, 100 U/ml streptomycin, and 0.1 mg/ml penicillin.

Isolation of ventricular myocytes. Excised hearts were cannulated and perfused retrogradely through the aorta with prrewarmed (37°C) Ca\(^{2+}\)-free Tyrode solution containing (in mM) 137 NaCl, 5.4 KCl, 1 MgCl\(_2\), 10 HEPES, and 10 glucose, pH 7.4 (adjusted with NaOH). After 5 min, the heart was perfused with Ca\(^{2+}\)-free Tyrode solution containing collagenase (type I, 560 U/ml) and protease (type XIV, 0.28 mg/ml). After a 12-min enzyme-recirculating period, the heart was washed with 0.2 mM Ca\(^{2+}\) Tyrode solution for 5 min to stop the enzymatic digestion. The left ventricle was then cut from the heart and placed in 20 ml of prrewarmed 0.2 mM Ca\(^{2+}\) Tyrode solution. Myocytes were dispersed by gentle agitation and stored at room temperature (~22°C) before use.

Measurement of Ca\(^{2+}\) sparks. Ca\(^{2+}\) sparks were visualized using the membrane-permeable Ca\(^{2+}\)-sensitive fluorescent dye fluo 3-AM (Amersham Pharmacia Biotech). Myocytes were loaded with 5–10 \(\mu\)M fluo 3-AM (dissolved in dimethyl sulfoxide (DMSO) with 20% pluronic acid) in normal Tyrode solution for 30–45 min and 15 min at room temperature (~22°C), respectively. Cells were then washed thoroughly with 2 mM Ca\(^{2+}\) Tyrode solution to remove extracellular fluo 3-AM and rested for 15–30 min in a cell chamber to allow for complete deesterification of cytotoxic dye. Confocal images were acquired using a Zeiss LSM-510 inverted confocal microscope (Carl Zeiss) with a Zeiss Plan-Neofluor \(\times40\) oil immersion objective (numeric aperture = 1.3). The confocal pinhole was set to render a spatial resolution of 0.4 \(\mu\)m in the x-y axes, and 1.7 \(\mu\)m in the z-axis. Fluo 3-AM was excited by the 488-nm line of an argon laser, and fluorescence was measured at >505 nm. Images were acquired in the linescan mode (digital zoom rendering a 38-\(\mu\)m scan line), scanning at 0.075 \(\mu\)m/pixel, 512 pixels/line at 2-ms intervals for 512 lines/image from different cells within the same culture dish before and after drug application. Photobleaching and laser damage to the cells were minimized by attenuating the laser to ~1% of its maximum power (25 mW) with an acousto-optical tunable filter. Only 10 images (once every 10 s) were taken for each cell. Cells that did not respond to an external solution containing 10 mM Ca\(^{2+}\) and 0.5 mM caffeine applied at the end of experiments were discarded. All experiments were performed at room temperature.

Spontaneous Ca\(^{2+}\) sparks were detected by an automated detection algorithm custom written using the Interactive Data Language (Research Systems, Boulder, CO) to minimize subjective selection bias by identifying Ca\(^{2+}\) sparks on the basis of their statistical deviation from background noise, similar to that described previously by Cheng et al. (13). In brief, local fluorescence signals (F) of the focal image were first normalized in terms of F/F\(_0\), where F\(_0\) is the baseline value of F in a region of the image without Ca\(^{2+}\) sparks, and the mean (m) and variance (\(\sigma^2\)) of the confocal image was estimated. Ca\(^{2+}\) sparks were then identified based on local fluorescence intensity greater than m + 3\(\sqrt{\sigma^2}\). The amplitude of sparks being selected was expressed as \(\Delta F/F_0\); in some cases, F\(_0\) was calibrated to absolute [Ca\(^{2+}\)]\(_i\) by a pseudoratio method (12), using the following equation: [Ca\(^{2+}\)]\(_i\) = (K\(_R\)/[K/([Ca\(^{2+}\)]\(_i\))\(_0\)]) + 1) - R, where R is F/F\(_0\), the dissociation constant (K) of fluo 3 is 400 nM, and resting Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\))\(_0\) is assumed to be 100 nM. The duration and size (or width) of Ca\(^{2+}\) sparks were quantified as the full-duration half-maximum (FDHM) and full-width half-maximum (FWHM), respectively, as depicted in Fig. 1. The spark frequency of each cell was defined as the number of sparks detected per second in the scan line. In some images, large increases in global [Ca\(^{2+}\)]\(_i\) (G-Ca) or clustering of sparks rendered individual sparks indiscernible. These G-Ca increases were not analyzed with the program, but their frequency of occurrence during each treatment was quantified in each cell as the number of G-Ca images divided by the total number of images being taken.

