Published in final edited form as:

J Mol Cell Cardiol. 2009 October ; 47(4): 436–444. doi:10.1016/j.yjmcc.2009.07.008.

Molecular and Biophysical Mechanisms of Ca²⁺ sparklets in Smooth Muscle

Luis F. Santana and Manuel F. Navedo

Department of Physiology & Biophysics, University of Washington, Box 357290, Seattle, WA USA 98195

Abstract

In this article, we review the biophysical basis and functional implications of a novel Ca²⁺ signal (called “Ca²⁺ sparklets”) produced by Ca²⁺ influx via L-type Ca²⁺ channels (LTCCs) in smooth muscle. Ca²⁺ sparklet activity is bimodal. In low activity mode, Ca²⁺ sparklets are produced by random, brief openings of solitary LTCCs. In contrast, small clusters of LTCCs can function in a high activity mode that create sites of continual Ca²⁺ influx called “persistent Ca²⁺ sparklets”. Low activity and persistent Ca²⁺ sparklets contribute to Ca²⁺ influx in arterial, colonic, and venous smooth muscle. Targeting of PKC α by the scaffolding protein AKAP150 to specific sarcolemmal domains is required for the activation of persistent Ca²⁺ sparklets. Calcineurin, which is also associated with AKAP150, opposes the actions of PKC α on Ca²⁺ sparklets. At hyperpolarized potentials, Ca²⁺ sparklet activity is low and hence does not contribute to global [Ca²⁺]_i. Membrane depolarization increases low and persistent Ca²⁺ sparklet activity, thereby increasing local and global [Ca²⁺]_i. Ca²⁺ sparklet activity is increased in arterial myocytes during hypertension, thus increasing Ca²⁺ influx and activating the transcription factor NFATc3. We discuss a model for subcellular variations in Ca²⁺ sparklet activity and their role in the regulation of excitation-contraction coupling and excitation-transcription coupling in smooth muscle.

Keywords

portal vein; Ca²⁺ sparklets; PKC; AKAP; arterial smooth muscle; NFAT

1. Introduction

L-type Ca²⁺ channels (LTCCs) play a critical role in the regulation of excitability, contraction, and gene expression in smooth muscle [1-6]. Ca²⁺ influx through these channels produce subcellular Ca²⁺ signals called “Ca²⁺ sparklets” [7,8]. In this review, we examine strategies for the recording, detection, and analysis of Ca²⁺ sparklets. Furthermore, we discuss recent work indicating that Ca²⁺ sparklet activity varies within the sarcolemma of smooth muscle cells and examine the potential mechanisms for these regional variations in LTCC activity. We conclude the review with a model that incorporates these findings and evaluate their implications on the regulation of local and global [Ca²⁺]_i as well as gene expression in smooth muscle under physiological and pathophysiological conditions.

Corresponding author: LFS (santana@u.washington.edu), Tel. 206-543-8681, Fax. 206-685-0619.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

2. Recording Ca^{2+} sparklets in smooth muscle cells

First, we would like to discuss some technical considerations related to the recording of Ca^{2+} sparklets. This is important because optical recording of Ca^{2+} sparklets and related Ca^{2+} influx events is a relatively new approach with great potential, but with associated complexities that are worth discussing for those interested in using these approaches. Due to space limitations, our discussion will be brief. Readers interested in a more extensive discussion of these issues are encouraged to read other papers by our group [8-10] and excellent articles by Ian Parker's group [11,12].

Recent advances in the imaging field have allowed investigators to record relatively small Ca^{2+} signals from single sarcolemmal Ca^{2+} channels with unprecedented speed and spatial resolution [9,11]. One particular example is the development of electron multiplying charged-coupled device (EMCCD) cameras. These cameras have quantum efficiencies >90% and are thus capable of detecting photons emitted from a single fluorescence molecule. EMCCD cameras can be used to acquire ultra fast (e.g. 100-500 Hz) 2-dimensional images of dim fluorescence events. This is particularly attractive for investigators interested in imaging rare and low amplitude Ca^{2+} signals like Ca^{2+} sparklets, as one could image relatively large areas of the cell (compared to a confocal line-scan imaging) at relatively fast acquisition rates and with high sensitivity. Indeed, EMCCD camera-based imaging systems — confocal and evanescent field total internal reflection fluorescence (TIRF) microscope — have recently been used to image Ca^{2+} influx events via single Ca^{2+} channels in multiple cell types [7,8, 11,13-16]. Figure 1A shows images of Ca^{2+} sparklets in arterial and portal vein smooth muscle obtained with an EMCCD camera (Navedo and Santana, unpublished work). Ca^{2+} sparklets have also been recorded in colon smooth muscle using TIRF microscopy [15].

To record Ca^{2+} sparklets, we use the following experimental conditions [8-10]. We begin experiments by allowing acutely dissociated smooth muscle cells to settle and adhere to the coverslip of a perfusion chamber. This is especially critical for those experiments involving TIRF microscopy in which one needs a relatively large portion of the cell within the evanescent field created during TIRF in order to increase the probability of observing Ca^{2+} sparklet sites. We strongly recommend that cells are patch-clamped in the whole-cell configuration so that membrane potential, and hence the open probability of LTCCs and the driving force for Ca^{2+} influx are controlled. Using the whole-cell patch-clamp technique in its conventional configuration has an additional advantage: it allows the introduction into the cell of a solution containing the relatively fast fluorescent Ca^{2+} indicator Fluo-5F (200 μM ; penta-potassium salt) and the slower and non-fluorescent Ca^{2+} buffer EGTA (10 mM).

We use this combination of Fluo-5F and EGTA to record Ca^{2+} sparklets for two important reasons. **First**, Ca^{2+} influx events produce a bright fluorescence signal that is confined to the volume near the site of entry [17]. This happens because as Ca^{2+} enters the cell it preferentially binds the fast Fluo-5F molecules, thus increasing their fluorescence. However, this fluorescence is short-lived, as Ca^{2+} dissociates from Fluo-5F and binds to the more abundant and non-fluorescent EGTA. Using this combination of Fluo-5F and EGTA, we have found that the average area of a Ca^{2+} sparklet was small: $\approx 0.8 \mu\text{m}^2$. **Second**, by using high concentrations of EGTA, global $[\text{Ca}^{2+}]_i$ is maintained relatively low, thus decreasing background fluorescence. This, in combination with the thin optical section ($\approx 100 \text{ nm}$ with a 1.45 NA lens and 488 nm excitation light [18]) of the evanescent field during TIRF microscopy minimizes background fluorescence, thus increasing the signal-to-noise ratio of this system and hence one's ability to detect small amplitude Ca^{2+} signals.