Measurement of membrane potential. Membrane potential of PASMCs was measured using the perforated-patch technique to avoid disturbance of Ca\(^{2+}\) dynamics resulting from intracellular dialysis. Gigaohm seals were first established with patch pipettes (~3 M\(\Omega\)) tip-filled with a solution containing (in mM) 35 KCl, 90 potassium gluconate, 10 NaCl, and 10 HEPES, pH 7.2 (adjusted with KOH) and were back-filled with the same solution containing 300 mg/ml amphotericin B. After an access resistance of ≤30 M\(\Omega\) was achieved with the cells voltage-clamped at a holding potential of ~50 mV with an Axopatch 200B amplifier (Axon Instruments, Union City, CA). Membrane potential was recorded by switching the amplifier to current-clamp mode (I = 0). Data were filtered at 5 kHz, digitized with a Digidata 1200 analog-to-digital converter, and analyzed with pCLAMP software. Complete exchanges of external solution were achieved in <1 s using a rapid concentration-clamp system.

Statistical analysis. Throughout this paper, data are expressed as means ± SE. The number of cells or Ca\(^{2+}\) sparks was specified in the text. Statistical significance (\(P < 0.05\)) of the changes in spark characteristics was assessed by paired or unpaired Student’s t-tests or by one-way ANOVA with Newman-Keuls post hoc analyses, nonparametric Mann-Whitney U-tests, or Kolmogorov-Smirnov tests, wherever applicable.
Chemicals and drugs. Collagenase, protease, papain, serum albumin, dithiothreitol, caffeine, 2-aminoethyl diphenyl borate (2-APB), amphotericin B, and antibiotics were purchased from Sigma Chemical (St. Louis, MO). FCS and Ham’s F-12 medium were purchased from Mediatech (Herndon, VA). Fluo 3-AM and pluronic acid were purchased from Molecular Probes (Eugene, OR). BAY K 8644, nifedipine, and ryanodine were purchased from Calbiochem (La Jolla, CA). Stock solutions of BAY K 8644, nifedipine, ryanodine, amphotericin B, and 2-APB were prepared in DMSO and diluted 1:400 or 1:1,000 in 2 mM Ca2+/H11001-Tyrode solution. High-K+/H11001 (20 mM) solution was prepared by equimolar replacement of NaCl.

RESULTS

Biophysical characterization of Ca2+/H11001 sparks in PASMCs. To characterize the biophysical properties of Ca2+/H11001 sparks in PASMCs and to allow for comparisons with Ca2+/H11001 sparks recorded in other tissues, the settings for linescan confocal imaging were first verified using spontaneous Ca2+/H11001 sparks in rat ventricular myocytes as the standard, since they have been characterized extensively in different laboratories. Random spontaneous Ca2+/H11001 sparks were observed in ~80% of ventricular myocytes during the 10-s recording period, giving an overall average frequency of 1.31 ± 0.21 s−1 (40 cells, 511 sparks; Fig. 2). The amplitude of sparks (ΔF/F0) was 0.93 ± 0.03, corresponding to a [Ca2+] increase of 217.4 ± 25.5 nM. The size (FWHM) and duration (FDHM) of Ca2+/H11001 sparks were 1.76 ± 0.03 μm and 33.9 ± 0.7 ms, respectively, values highly consistent with those reported previously (9, 12, 20), indicating that both the acquisition parameters and the analysis algorithm used in the present study are comparable with other studies.

In contrast to ventricular myocytes, spontaneous Ca2+/H11001 sparks occurred rather infrequently in PASMCs (Fig. 2), with only 49% of cells exhibiting Ca2+/H11001 sparks. The overall spark frequency was 0.30 ± 0.04 s−1 (152 cells, 423 sparks; P < 0.05), ~75% lower than in cardiac myocytes. The average amplitude of Ca2+/H11001 sparks, also significantly lower in PASMCs, was 0.51 ± 0.01, equivalent to a Δ[Ca2+] of 74.7 ± 1.7 nM. On the other hand, the size (1.55 ± 0.03 μm) and duration (35.4 ± 1.0 ms) of Ca2+/H11001 sparks in PASMCs were comparable to those of cardiac sparks, although the former was slightly (~10%) smaller than cardiac sparks.

Fig. 1. Measurement of Ca2+ sparks. A: spark amplitude is defined as the change in peak fluorescence (F) normalized by the basal fluorescence (F0). Points a → a’ and b → b’ refer to the temporal and spatial profiles, respectively, for the determination of the full-duration half-maximum (FDHM) and full-width half-maximum (FWHM). B: FDHM, the duration of the spark that exceeds 50% of peak amplitude. C: FWHM, the width of the spark that exceeds 50% of the peak amplitude.

Fig. 2. Comparison of spontaneous Ca2+/H11001 sparks from pulmonary artery smooth muscle cells (PASMCs) and cardiac cells. A: representative line-scan images generated from PASMCs (left) and ventricular cells (right) under control conditions. B: bar graphs summarizing spark characteristics obtained from PASMCs (open bars) and cardiac cells (filled bars). Change (Δ) in intracellular Ca2+ concentration ([Ca2+]i) values calculated from the change in the ratio of fluorescence (F) to baseline fluorescence (F0) are given in the text. *Significant difference from PASMC sparks.
To further characterize the properties of Ca$^{2+}$ sparks, frequency distributions of ΔF/Φ₀, size, and duration of Ca$^{2+}$ sparks of PASMC and heart cells were generated (Fig. 3). Both frequency distributions of ΔF/Φ₀ from PASMCs and cardiac myocytes were non-Gaussian, highly left-skewed, with monotonic decreasing amplitude distributions as the result of off-center sampling in linescan confocal imaging (47, 62). Eighty-eight percent of PASMC sparks ranged between 0.29 and 0.72 in amplitude (40–110 nM Δ[Ca$^{2+}$]i) with a mode of 0.38 (53 nM) and a median of 0.46 (65 nM). Cardiac sparks had a wider range of amplitude distribution. Ninety-four percent of ventricular sparks had a ΔF/Φ₀ ranging from 0.2 to 2.0 (30–500 nM), with a mode of 0.55 (80 nM) and a median of 0.72 (110 nM). Because of the non-Gaussian distribution of spark amplitude, a nonparametric analysis was applied. The Mann-Whitney U-test indicated that the amplitude of Ca$^{2+}$ sparks was significantly lower in PASMCs than in cardiac myocytes ($P = 0.02$).

The distributions of spark duration measured at the 50% maximum amplitude level were also left skewed in both PASMCs and cardiac myocytes. The duration of sparks recorded in PASMCs ranged from 10 ms to 136 ms, with a mode of 20 ms and a median of 28 ms, almost identical to that of cardiac sparks, which ranged from 6 to 110 ms, with a mode of 20 ms and a median of 30 ms ($P = 0.08$, Mann-Whitney U-test). The size of both pulmonary and cardiac sparks displayed apparent modal distributions that could be described somewhat adequately by a single Gaussian function, as indicated by the Kolmogorov-Smirnov two-sample test ($P > 0.1$). This apparent modal distribution of spark size is consistent with a previous report on cardiac Ca$^{2+}$ sparks (64) but is in contrast to a theoretical study, which predicts more left skewing in the distribution curve (62). The size of pulmonary sparks distributed over a 90% range of 0.55 to 2.35 μm, with a mode of 1.35 μm and a median of 1.50 μm, similar to cardiac sparks that had a 90% range of 1.00–2.80 μm with a mode of 1.60 μm and a median of 1.73 μm. Comparison of means using a Student’s t-test indicated a small but significant difference ($P < 0.001$) between the mean size of sparks of PASMCs and cardiac myocytes, but nonparametric comparison of medians failed to detect a significant difference ($P = 0.38$) in the distribution between the two cell types.

Spontaneous PASMC Ca$^{2+}$ sparks and ryanodine-sensitive Ca$^{2+}$ stores. To examine if spontaneous Ca$^{2+}$ sparks in PASMCs were due entirely to the activation of RyRs, Ca$^{2+}$ sparks were recorded in the same population of PASMCs before and after exposure to 10 or 50 μM ryanodine for ≥15 min to inhibit RyRs. Ryanodine reduced spontaneous spark frequency in a concentration-dependent manner from a control value of 0.39 ± 0.06 s$^{-1}$ (75 cells, 305 sparks) to 0.20 ± 0.04 s$^{-1}$ (47 cells, 93 sparks; $P < 0.05$) and 0.05 ± 0.02 s$^{-1}$ (27 cells, 14 sparks; $P < 0.05$) with 10 and 50 μM ryanodine (Fig. 4A). Ryanodine did not evoke any changes in
spark amplitude, duration, or size (Table 1). In a separate series of experiments (Fig. 4B, Table 1), 50 μM 2-APB, an inhibitor of IP₃-sensitive Ca²⁺ stores, had no effect on the frequency of spontaneous sparks (control = 0.40 ± 0.05 s⁻¹, 53 cells, 216 sparks; 2-APB = 0.40 ± 0.10 s⁻¹, 37 cells, 154 sparks) nor on their amplitude, size, or duration. In the presence of 50 μM ryanodine, coapplication of 50 μM 2-APB did not cause further reduction of spark frequency (ryanodine: 0.06 ± 0.02 s⁻¹, 24 cells, 15 sparks; 2-APB + ryanodine: 0.02 ± 0.01 s⁻¹, 21 cells, 5 sparks). Moreover, 100 μM xestospongin C, another inhibitor of IP₃ receptors, also failed to inhibit Ca²⁺ sparks (Zhang and Sham, unpublished data).

To further determine if the occurrence of PASMC Ca²⁺ sparks could be enhanced by activating RyRs, a subthreshold (0.5 mM) concentration of caffeine was used to provide moderate stimulation of RyRs without evoking regenerative Ca²⁺ release and depletion of SR Ca²⁺ stores. In different cells and/or scans, caffeine activated readily discernible individual sparks, clusters of Ca²⁺ sparks, and in some cases even G-Ca increases during which Ca²⁺ sparks were not discernible (Fig. 5A). Caffeine caused a significant increase in spark frequency from 0.30 ± 0.04 s⁻¹ (152 cells, 423 sparks) to 1.07 ± 0.21 s⁻¹ (23 cells, 251 sparks; P < 0.05). Caffeine did not alter the spark amplitude (control = 0.51 ± 0.01; caffeine = 0.55 ± 0.01) but significantly prolonged the duration (control = 35.4 ± 1.0 ms; caffeine = 45.5 ± 1.9 ms; P < 0.05) and the size of sparks (control = 1.55 ± 0.03 μm; caffeine = 2.04 ± 0.06 μm; P < 0.05). The increase in spark frequency induced by caffeine was completely blocked by 10 μM ryanodine (0.30 ± 0.09 s⁻¹, 22 cells, 66 sparks; Fig. 5B). All of these observations indicate that spontaneous Ca²⁺ sparks are mediated exclusively by RyRs, whereas IP₃-sensitive Ca²⁺ stores are not involved in the process.