A limitation of TIRF microscopy is that imaging is restricted to the region of the cell within the fixed evanescent field that decays exponentially from the coverslip. The use of a confocal

microscope could overcome this limitation. Note, however, that the thicker optical section of the confocal microscope — typically about 1 μm in the z-axis (≈ 10 -fold thicker than in TIRF) — will increase background fluorescence, potentially limiting detection of small Ca^{2+} sparklets events.

Although we [10], and others [15], have been able to record Ca^{2+} sparklets in the absence of an exogenous Ca^{2+} buffer such as EGTA in smooth muscle cells, at least in our hands, recording small Ca^{2+} sparklet events under these experimental conditions has been very difficult. Thus, whenever possible, we recommend that the combination of Fluo-5F and EGTA is used to record Ca^{2+} sparklets.

3. L-type Cav1.2 channels underlie Ca^{2+} sparklets in arterial smooth muscle

Molecular identification of ion channels is based on pharmacological, biophysical, and molecular biological criteria. Thus, Ca^{2+} sparklets meet all the generally accepted pharmacological, biophysical, and molecular biological criteria used to identify LTCCs. Ca^{2+} sparklets are activated by the dihydropyridine agonist Bay-K 8644 and are eliminated by the dihydropyridine antagonists nifedipine and nisoldipine [10,14,15]. Simultaneous recordings of Ca^{2+} signals and LTCC currents indicate that Ca^{2+} sparklets are associated with an inward Ca^{2+} current (Figure 1B). Importantly, Ca^{2+} sparklets have similar voltage dependencies of activity and amplitude as LTCCs. Furthermore, Ca^{2+} sparklets in tsA-201 cells expressing L-type Cav1.2 channels reproduce all the basic features of native Ca^{2+} sparklets including block by nifedipine, activation by Bay-K 8644, bimodal gating modalities, amplitude of quantal event and voltage dependencies (Table 1) [9]. Finally, arterial myocytes expressing a mutant Cav1.2 channel that is insensitive to inhibition by dihydropyridines [19, 20], produced dihydropyridine-insensitive low activity and persistent Ca^{2+} sparklets [14]. This finding is important because it eliminates the possibility that Ca^{2+} sparklets are produced by a TRP or store-operated channel in arterial myocytes.

4. Ca^{2+} sparklet activity is quantal

As Heping Cheng's group demonstrated for Ca^{2+} sparks [21], manual detection of small amplitude Ca^{2+} signals such as Ca^{2+} sparks and Ca^{2+} sparklets is difficult and prone to bias. Thus, we developed an algorithm for the automatic detection of Ca^{2+} sparklets [8,9]. The software, written in IDL language, is available to any investigator interested in studying Ca^{2+} sparklets and can be requested via email.

Briefly, the program uses spatial, temporal, and amplitude criteria for the detection of Ca^{2+} sparklet sites and events. The program has an extremely low ($< 5\%$) false-positive rate of Ca^{2+} sparklet detection. We highly recommend that this or similar automatic detection methods [21-24] be used over a visual detection approach to facilitate the identification and later classification of Ca^{2+} sparklet sites. Not using an automatic detection algorithm may lead to underestimation of Ca^{2+} sparklet events and to human bias towards rare, high amplitude, and high activity events [21].

Using the imaging and analytical approaches described above, we recorded Ca^{2+} sparklets in the presence of 2 mM external Ca^{2+} at -70 mV and physiological -40 mV [10]. Figure 2A shows an all-points histogram generated from representative blank TIRF $[\text{Ca}^{2+}]_i$ records. This histogram shows a large peak at a change in $[\text{Ca}^{2+}]_i$ of 0, which represents the mean basal $[\text{Ca}^{2+}]_i$. This peak could be fitted with a Gaussian function with a mean of 0 and standard deviation (SD) of 6.1 nM. The width of this peak is useful because it provides an indication of the “noise” of our system, which we used to set a threshold of mean basal of $\Delta[\text{Ca}^{2+}]_i$ (i.e. $0 + 3 * \text{SD}$ (i.e. 18 nM) for the detection of Ca^{2+} sparklets. Readers will appreciate that the width of this peak, and hence the amplitude detection threshold, would critically depend on

experimental conditions. For example, conditions that increase background fluorescence (e.g. absence of intracellular EGTA or epifluorescence imaging) would increase the baseline SD, therefore decreasing one's ability to resolve small amplitude Ca^{2+} sparklets.

At -70 mV and with 2 mM external Ca^{2+} , Ca^{2+} sparklet amplitude range from 18 to 280 nM [8]. Note, however, that under these experimental conditions the vast majority of these events had amplitudes close to or at the amplitude detection threshold of 18 nM, suggesting frequent sub-threshold events. This is the reason why we decided to increase the driving force for Ca^{2+} influx by increasing external Ca^{2+} from 2 to 20 mM. In Figure 2B, we show an all-points histogram generated from representative Ca^{2+} sparklet records obtained using 20 mM external Ca^{2+} . The histogram could be fit with a multi-component Gaussian function with a quantal unit of Ca^{2+} influx of ≈ 35 nM. Furthermore, note that the amplitude of the various peaks of the histogram progressively decreases with amplitude, suggesting that Ca^{2+} entry through Ca^{2+} sparklet sites is quantal in nature and that multi-quantal events “likely arise from random overlapping openings of adjacent” LTCCs [8].