Effects of Ca²⁺ channel activity and SR loading on Ca²⁺ sparks. Under physiological conditions, Ca²⁺ is the major stimulus for RyR activation, and the L-type voltage-gated Ca²⁺ channel is the major pathway of Ca²⁺ influx in PASMCs. To understand the relationship between sarcolemmal Ca²⁺ channel activity and Ca²⁺ sparks, we first examined if spontaneous Ca²⁺ spark activity was related to the activation of RyRs by Ca²⁺ influx via Ca²⁺ channels. Inhibition of Ca²⁺ channels by 10 μM nifedipine (>15 min exposure; Fig. 6A) did not alter the frequency (control = 0.38 ± 0.07 s⁻¹, 38 cells, 142 sparks; nifedipine = 0.24 ± 0.06 s⁻¹, 42 cells, 110 sparks) or duration of spontaneous Ca²⁺ sparks (Table 1).
sparks (control = 41.8 ± 2.0 ms; nifedipine = 40.3 ± 2.1 ms) but slightly augmented the amplitude (control = 0.46 ± 0.01; nifedipine = 0.54 ± 0.02; P < 0.05) and size (control = 1.62 ± 0.05 μm; nifedipine = 1.99 ± 0.08 μm; P < 0.05) of Ca²⁺ sparks. The increase in ΔF/F₀ could be because of a decrease in the resting [Ca²⁺] (F₀) in the presence of nifedipine, in which case an equivalent Ca²⁺ release (ΔF) would result in a greater ΔF/F₀. Nonetheless, under resting conditions, Ca²⁺ influx via Ca²⁺ channels did not seem to contribute to the activation of spontaneous Ca²⁺ sparks in PASMCs.

To examine whether enhancing Ca²⁺ influx via Ca²⁺ channels could activate Ca²⁺ sparks, Ca²⁺ channel activity was increased with BAY K 8644, an L-type Ca²⁺ channel agonist, or a depolarizing external solution containing 20 mM K⁺. Application of 3 μM BAY K 8644 caused a significant increase in Ca²⁺ spark frequency (Fig. 6B) from 0.26 ± 0.05 s⁻¹ in control (67 cells, 164 sparks) to 0.53 ± 0.13 s⁻¹ (59 cells, 285 sparks; P < 0.05). In addition, global increases in [Ca²⁺] or Ca²⁺ oscillations were observed more frequently in BAY K 8644-treated cells (control = 0.09 ± 0.02 s⁻¹; BAY K 8644 = 0.26 ± 0.03 s⁻¹; P < 0.05). The enhancement in spark frequency and Ca²⁺ oscillations was completely reversed by the addition of 10 μM nifedipine (32 cells, 69 sparks). BAY K 8644 had no noticeable effect on the amplitude, duration, or size of Ca²⁺ sparks. Similarly, increasing external K⁺ concentration ([K⁺]) from 5.4 (control) to 20 (Fig. 6C) mM caused a significant increase in spark frequency from a control value of 0.38 ± 0.09 s⁻¹ (45 cells, 170 sparks) to 0.76 ± 0.13 s⁻¹ (49 cells, 377 sparks; P < 0.05) without altering the spark amplitude and duration. Spark size was also increased slightly by 20 mM K⁺ from 1.85 ± 0.07 to 2.02 ± 0.04 μm (P < 0.05).

Furthermore, the effects of increasing global Ca²⁺ influx on Ca²⁺ sparks were examined by increasing external [Ca²⁺] from 2 to 10 mM (Fig. 6D). A high concentration of external Ca²⁺ evoked significant increases in spark frequency (2 mM = 0.30 ± 0.04 s⁻¹, 152 cells, 423 sparks; 10 mM = 1.07 ± 0.22 s⁻¹, 28 cells, 307 sparks; P < 0.05), size (2 mM = 1.54 ± 0.03 μm; 10 mM = 1.99 ± 0.05 μm; P < 0.05), and amplitude (2 mM = 0.51 ± 0.01; 10 mM = 0.58 ± 0.01; P < 0.05) without significantly altering the duration of Ca²⁺ sparks. These observations consistently indicate that enhancing Ca²⁺ influx by increasing either L-type Ca²⁺ channel activity or the driving force for Ca²⁺ influx could effectively activate Ca²⁺ sparks in PASMCs.

Ca²⁺ sparks and membrane potential. The modulatory effect of Ca²⁺ sparks on the membrane potential of PASMCs was examined using the perforated-patch technique to avoid disturbing subcellular Ca²⁺ dynamics. Under control conditions, the membrane potential of PASMCs appeared to be less quiescent when measured using the perforated-patch technique compared with those measured under the conventional whole cell configuration in our previous studies (55); small sporadic depolarizations were occasionally observed. Application of a subthreshold concentration of caffeine (0.5 mM) to PASMCs, which had enhanced the occurrence of Ca²⁺ sparks in prior experiments, caused an immediate membrane depolarization in 8 out of 10 cells (Fig. 7), from an average resting membrane potential of −45.2 ± 5.4 mV to a slightly depolarized potential of −37.8 ± 5.9 mV (10 cells, P < 0.003). In contrast, in PASMCs pretreated with 50 μM ryanodine for 15 min to inhibit Ca²⁺ sparks, application of caffeine did not alter membrane potential in all six cells tested (before caffeine: −38.5 ± 5.8 mV; with caffeine: −40.3 ± 6.0 mV). These results suggested that activation of Ca²⁺ sparks in PASMCs could lead to membrane depolarization, as opposed to the hyperpolarization observed previously in systemic arterial smooth muscle cells (23, 27, 29, 39, 40, 43).

Vasoconstrictors and Ca²⁺ sparks. Because PASMCs are under the influences of a wide variety of vasoactive factors, some of these vasoactive factors may exert their effects in part by modulating Ca²⁺ sparks. To test this possibility, the effects of two vasoconstricting agents, NE and ET-1, on Ca²⁺ sparks were examined. Application of 10 nM NE (Fig. 8A) elicited a global increase in [Ca²⁺]; however, under steady-state conditions, there was a decrease in the spark frequency from a control of 0.39 ± 0.09 s⁻¹ (40 cells, 151 sparks) to 0.17 ± 0.06 s⁻¹ (30 cells, 53 sparks, P = 0.053). There was also a slight increase in the amplitude (control = 0.45 ± 0.02; NE = 0.55 ± 0.03, P < 0.005) and the size (control = 1.60 ± 0.04 μm; NE = 1.86 ± 0.1 μm, P <
significant lower spark frequency and amplitude in sparks of PASMCs and cardiac myocytes were the same. In contrast, RyRs are distributed diffusely in cardiac myocytes, cytoplasmic Ca$^{2+}$ is removed by reuptake in the SR via a Ca$^{2+}$-ATPase (20) and sarcolemmal extrusion via Na$^{+}$/Ca$^{2+}$ exchange (3, 55). Although the relative contribution of Ca$^{2+}$ removal systems on spark duration has not been investigated systematically in smooth muscle, the near-identical spark duration in PASMCs and cardiac myocytes suggests that the combined influence of Ca$^{2+}$ diffusion and removal on Ca$^{2+}$ spark duration is very similar in the two cell types. Recent theoretical studies have suggested that spark size is determined by the magnitude of SR Ca$^{2+}$ release fluxes (24), in addition to the cytosolic Ca$^{2+}$ diffusion and buffering capacity (20, 62). The slightly smaller spark size in PASMCs is consistent with this notion because their amplitude is several times smaller than that of cardiac myocytes.

Pulmonary vs. cardiac sparks. In the present study, we have provided definitive evidence showing that Ca$^{2+}$ sparks are present in rat intralobar PASMCs. The biophysical characteristics of these pulmonary Ca$^{2+}$ sparks are first evaluated with those of cardiac Ca$^{2+}$ sparks as the benchmark for comparison (12). The major differences between spontaneous Ca$^{2+}$ sparks of PASMCs and cardiac myocytes were the significantly lower spark frequency and amplitude in PASMCs. The 70–80% lower spark frequency in PASMCs is likely related to a smaller number of individual or clustered RyRs. In cardiac myocytes, diadic junctions are abundant, covering ~50% of the cytoplasmic surface of the transverse tubules (42), and the number of RyRs is estimated to be as high as 10$^6$/cell (12). In contrast, RyRs are distributed diffusely in vascular smooth muscle, associating with the sparsely located peripheral junctions and the mesh-like central SR network (33). Radioligand binding studies have shown that the number of RyRs per gram of tissue is several times lower in smooth muscle than in cardiac muscle (38, 66). Electron micrographs have also revealed that the density of “foot processes” (RyRs) in the diadic junctions of arterial smooth muscle is less than in striated muscles (33), suggesting that there are fewer RyRs within a release unit. Moreover, electron-probe X-ray microanalyses have indicated that SR Ca$^{2+}$ content is less in vascular smooth muscle than in cardiac muscle (31, 32). The combination of fewer RyRs and lower SR Ca$^{2+}$ content in PASMCs could restrict the activity of RyRs and the amount of Ca$^{2+}$ released from an SR Ca$^{2+}$ release unit, resulting in lower spark frequency and amplitude (50). Furthermore, differential modulation of basal RyR activity and open time duration by kinase-dependent processes (7, 44) might also contribute to the differences in the frequency and amplitude of PASMC and cardiac sparks, even though the RyR isoforms involved are presumably the same.