Two observations suggest that quantal Ca^{2+} sparklets likely result from the opening of a single LTCC. First, simultaneous recordings of Ca^{2+} signals and membrane currents indicate that quantal Ca^{2+} sparklets are associated with elementary LTCC currents (Figure 1B) [8]. Second, consistent with these imaging and electrophysiological data, analysis of the signal mass (i.e. total Ca^{2+} charge in coulombs) of Ca^{2+} sparklets suggest that, at least in principle, these signals could be produced by the opening of a single LTCC. Briefly, the smallest and briefest Ca^{2+} sparklets detected at -40 mV with 2 mM external Ca^{2+} had a signal mass of 7 fC (Table 1) [10]. This signal mass could be produced by a single LTCC channel producing a -0.2 pA current [25] with a duration of 35 ms: $0.2 \times 10^{-12} \text{ C/s} \times 0.035 \text{ s} = 7 \times 10^{-15} \text{ C}$.

5. Smooth muscle cells have low and high activity, persistent Ca^{2+} sparklets

An interesting feature of Ca^{2+} sparklets is that their activity is heterogeneous [8,9]. Tour et al. [13] and McCarron et al. [15] recently reported similar findings in Hela cells expressing Cav1.2 channels and colonic smooth muscle, respectively. This has important functional implications, but also poses analytical challenges. Let us deal with the later issue first. We developed a method for the quantitative analysis of sparklet activity. Two important features of Ca^{2+} sparklets helped us develop an analytical framework for the quantification of Ca^{2+} sparklet activity: (a) Ca^{2+} sparklet activity is quantal and (b) under TIRF and with Fluo-5F/EGTA these records resemble single channel recordings. Thus, analogous to single channel analysis we decided to quantify Ca^{2+} sparklet activity as nP_s , where n is the number of quantal levels and P_s is the probability of Ca^{2+} sparklet occurrence. Details of this analysis could be found elsewhere [9]. An example of this type of analysis is shown in Figure 2C. Briefly, the first step is to determine the quantal unit of Ca^{2+} influx using an approach similar to the one developed by del Castillo and Katz [26] for neurotransmitter release. To estimate nP_s , we import Ca^{2+} sparklet records into Clampex 9 software. Individual Ca^{2+} sparklet events can be detected using this program's “threshold detection analysis” using no duration constraints and the quantal unit of Ca^{2+} entry determined above as a starting point for event detection. Traces can then be fitted (green lines, Figure 2C) with these initial parameters.

This analysis revealed that Ca^{2+} sparklet activity was bimodal [8,9]. Thus, Ca^{2+} sparklets were grouped three categories. By default, **silent** sites had no activity ($nP_s = 0$). Note that a silent Ca^{2+} sparklet site represents a site that is typically inactive, but can be activated by an agonist. Second, **low activity Ca^{2+} sparklet** sites had a relatively low probability of activation (nP_s between 0.0-0.2). Third, **high activity, persistent Ca^{2+} sparklet** sites ($nP_s > 0.2$) were characterized by frequent LTCC openings. Examination of the amplitude and duration histograms of Ca^{2+} sparklet sites suggest that low activity sparklets are produced by rare, brief

openings of single LTCCs (i.e. single mode amplitude histogram) while persistent Ca^{2+} sparklets are produced by frequent, longer openings of a small clusters of LTCCs (i.e. multi modal amplitude histogram) [8,9].

Ca^{2+} sparklet amplitude and duration is highly variable [8]. Thus, the nP_s analysis provides a better indicator of Ca^{2+} influx than amplitude and duration plots alone. We suggest that amplitude and duration histograms are accompanied by nP_s data for each site analyzed. An attractive feature of calculating the nP_s value of a sparklet site is that it is proportional to the nP_o (P_o = open probability) of the underlying Ca^{2+} channel(s). L-type Ca^{2+} channels in mode 1 and mode 2 have mean open times of about 2 and 11 ms, respectively [27] (Table 1). Thus, if as proposed [14], LTCCs in persistent Ca^{2+} sparklet sites are in mode 2 and have an open time ≥ 11 ms, the nP_s values of these sites at acquisition rates >90 Hz should approximate the nP_o of the channels in these sites. Because the mean open time of LTCCs in mode 1 is ≈ 2 ms, acquisition rates of 1 KHz or higher are required to convert their nP_s to nP_o values.

Quantitative approaches and automatic detection algorithms like the one described above are absolutely necessary for the detection, analysis, and classification of Ca^{2+} sparklet activity. Indeed, in our opinion, in the absence of an nP_s or similar quantitative approach to determine Ca^{2+} sparklet activity, conclusions about the frequency or type of Ca^{2+} sparklet site in any particular cell are difficult to interpret [15] as it may lead to biased classification of Ca^{2+} sparklet events.

6. Ca^{2+} sparklet activity varies within and between cells

The finding that sparklet activity varies within cells is interesting because Cav1.2 channels are broadly distributed throughout the sarcolemma of smooth muscle [8,25,28]. Indeed, we performed a Chi square (χ^2) test to test the null hypothesis that the spatial distribution of Ca^{2+} sparklet sites followed a Poisson distribution (i.e. was random) [8]. Although the specific location of Ca^{2+} sparklets differed between cells, the χ^2 tests indicated there were a significant difference between the spatial distribution of Ca^{2+} sparklets and the predicted Poisson distribution in each cell. This indicates that the *spatial distribution* of Ca^{2+} sparklets within a cell is **not random**.

Work by McCarron et al. [15] gives credence to this view. Although, unlike Navedo et al. [8], they did not perform a statistical test for randomness of the spatial distribution of Ca^{2+} sparklet sites, they found that in colon smooth muscle membrane depolarization activated what seems like a persistent Ca^{2+} sparklet (whether or not this is a persistent sparklet site is unclear since the authors did not quantify the activity of this or other sites) within a small region of the sarcolemma ($\approx 1 \mu\text{m}$), while the rest of the analyzed regions of the membrane remained optically silent. This suggests that, as in arterial myocytes, Ca^{2+} sparklet activity in colon smooth muscle cells is not uniform throughout their sarcolemma. Thus, analysis of the gating and spatial distribution of Ca^{2+} sparklets leads to two fundamentally different conclusions regarding these local Ca^{2+} signals that should not be confused: while quantal analysis of Ca^{2+} sparklets suggests that their gating is predominantly stochastic, independent (i.e. multi-modal amplitude histogram with a progressively decreasing probability of multi-quantal sparklet occurrence); the spatial distribution of Ca^{2+} sparklet activity is not random (i.e. non-Poisson distribution), being higher at specific sites within the sarcolemma of smooth muscle cells [8].