Despite the differences in spark frequency and amplitude, there was no difference in spark duration and only a minor difference in spark size between PASMCs and cardiac cells. Previous studies have suggested that Ca$^{2+}$ spark duration is determined predominantly by Ca$^{2+}$ diffusion (~75%) and, to a lesser extent, by removal of Ca$^{2+}$ from the cytoplasm (20). In cardiac myocytes, cytoplasmic Ca$^{2+}$ is removed by reuptake in the SR via a Ca$^{2+}$-ATPase (20) and sarcolemmal extrusion via Na$^{+}$/Ca$^{2+}$ exchange (3, 55). Although the relative contribution of Ca$^{2+}$ removal systems on spark duration has not been investigated systematically in smooth muscle, the near-identical spark duration in PASMCs and cardiac myocytes suggests that the combined influence of Ca$^{2+}$ diffusion and removal on Ca$^{2+}$ spark duration is very similar in the two cell types. Recent theoretical studies have suggested that spark size is determined by the magnitude of SR Ca$^{2+}$ release fluxes (24), in addition to the cytosolic Ca$^{2+}$ diffusion and buffering capacity (20, 62). The slightly smaller spark size in PASMCs is consistent with this notion because their amplitude is several times smaller than that of cardiac myocytes.

PASMCs vs. other smooth muscle cells. Even though Ca$^{2+}$ sparks have not been studied in detail in PASMCs (30), they have been examined in a variety of vascular smooth muscle cells from the cerebral arteriole (28, 35, 40), coronary artery (16), portal vein (2, 39), and mesenteric artery (6), as well as nonvascular smooth muscle cells from the trachea (41, 72), ileum (6, 21), and urinary bladder (23). Similar to our results in rat PASMCs, resting spontaneous spark frequency is generally low in smooth muscle cells. It ranges between 0.1 and 0.4 s$^{-1}$ [with the exception of Lohn et al.
(35), \( \sim 0.9 \text{ s}^{-1} \); see Refs. 7, 39, 44] when measured using a scan line of length similar to our study and between 0.1 and 0.7 cells/s when recorded using high-speed frame scanning (19, 23, 28, 30, 41, 43). A somewhat higher spark frequency has been recorded in intact mesenteric arteries (0.025 \( \mu \text{m/s} \); see Ref. 37). In addition, \( \text{Ca}^{2+} \) spark frequency may be further enhanced by an increase in intraluminal pressure, as reported in cerebral artery preparations (40). The spark frequency (\( \sim 0.3 \text{ s}^{-1} \) or 0.01 \( \mu \text{m/s} \)) recorded in our rat PASMCs is well within the range reported in the above-mentioned studies, suggesting that the number of \( \text{Ca}^{2+} \) release sites and the activity of RyRs are similar in PASMCs and other systemic smooth muscle cells.

In contrast, spark amplitudes are variable among different types of smooth muscle cells. Averaged spark amplitudes, in terms of \( \Delta[\text{Ca}^{2+}]_{i} \), vary from 30 nM in rat portal vein (2, 39) to 200–300 nM in rat cerebral artery (7, 28, 35, 40, 43) and guinea pig urinary bladder.
smooth muscle cells (23). The average PASMC spark amplitude of 75 nM recorded in this study is similar to the 50–80 nM measured in airway smooth muscle cells (41, 60). The large variability of Ca$^{2+}$ spark amplitude suggests significant heterogeneity of the ryanodine-sensitive SR Ca$^{2+}$ stores in different smooth muscles.

The average duration (35 ms) and size (1.6 μm) of Ca$^{2+}$ sparks in our PASMCs is similar to those of other smooth muscles, ranging from 30 to 65 ms (2, 6, 7, 30, 35, 39, 40, 43) and 1.5 to 2.4 μm (2, 6, 19, 21, 28, 39, 40), respectively. However, there are reports of Ca$^{2+}$ sparks of much longer duration (100–600 ms) in tracheal and urinary bladder smooth muscle cells (23, 60, 72) and much larger size (3–4 μm) in cerebral arterial smooth muscle cells (7, 35). It is interesting to note that microsparks of much smaller amplitude, size, and duration have been found along with the typical Ca$^{2+}$ sparks in guinea pig ileum smooth muscle cells, possibly representing the Ca$^{2+}$ release signals arising from single RyRs (6, 21). However, we could not confirm the existence of microsparks in rat PASMCs under our experimental settings.

**Origin of spontaneous Ca$^{2+}$ sparks.** Consistent with previous studies in other cell types (9, 12, 27, 40), our results indicate that spontaneous Ca$^{2+}$ sparks in rat PASMCs originate exclusively from ryanodine-sensitive SR Ca$^{2+}$ stores. This is based on evidence that 1) ryanodine concentration dependently abolished spontaneous Ca$^{2+}$ sparks, and 2) enhancement of RyR activity using a subthreshold concentration of caffeine increased Ca$^{2+}$ spark frequency, an effect reversed by ryanodine. The IP$_3$-sensitive Ca$^{2+}$ store is supposed to be the major intracellular Ca$^{2+}$ store in smooth muscle cells (63, 66), and local Ca$^{2+}$ release events (known as “Ca$^{2+}$ puffs”) originating from IP$_3$ receptors have been identified in nonvascular smooth muscle cells (5). The finding that ryanodine completely abolishes spontaneous Ca$^{2+}$ sparks and that 2-APB or xestospongin C does not significantly alter spark frequency suggests that IP$_3$-sensitive Ca$^{2+}$ stores do not contribute to the spontaneous local Ca$^{2+}$ release in PASMCs under resting conditions. However, enhanced IP$_3$ production during an agonist stimulation in smooth muscle may elicit additional local Ca$^{2+}$ release events via IP$_3$ receptors in the form of Ca$^{2+}$ puffs or Ca$^{2+}$ waves (5, 30), depending on the spatial association, activation, and coordination of IP$_3$ receptor release channels (8). We noted that a higher concentration of ryanodine (50 μM) was required to completely block PASMC sparks. This may be because of the lower activity of RyRs in resting PASMCs than in cardiac myocytes, since ryanodine blocks the receptors only at the open state. A similar high concentration of ryanodine was found necessary to block ryanodine-sensitive stores in other smooth muscle preparations (30).