It is important to note that although the analysis described above suggests that Ca^{2+} sparklet gating is predominantly stochastic, the possibility that small clusters of LTCCs in persistent sparklet sites could gate in a coordinated, non-stochastic manner cannot be completely ruled out. Future studies should examine the whether at least a fraction of persistent Ca^{2+} sparks are produced by coupled gating of small clusters of adjacent LTCCs.

7. PKC α targeting by AKAP150 to specific sarcolemmal domains is required for persistent Ca²⁺ sparklet activity

What are the mechanisms underlying regional variations in Ca²⁺ sparklet activity? The diagram in Figure 3 summarizes a model for heterogeneous Ca²⁺ sparklet function in smooth muscle. It proposes that regional variations in Ca²⁺ sparklet activity are not necessarily due to clustering of functional Cav1.2 channels, as immunofluorescence, electrophysiological, and imaging data suggest that these channels are broadly distributed throughout the sarcolemma of smooth muscle cells [8,25,28]. Instead, membrane targeting of PKC α by the scaffolding protein AKAP150 (the rodent ortholog of human AKAP79) to specific regions of the surface membrane likely underlies heterogeneous Ca²⁺ sparklet activity in these cells [29]. Local targeting of PKC α by AKAP150 to specific regions of the surface membrane likely underlies heterogeneous Ca²⁺ sparklet activity in these cells [29]. Local targeting of PKC α by AKAP150 to specific regions of the surface membrane allows this kinase to phosphorylate nearby LTCCs thus promoting persistent Ca²⁺ sparklet activity. Two important observations support this view [29]. **First**, AKAP150 and PKC α co-localize in specific regions at or near the sarcolemma of arterial myocytes. **Second**, loss of AKAP150 prevents PKC α targeting to the sarcolemma of arterial myocytes and thus induction of persistent Ca²⁺ sparklet activity.

AKAP150 also targets calcineurin to the surface membrane [30]. In our model calcineurin and PKC α have opposing actions on Ca²⁺ sparklets and the level of Ca²⁺ sparklet activity varies regionally depending on the relative activities of PKC α and calcineurin [9]. Recent work by John Scott's lab suggests a potential mechanism for local Ca²⁺-dependent activation of PKC α [31]. They found that as Ca²⁺ enters the cell it recruits Ca²⁺/calmodulin to AKAP150 [31]. This releases PKC α from the AKAP150 complex allowing it to phosphorylate nearby targets including LTCCs. Because in this model PKC α is associated with AKAP150 in the sarcolemma of arterial myocytes and hence readily available for activation by a nearby local increase in Ca²⁺, a global increase in [Ca²⁺]_i is **not** necessary to induce the translocation of this kinase to the membrane.

8. Ca²⁺ sparklets regulate local and global [Ca²⁺]_i at physiological membrane potentials

LTCCs are critical regulators of [Ca²⁺]_i in smooth muscle [4,5]. It is therefore important to consider the impact of Ca²⁺ sparklets on [Ca²⁺]_i in these cells. We will focus on whether Ca²⁺ sparklets influence global [Ca²⁺]_i as their impact on local [Ca²⁺]_i is evident. Determining the relative contribution of low activity and persistent Ca²⁺ sparklets to global Ca²⁺ would be relatively easy if a specific blocker of each one of these events were available. There are none.

LTCCs antagonists block low activity and persistent Ca²⁺ sparklets. Thus, a multi-pronged approach must be used to determine the contribution of low activity and persistent Ca²⁺ sparklets to global [Ca²⁺]_i. As a first step, we determined the signal mass associated with Ca²⁺ sparklets (Figure 4). The Walsh's [32] and Singer's [33] groups developed a method for estimating the signal mass of local Ca²⁺ signals in coulombs from 2d images. The strength of this approach is that one could attribute signal mass to specific Ca²⁺ sparklet sites from which we have previously determined their nP_s (i.e. activity).

The signal mass of low and high nP_s Ca²⁺ sparklets was measured at -70 mV and physiological -40 mV using 2 mM external Ca²⁺ (Figure 4) [10]. The median signal mass of low and high nP_s , persistent Ca²⁺ sparklets at -70 mV was 63 and 189 fC, respectively (Figure 4A and C). As expected for an LTCC, membrane depolarization increased the signal mass of Ca²⁺ sparklets. At -40 mV, the median signal mass of low and high nP_s Ca²⁺ sparklets was 292 and 452 fC, respectively (Figure 4B and C). These signal mass values could be used to provide an

indication of the potential impact of low and high activity Ca^{2+} sparklets on global $[\text{Ca}^{2+}]_i$ using the following equation:

$$\Delta[\text{Ca}^{2+}]_i = \frac{\Delta Q_{\text{Ca}}}{2FBV}$$

where ΔQ_{Ca} is the change in calcium influx (in coulombs, C; as determined by the signal mass analysis), F is the Faraday constant, V is the accessible cytosolic volume, and B is the buffering capacity of the cell. Assuming that smooth muscle cells have an accessible cytosolic volume of 0.9 pL and a buffering capacity of 80, we determined that at -70 mV median low and high nP_s Ca^{2+} sparklets could increase $[\text{Ca}^{2+}]_i$ by as much as 5 and 14 nM, respectively [10]. As expected, membrane depolarization increases Ca^{2+} sparklet activity [7,10,15]. At -40 mV, median low and high nP_s sites could increase global $[\text{Ca}^{2+}]_i$ by as much as 21 and 38 nM, respectively [10]. Because the driving force for Ca^{2+} entry decreases with depolarization, this voltage-induced increase in Ca^{2+} entry is likely to be due to an increase in the activity of LTCCs. These data indicate that Ca^{2+} influx via low activity and persistent Ca^{2+} sparklets is voltage dependent and could influence local and global $[\text{Ca}^{2+}]_i$ at physiological -40 mV and 2 mM external Ca^{2+} .