**Sarcotemmal Ca$^{2+}$ channel activation and Ca$^{2+}$ sparks.** In cardiac myocytes, L-type Ca$^{2+}$ channels are closely associated with and are functionally coupled to a cluster of RyRs, forming a Ca$^{2+}$ release unit (52, 53). Upon stimulation, Ca$^{2+}$ influx through these Ca$^{2+}$ channels increases local [Ca$^{2+}$] in the junctional clefts, activating RyRs via the Ca$^{2+}$-induced Ca$^{2+}$ release mechanism and generating Ca$^{2+}$ sparks (59). In quiescent cardiac cells, however, the generation of spontaneous sparks does not appear to require Ca$^{2+}$ influx through L-type Ca$^{2+}$ channels (12). In our experiments, the occurrence of Ca$^{2+}$ spark was not significantly affected by nifedipine, suggesting that spontaneous Ca$^{2+}$ sparks in resting PASMCs are not triggered by an L-type Ca$^{2+}$ current but are initiated by the spontaneous stochastic activity of RyRs instead. This concurs with previous observations in cardiac and other smooth muscle cells (2, 6, 14, 21, 39–41, 60).

In contrast, enhanced Ca$^{2+}$ influx through Ca$^{2+}$ channels can effectively modulate Ca$^{2+}$ spark frequency in PASMCs. This was demonstrated unequivocally since enhancing Ca$^{2+}$ influx by 1) direct activation of Ca$^{2+}$ channels with a Ca$^{2+}$ channel agonist, 2) membrane depolarization (elevated [K$^+$]), and 3) increasing extracellular [Ca$^{2+}$] all enhanced Ca$^{2+}$ spark frequency. The twofold increase in Ca$^{2+}$ spark frequency induced with BAY K 8644 and with 20 mM extracellular K$^+$ concentration ([K$^+$]) was reversed by nifedipine, clearly indicating that Ca$^{2+}$ influx through Ca$^{2+}$ channels activated Ca$^{2+}$ sparks rather than activation of RyRs by a nonspecific effect of BAY K 8644 (51) or by reverse Na$^+$/Ca$^{2+}$ exchange at depolarized potentials (50). These results are in agreement with studies on other smooth muscle cells showing that both BAY K 8644 (21, 40) and 30 mM [K$^+$], (19, 28) enhanced Ca$^{2+}$ spark frequency.

Our results, however, do not discriminate between the possibility that Ca$^{2+}$ influx via Ca$^{2+}$ channels activates PASMC RyRs directly because of close coupling of the two sets of channels, as in ventricular myocytes, or indirectly via increasing SR Ca$^{2+}$ load (11, 50, 73). Circumstantial evidence has suggested that Ca$^{2+}$ channels and RyRs may be colocalized in caveolae in cerebral smooth muscle cells and that Ca$^{2+}$ sparks may arise within these regions (29, 35, 43). Voltage-clamp experiments in portal vein and mesenteric smooth muscle cells showed that depolarizing pulses could trigger Ca$^{2+}$ sparks (2, 6, 15). However, similar experiments in urinary bladder smooth muscle cells showed that Ca$^{2+}$ sparks triggered by Ca$^{2+}$ currents occurred with a longer latency and that the efficacy of Ca$^{2+}$ current to activate Ca$^{2+}$ sparks was related to an increase in global [Ca$^{2+}$], suggesting “loose” coupling or uncoupling of Ca$^{2+}$ channels and RyRs in these smooth muscle cells (14). The type of coupling between Ca$^{2+}$ channels and RyRs, whether tight or loose, has yet to be determined in PASMCs and warrants further investigation. Nevertheless, increasing Ca$^{2+}$ influx without enhancing Ca$^{2+}$ channel activity, e.g., by elevating external [Ca$^{2+}$] (Fig. 6D and Refs. 11, 62, 73), can enhance Ca$^{2+}$ spark occurrence. This raises the possibility that other Ca$^{2+}$ entry pathways, such as reverse Na$^+$/Ca$^{2+}$ exchange, receptor-operated Ca$^{2+}$ channels, capacitative Ca$^{2+}$ entry, and nonsel...
Physiological functions of Ca\(^{2+}\) sparks in PASMCs.