Do Ca^{2+} sparklets contribute to global $[\text{Ca}^{2+}]_i$ at hyperpolarized membrane potentials (i.e. -70 mV)? Because low nP_s Ca^{2+} sparklets are rare, the probability of coincidental activation of independent events is low [8]. Thus, it is reasonable to predict that infrequent, random, non-overlapping Ca^{2+} sparklets with a signal mass similar to that of the mode (100 pC) or median of the distribution would have a small (< 5 nM) impact on global $[\text{Ca}^{2+}]_i$ at -70 mV. However, because the signal mass of persistent Ca^{2+} sparklets at this potential is larger than that of low nP_s events, the predicted impact of these events on global $[\text{Ca}^{2+}]_i$ is potentially larger than the typical low nP_s sparklet. Persistent Ca^{2+} sparklets with a signal mass similar to that of the mode (100 pC) or median of the distribution could have a relatively small (< 14 nM) impact on global $[\text{Ca}^{2+}]_i$ at -70 mV.

To provide further support to this conclusion, we modeled the effects of LTCC inhibition on global Ca^{2+} measured in a cell loaded with Fluo-5F using epifluorescence or TIRF microscopy (Figure 5). As above, the model assumes 1 persistent Ca^{2+} sparklet contributing to a steady-state increase in $[\text{Ca}^{2+}]_i$ of 14 nM Ca^{2+} (median signal mass) to $[\text{Ca}^{2+}]_i$ at -70 mV [8]. In the model, noise was simulated assuming a Gaussian distribution using experimentally determined values from arterial myocytes loaded with Fluo-5F and imaged in TIRF or epifluorescence mode using an 60 $\times$ (1.45 NA) lens and an EMCCD camera. As expected, the standard deviation of the basal fluorescence signal was larger in epifluorescence (24 nM) than in TIRF (6 nM) imaging, due to the thicker optical section in epifluorescence than in TIRF mode. We generated all-points histograms from $[\text{Ca}^{2+}]_i$ records undergoing a decrease of 14 nM $[\text{Ca}^{2+}]_i$ in epifluorescence (Figure 5A) and TIRF mode (Figure 5B). Note that both histograms could be fitted with single Gaussian function, suggesting that a change in $[\text{Ca}^{2+}]_i$ of <14 nM cannot be distinguished from baseline using Fluo-5F and epifluorescence or TIRF microscopy. These findings are consistent with the observation by McCarron et al. [15] that application of 1 μM nisoldipine did not change global $[\text{Ca}^{2+}]_i$ in colonic smooth muscle at -70 mV. Importantly, these findings indicate that due to the relatively low frequency of persistent Ca^{2+} sparklets at hyperpolarized potentials, epifluorescence imaging cannot be used to determine their contribution to global $[\text{Ca}^{2+}]_i$.

As noted above, we demonstrated that depolarization from -70 to -40 mV increases the signal mass of low activity and persistent Ca^{2+} sparklets due to an increase in Ca^{2+} sparklet activity [10]. This brings up the following question: What is the relative contribution of low and high

nP_s sites to global $[Ca^{2+}]_i$ in smooth muscle at the physiological membrane potential of -40 mV? Answering this question is difficult due to the absence of specific blockers of low activity and persistent Ca^{2+} sparklets. Two recent studies in AKAP150 [29] and PKC α null [9] myocytes provide insights on this issue.

Loss of AKAP150 and PKC α expression eliminated persistent but not low activity Ca^{2+} sparklets. Indeed, we found that the dihydropyridine-sensitive component of steady-state L-type Ca^{2+} current and the global $[Ca^{2+}]_i$ at -40 mV was $\approx 50\%$ lower in cells lacking AKAP150 or PKC α expression. On the basis of these findings and the signal mass data above, we proposed that at the membrane potential of -40 mV and with 2 mM external Ca^{2+} , persistent Ca^{2+} sparklets (i.e. AKAP150 and PKC α -dependent events) contribute $\approx 50\%$ of the dihydropyridine-sensitive component of Ca^{2+} influx and could therefore be involved in the activation of myosin light chain kinase during excitation-contraction coupling in smooth muscle.

Our Ca^{2+} sparklet model also provides insight into the mechanisms by which LTCC agonists like Bay-K 8644 increases Ca^{2+} influx into arterial myocytes [9]. Note that application of Bay-K 8644 induced persistent Ca^{2+} sparklet activity in tsA-201 expressing Cav1.2 and PKC α . In contrast, tsA-201 cells co-expressing Cav1.2 channels only, Bay-K 8644 increases the frequency and duration of quantal Ca^{2+} sparklets, but did not induce persistent Ca^{2+} sparklet activity. Based on our model, we propose that application of Bay-K 8644 to arterial myocytes increases Ca^{2+} sparklet activity by increasing the mean open time and/or open probability of LTCCs. If any of these events occurs in the vicinity of an AKAP150-targeted, Ca^{2+} -sensitive PKC α , it could activate this kinase. Once activated, PKC α can induce multiple adjacent LTCCs to operate in a persistent gating mode (i.e. mode 2), thus causing further Ca^{2+} influx, which could presumably maintain PKC α activity and hence persistent Ca^{2+} sparklet activity. Bay-K 8644 has little or no effect on previously active (i.e. during control conditions) persistent Ca^{2+} sparklet sites presumably because the majority of the LTCCs in these clusters were already in mode 2 before application of the drug. An interesting implication of our model is that while Ca^{2+} sparklets could be evoked by voltage or pharmacological means wherever Cav1.2 channels are expressed, persistent Ca^{2+} channel activity would only occur in regions of the cell membrane where Cav1.2, AKAP150, and PKC α are located.

9. Persistent Ca^{2+} sparklets activate NFATc3 in arterial myocytes

LTCC activity is increased in arterial smooth muscle during the development of hypertension [34]. Accordingly, the number and probability of activation of low activity and persistent Ca^{2+} sparklet sites — not an increase in the amplitude of quantal Ca^{2+} sparklets or their duration — underlies increased Ca^{2+} influx via LTCCs into arterial myocytes during hypertension. This is the result of an increase in Cav1.2 channel expression [34] and PKC α activity in arterial smooth muscle during hypertension [1].