Despite the similarity in their basic properties, our results suggest that the physiological functions of Ca\(^{2+}\) sparks may be quite different in pulmonary and systemic vascular myocytes. In systemic vascular smooth muscle cells, it has been proposed that Ca\(^{2+}\) sparks modulate membrane potential by activating K\(_{Ca}\) channels to generate STOCs, leading to membrane hyperpolarization, closure of L-type Ca\(^{2+}\) channels, decreased [Ca\(^{2+}\)], and vasodilatation (23, 27, 29, 39, 40, 43). In contrast, we found that activation of Ca\(^{2+}\) sparks in PASMCs by a subthreshold concentration of caffeine elicited a small but consistent membrane depolarization, which was completely abolished by ryanodine. This raises the possibility that sparks may contribute to vasoconstriction, rather than vasorelaxation, in PASMCs. The disparity from systemic vessels could be because of a diminished influence of K\(_{Ca}\) channels on membrane potential in adult PASMCs. Previous studies showed that inhibition of K\(_{Ca}\) channels with charybotoxin or tetraethylammonium had no effect on resting membrane potential in adult rat PASMCs (1, 56, 68), resting tension of isolated vessels (67), or baseline perfusion pressure in isolated perfused lungs (22). Recent developmental studies have shown that Ca\(^{2+}\) sparks and STOCs are very active in fetal PASMCs (45, 46). However, the occurrence of STOCs, the responses of [Ca\(^{2+}\)]\(_i\) to iberyotoxin, and the expression of K\(_{Ca}\) channel proteins and mRNA in distal PASMCs diminish with maturation (46, 48). The lower K\(_{Ca}\) channel expression in adult PASMCs may therefore compromise the ability of the Ca\(^{2+}\) spark to induce membrane hyperpolarization. However, K\(_{Ca}\) currents have been unequivocally demonstrated in adult PASMCs of different species, including rats (57, 70). It is possible that K\(_{Ca}\) channels, in addition to their lower channel protein expression, are coupled less efficiently to RyRs in adult PASMCs. Moreover, prominent Cl\(_{Ca}\) currents (STICs) have been found in PASMCs (69). Activation of Cl\(_{Ca}\) channels similar to those observed in tracheal smooth muscle cells (72), and, perhaps, modulation of other Ca\(^{2+}\)-sensitive ion transport processes (such as inhibition of voltage-gated K\(^+\) (K\(_V\)) channels (18) and Ca\(^{2+}\) extrusion by Na\(^+\)/Ca\(^{2+}\) exchange (4)) by Ca\(^{2+}\) sparks might override the compromised hyperpolarization of K\(_{Ca}\) channels leading to membrane depolarization. However, the exact contributions of K\(_{Ca}\), Cl\(_{Ca}\), and other channels or transporters to the membrane depolarization induced by Ca\(^{2+}\) sparks in PASMCs require future investigation. Additionally, it has been shown that expressions of K\(_V\) and Cl\(_{Ca}\) channels are more prominent and K\(_{Ca}\) channel expression is much reduced in PASMCs of the distal resistant artery (1, 61). Because our PASMCs were isolated from relatively proximal arteries, it is possible that Ca\(^{2+}\) spark may elicit an even greater depolarization in distal pulmonary arteries.

Under basal conditions, Ca\(^{2+}\) sparks are unlikely to elicit vasoconstriction because spark frequency is low. However, they may contribute to pulmonary vascular reactivity during active vasoconstriction. Our experiments using NE and ET-1 showed, for the first time [see preliminary reports (71)], that ET-1 caused a significant increase in Ca\(^{2+}\) spark occurrence in PASMCs. This is consistent with previous findings in rat PASMCs that ET-1 activated STOCs and STICs (likely activated by Ca\(^{2+}\) sparks), both of whose currents were abolished by pretreatment with caffeine (49). The increase in spark frequency by ET-1 may contribute to the ET-1-induced vasoconstriction by enhancing Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels as the result of membrane depolarization and may even by providing Ca\(^{2+}\) for direct activation of myofilaments. The enhancement of Ca\(^{2+}\) spark frequency by ET-1 appears to be agonist specific because NE, which caused a significant increase in global [Ca\(^{2+}\)], decreased rather than enhanced spark frequency in PASMCs. The reduction of Ca\(^{2+}\) sparks by NE is similar to that in systemic vascular smooth muscle where vasoconstrictors typically reduce Ca\(^{2+}\) spark frequency via a protein kinase C-dependent mechanism (7, 26, 37). The differential regulation of Ca\(^{2+}\) sparks in PASMCs by vasoactive agonists may provide a mechanism for the agonists to perform specific physiological functions in addition to vasoconstriction. It is interesting to note that ET-1 has been implicated as an important modulator/mediator of acute and chronic hypoxic pulmonary vasoconstriction (10, 54) and that acute hypoxic responses in PASMCs have been abolished by ryanodine in several studies (17, 25, 34, 65). This certainly raises the speculation that ET-1 (or other agonists)-specific regulation of Ca\(^{2+}\) sparks may be involved in hypoxic responses of PASMCs.

In conclusion, we have provided a detailed characterization of the physiological properties and functions of Ca\(^{2+}\) sparks in PASMCs (see Table 2 for summary). We have found that the spatial and temporal properties of these sparks are similar to those observed in systemic vascular smooth muscle cells. However, their regulations and functions appear to be different and may play specific roles in the control of pulmonary vascular reactivity.

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CA\(^{2+}\) SPARKS IN RAT PASMCs