The increase in persistent Ca^{2+} sparklet activity had important functional consequences in arterial myocytes during hypertension: they activate the transcription factor NFATc3 via calcineurin [1]. Thus, the Ca^{2+} sparklet model described above has important consequences on Ca^{2+} influx and gene expression (Figure 3). Activation of PKC α induces persistent Ca^{2+} sparklet, producing an increase in local Ca^{2+} influx that activates nearby AKAP150-targeted calcineurin. Upon activation, calcineurin dephosphorylates NFATc3 allowing this transcription factor to translocate into the nucleus of arterial myocytes where it modulates gene expression (Figure 3). Calcineurin and NFATc3 activities are low in arterial smooth muscle under physiological conditions [35] because of low levels of persistent Ca^{2+} sparklet activity (≈ 1 persistent Ca^{2+} sparklet site per cell) [8] and relatively high nuclear NFATc3 export rates [36]. However, during hypertension, an increase in PKC α activity increases persistent Ca^{2+}

sparklet activity thereby increasing calcineurin activity and consequently NFATc3 nuclear import rate, which if coupled with a decrease in export rate [36], would lead to high nuclear accumulation of this transcription factor. This, in turn, leads to down-regulation of Kv2.1 transcript expression [2,37]. As suggested previously [2,38,39], down-regulation of Kv2.1 decreases voltage-gated K⁺ currents and contributes to arterial smooth muscle depolarization during hypertension, which indirectly increases Ca²⁺ sparklet activity, global [Ca²⁺]_i, and tone (Figure 3). Consistent with this model, loss of AKAP150 and PKCα not only eliminated persistent Ca²⁺ sparklet activity, but also protected against the development angiotensin II-induced hypertension [29].

10. Conclusions and Future studies

The work discussed in this review suggests multiple strategies for the recording, detection, and later analysis of Ca²⁺ sparklets. Implementation of these approaches has revealed an interesting feature of LTCCs: their opening probability can vary regionally within the sarcolemma of smooth muscle cells. This is not the result of massive clustering of functional LTCCs, as multiple independent studies suggest they are broadly distributed throughout the sarcolemma of smooth muscle cells. Instead, heterogeneous Ca²⁺ sparklet activity results from regional targeting of PKCα and calcineurin by AKAP150 as well as the relative activities of this kinase and phosphatase.

Although it is persistent Ca²⁺ sparklets are critical for excitation-contraction coupling and excitation-transcription coupling in smooth muscle multiple questions about these Ca²⁺ events remain unanswered. For example, what are the relationships among sarcoplasmic reticulum Ca²⁺ load, ryanodine receptor function, and Ca²⁺ sparklet activity? What is the spatial and functional relationship between PKCα and Ca²⁺ sparklet activity in living cells? Is the number of persistent Ca²⁺ sparklet per cell limited by the level of AKAP150 expression in it? What are the factors determining the size of Ca²⁺ sparklet sites? What is the relationship between gating modalities and Ca²⁺ influx in individual LTCCs within a persistent Ca²⁺ sparklet site? Finally, what is the relationship between persistent Ca²⁺ sparklet activity and NFATc3 translocation? Future studies should address these important questions.

Acknowledgments

The work from the author's laboratory was supported by the AHA and NIH.

References

1. Nieves-Cintrón M, Amberg GC, Navedo MF, Molkentin JD, Santana LF. The control of Ca²⁺ influx and NFATc3 signaling in arterial smooth muscle during hypertension. *Proc Natl Acad Sci U S A* 2008;105(40):15623–8. [PubMed: 18832165]
2. Amberg GC, Rossow CF, Navedo MF, Santana LF. NFATc3 regulates Kv2.1 expression in arterial smooth muscle. *J Biol Chem* 2004;279(45):47326–34. [PubMed: 15322114]
3. Cartin L, Lounsberry KM, Nelson MT. Coupling of Ca(2+) to CREB activation and gene expression in intact cerebral arteries from mouse : roles of ryanodine receptors and voltage-dependent Ca(2+) channels. *Circ Res* 2000;86(7):760–7. [PubMed: 10764409]
4. Knot HJ, Nelson MT. Regulation of arterial diameter and wall [Ca²⁺] in cerebral arteries of rat by membrane potential and intravascular pressure. *J Physiol* 1998;508(Pt 1):199–209. [PubMed: 9490839]
5. Harder DR, Gilbert R, Lombard JH. Vascular muscle cell depolarization and activation in renal arteries on elevation of transmural pressure. *Am J Physiol* 1987 Oct;253(4 Pt 2):F778–81. [PubMed: 2444115]
6. Guia A, Wan X, Courtemanche M, Leblanc N. Local Ca²⁺ entry through L-type Ca²⁺ channels activates Ca²⁺-dependent K⁺ channels in rabbit coronary myocytes. *Circ Res* 1999 May 14;84(9):1032–42. [PubMed: 10325240]

7. Wang SQ, Song LS, Lakatta EG, Cheng H. Ca^{2+} signalling between single L-type Ca^{2+} channels and ryanodine receptors in heart cells. *Nature* 2001;410(6828):592–6. [PubMed: 11279498]
8. Navedo MF, Amberg G, Votaw SV, Santana LF. Constitutively active L-type Ca^{2+} channels. *Proc Natl Acad Sci U S A* 2005 August 2;102(31):11112–7. [PubMed: 16040810]
9. Navedo MF, Amberg GC, Nieves M, Molkentin JD, Santana LF. Mechanisms Underlying Heterogeneous Ca^{2+} Sparklet Activity in Arterial Smooth Muscle. *Journal of General Physiology* 2006;127(6):611–22. [PubMed: 16702354]
10. Amberg GC, Navedo MF, Nieves-Cintrón M, Molkentin JD, Santana LF. Calcium Sparklets Regulate Local and Global Calcium in Murine Arterial Smooth Muscle. *J Physiol* 2007 Dec 7;579(1):187–201. [PubMed: 17158168]
11. Demuro A, Parker I. “Optical patch-clamping”: single-channel recording by imaging Ca^{2+} flux through individual muscle acetylcholine receptor channels. *J Gen Physiol* 2005 Sep;126(3):179–92. [PubMed: 16103278]
12. Shuai J, Parker I. Optical single-channel recording by imaging Ca^{2+} flux through individual ion channels: theoretical considerations and limits to resolution. *Cell Calcium* 2005 Apr;37(4):283–99. [PubMed: 15755490]
13. Tour O, Adams SR, Kerr RA, Meijer RM, Sejnowski TJ, Tsien RW, et al. Calcium Green FAsH as a genetically targeted small-molecule calcium indicator. *Nature chemical biology* 2007 Jul;3(7):423–31.
14. Navedo MF, Amberg GC, Westenbroek RE, Sinnegger-Brauns MJ, Catterall WA, Striessnig J, et al. Cav1.3 channels produce persistent calcium sparklets, but Cav1.2 channels are responsible for sparklets in mouse arterial smooth muscle. *Am J Physiol Heart Circ Physiol* 2007 May 25;293(3):H1359–70. [PubMed: 17526649]
15. McCarron JG, Olson ML, Currie S, Wright AJ, Anderson KI, Girkin JM. Elevations of intracellular calcium reflect normal voltage-dependent behavior, and not constitutive activity, of voltage-dependent calcium channels in gastrointestinal and vascular smooth muscle. *J Gen Physiol* 2009 Mar 16;133(4):439–57. [PubMed: 19289573]
16. Santana LF, Navedo MF, Amberg GC, Nieves-Cintrón M, Votaw VS, Ufret-Vincenty CA. Calcium sparklets in arterial smooth muscle. *Clin Exp Pharmacol Physiol* 2008 Sep;35(9):1121–6. [PubMed: 18215181]
17. Zenisek D, Davila V, Wan L, Almers W. Imaging calcium entry sites and ribbon structures in two presynaptic cells. *J Neurosci* 2003;23(7):2538–48. [PubMed: 12684438]
18. Steyer JA, Almers W. A real-time view of life within 100 nm of the plasma membrane. *Nat Rev Mol Cell Biol* 2001 Apr;2(4):268–75. [PubMed: 11283724]
19. Sinnegger-Brauns MJ, Hetzenauer A, Huber IG, Renstrom E, Wietzorrek G, Berjukov S, et al. Isoform-specific regulation of mood behavior and pancreatic beta cell and cardiovascular function by L-type Ca^{2+} channels. *J Clin Invest* 2004 May;113(10):1430–9. [PubMed: 15146240]
20. Koschak A, Reimer D, Huber I, Grabner M, Glossmann H, Engel J, et al. $\alpha 1\text{D}$ (Cav1.3) subunits can form L-type Ca^{2+} channels activating at negative voltages. *J Biol Chem* 2001 Jun 22;276(25):22100–6. [PubMed: 11285265]
21. Cheng H, Song LS, Shirokova N, Gonzalez A, Lakatta EG, Rios E, et al. Amplitude distribution of calcium sparks in confocal images: theory and studies with an automatic detection method. *Biophys J* 1999;76(2):606–17. [PubMed: 9929467]
22. Sebillé S, Cantereau A, Vandebrouck C, Balghi H, Constantin B, Raymond G, et al. Calcium sparks in muscle cells: interactive procedures for automatic detection and measurements on line-scan confocal images series. *Comput Methods Programs Biomed* 2005 Jan;77(1):57–70. [PubMed: 15639710]
23. v Wegner F, Both M, Fink RH. Automated detection of elementary calcium release events using the a trous wavelet transform. *Biophys J* 2006 Mar 15;90(6):2151–63. [PubMed: 16387777]
24. Picht E, Zima AV, Blatter LA, Bers DM. SparkMaster: automated calcium spark analysis with ImageJ. *Am J Physiol Cell Physiol* 2007 Sep;293(3):C1073–81. [PubMed: 17376815]
25. Rubart M, Patlak JB, Nelson MT. Ca^{2+} currents in cerebral artery smooth muscle cells of rat at physiological Ca^{2+} concentrations. *J Gen Physiol* 1996;107(4):459–72. [PubMed: 8722560]

26. del Castillo J, Katz B. Quantal components of the end-plate potential. *J Physiol* 1954;124(3):560–73. [PubMed: 13175199]
27. Hess P, Lansman JB, Tsien RW. Different modes of Ca channel gating behaviour favoured by dihydropyridine Ca agonists and antagonists. *Nature* 1984;311(5986):538–44. [PubMed: 6207437]
28. Moore ED, Voigt T, Kobayashi YM, Isenberg G, Fay FS, Gallitelli MF, et al. Organization of Ca^{2+} release units in excitable smooth muscle of the guinea-pig urinary bladder. *Biophys J* 2004;87(3):1836–47. [PubMed: 15345562]
29. Navedo MF, Nieves-Cintrón M, Amberg GC, Yuan C, Votaw VS, Lederer WJ, et al. AKAP150 Is Required for Stuttering Persistent Ca^{2+} Sparklets and Angiotensin II Induced Hypertension. *Circ Res* 2008 Feb 1;102:e1–e11. [PubMed: 18174462]
30. Klauck TM, Faux MC, Labudda K, Langeberg LK, Jaken S, Scott JD. Coordination of three signaling enzymes by AKAP79, a mammalian scaffold protein. *Science* 1996 Mar 15;271(5255):1589–92. [PubMed: 8599116]
31. Faux MC, Scott JD. Regulation of the AKAP79-protein kinase C interaction by Ca^{2+} /Calmodulin. *J Biol Chem* 1997 Jul 4;272(27):17038–44. [PubMed: 9202019]
32. ZhuGe R, Fogarty KE, Tuft RA, Lifshitz LM, Sayar K, Walsh JV Jr. Dynamics of signaling between Ca^{2+} sparks and Ca^{2+} -activated K^{+} channels studied with a novel image-based method for direct intracellular measurement of ryanodine receptor Ca^{2+} current. *J Gen Physiol* 2000 Dec;116(6):845–64. [PubMed: 11099351]
33. Zou H, Lifshitz LM, Tuft RA, Fogarty KE, Singer JJ. Visualization of Ca^{2+} entry through single stretch-activated cation channels. *Proc Natl Acad Sci U S A* 2002 Apr 30;99(9):6404–9. [PubMed: 11983921]
34. Pratt PF, Bonnet S, Ludwig LM, Bonnet P, Rusch NJ. Upregulation of L-type Ca^{2+} channels in mesenteric and skeletal arteries of SHR. *Hypertension* 2002 Aug;40(2):214–9. [PubMed: 12154116]
35. Nieves-Cintrón M, Amberg GC, Nichols CB, Molkentin JD, Santana LF. Activation of NFATc3 down-regulates the $\beta 1$ subunit of large conductance, calcium-activated K^{+} channels in arterial smooth muscle and contributes to hypertension. *J Biol Chem* 2007 Feb 2;282(5):3231–40. [PubMed: 17148444]
36. Gomez MF, Bosc LV, Stevenson AS, Wilkerson MK, Hill-Eubanks DC, Nelson MT. Constitutively elevated nuclear export activity opposes Ca^{2+} -dependent NFATc3 nuclear accumulation in vascular smooth muscle: role of JNK2 and Crm-1. *J Biol Chem* 2003 Nov 21;278(47):46847–53. [PubMed: 12954637]
37. Layne J, Werner ME, Hill-Eubanks D, Nelson MT. NFATc3 Regulates BK Channel Function in Murine Urinary Bladder Smooth Muscle. *Am J Physiol Cell Physiol*. 2008 Jun 25;
38. Cox RH. Comparison of K^{+} channel properties in freshly isolated myocytes from thoracic aorta of WKY and SHR. *Am J Hypertens* 1996 Sep;9(9):884–94. [PubMed: 8879345]
39. Wellman GC, Cartin L, Eckman DM, Stevenson AS, Saundry CM, Lederer WJ, et al. Membrane depolarization, elevated Ca^{2+} entry, and gene expression in cerebral arteries of hypertensive rats. *Am J Physiol Heart Circ Physiol* 2001 Dec;281(6):H2559–67. [PubMed: 11709423]

Figure 1. Ca^{2+} sparklets in arterial and venous myocytes

(A) TIRF images of Ca^{2+} sparklets from representative arterial (*left*) and portal vein (*right*) smooth muscle cells (-70 mV; external Ca^{2+} = 20 mM). Traces below each image show the time-course of $[Ca^{2+}]_i$ in the sites marked by the green circles. (B) Simultaneous recordings of Ca^{2+} sparklets and unitary LTCC currents. Redrawn from [8].

Figure 2. Ca^{2+} sparklet activity is quantal

All-points histogram generated from 25 representatives blank TIRF traces (A) or Ca^{2+} sparklet traces (B) similar to those in the inserts. (C) Description of Ca^{2+} sparklet nP_s analysis. Solid lines represent fit of the $[\text{Ca}^{2+}]_i$ traces using the single channel detection and analysis algorithms of Clampex 9.0. Dotted lines represent quantal levels. Figure redrawn from [9].

Figure 3. Proposed model for heterogeneous Ca^{2+} sparklet activity and NFATc3 activation in arterial myocytes

Activation of hetero-trimeric $\text{G}_{q/11}$ proteins by a membrane receptor like the angiotensin receptor type 1 (AT1R) activates phospholipase C (PLC), which increases diacylglycerol (DAG) levels and activates PKC α . AKAP150 targets PKC α and calcineurin to specific regions of the sarcolemma where they can modulate the activity of nearby LTCCs. Ca^{2+} entering the cell recruits calmodulin, thus releasing PKC α from its interaction with AKAP150 allowing it to phosphorylate neighboring LTCCs. LTCCs associated with the AKAP150/PKC α /calcineurin complex can develop persistent Ca^{2+} sparklet activity, forming a signaling unit that modulates Ca^{2+} influx and NFATc3-dependent gene expression in smooth muscle. Initiation or continuation of this signaling unit could be prevented by inhibiting PKC α , calcineurin, or NFATc3 activation or with L-type Ca^{2+} channel blockers. Green arrows indicate activation; red arrows indicate inhibition/down-regulation.

Figure 4. Signal mass of Ca^{2+} sparklets in smooth muscle

Amplitude histograms of ΔQ_{Ca} from low and high nP_s Ca^{2+} sparklet sites at -70 (A) and -40 mV (B). (C) Scatter plot of ΔQ_{Ca} amplitudes from low and high nP_s Ca^{2+} sparklet sites at -70 and -40 mV; the red line represents the median value for each column of values. Figure redrawn from [10].

Figure 5. The effects of blocking Ca^{2+} sparklets on global $[\text{Ca}^{2+}]_i$ at -70 mV
 Simulation of the effects of blocking persistent Ca^{2+} sparklets on global $[\text{Ca}^{2+}]_i$, measured in a cell loaded with Fluo-5F using epifluorescence (A) or TIRF (B) microscopy. *Right side panels* show all-points histograms from the $[\text{Ca}^{2+}]_i$ records undergoing a decrease of 14 nM $[\text{Ca}^{2+}]_i$. Solid lines in the histograms represent the best fit of the data using a Gaussian function.

Table 1
Properties of Ca²⁺ sparklets and L-type Ca²⁺ channels

Sparklets Parameters	Calcium sparklets		Unitary I _{Ca}		Unitary LTCC Parameters
	arterial myocytes	Cav1.2 (tsA-201)	arterial myocytes	cardiac myocytes	
modal gating	yes ^a	yes ^b	yes ^d	yes ^e	Modal gating
voltage dependent changes in amplitude and activity	yes ^a	yes ^b	6.3 ^d	9.7 ^e	Conductance (pS)
quantal level (mM)	38 ^a	36 ^b	0.2 (2 mM Ca ²⁺) ^d	~ 0.6 (10 mM Ca ²⁺) ^e	Amplitude of unitary current at -40 mV (pA)
duration (ms)	low nP_s $\tau = 24$ high nP_s $\tau_{fast} = 23$ $\tau_{slow} = 104f$	ND	$\tau = 14, 3$ ^d	mode 1 $\tau = 2.34$ mode 2 $\tau = 11f$	Open times (ms)
Minimal signal mass (fC)	7 ^e (-40 mV; 2 mM Ca ²⁺)	ND	7 (for a 0.2 pA unitary current of 35 ms duration)	ND	Total charge movement at -40 mV and 2 mM external Ca ²⁺ (fC)

^aNavedo et al., *PNAS* 102: 11112-7;
^bNavedo et al., *JGP* 127: 221-3;
^cAmberg et al., *J Physiol* 579: 187-201;
^dRubart et al., *JGP* 107: 459-72;
^eYue and Marbal, *JGP* 95: 911-39;
^fCostantin et al., *J Physiol* 507: 93-103.

ND: not determined.