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Ca_v1.2 sparklets in heart and vascular smooth muscle

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Abstract

Ca_v1.2 sparklets are local elevations in intracellular Ca²⁺ ([Ca²⁺]_i) resulting from the opening of a single or small cluster of voltage-gated, dihydropyridine-sensitive Ca_v1.2 channels. Activation of Ca_v1.2 sparklets is an early event in the signaling cascade that couples membrane depolarization to contraction (i.e., excitation-contraction coupling) in cardiac and arterial smooth muscle. Here, we review recent work on the molecular and biophysical mechanisms that regulate Ca_v1.2 sparklet activity in these cells. Ca_v1.2 sparklet activity is tightly regulated by a cohort of protein kinases and phosphatases that are targeted to specific regions of the sarcolemma by the anchoring protein AKAP150. We discuss a model for the local control of Ca²⁺ influx via Ca_v1.2 channels in which a signaling complex formed by AKAP79/150, protein kinase C, protein kinase A, and calcineurin regulates the activity of individual Ca_v1.2 channels and also facilitates the coordinated activation of small clusters of these channels. This results in amplification of Ca²⁺ influx, which strengthens excitation-contraction coupling in cardiac and vascular smooth muscle.

Introduction

On average, a heart will beat about 3 billion times during the lifetime of a human. With each beat, the heart pumps blood to the pulmonary and systemic circulation. To achieve this feat, the heart must contract in a highly coordinated fashion that allows for the sequential activation of its atria and ventricles. Ca²⁺ influx via Ca_v1.2 channels during the action potential (AP) serves as the signal that triggers contraction in atrial and ventricular myocytes. The chain of events that links membrane depolarization to the increase in intracellular Ca²⁺ ([Ca²⁺]_i) that triggers contraction is known as excitation-contraction (EC) coupling [1]. The fidelity of EC coupling in the heart is remarkable, with every AP triggering a [Ca²⁺]_i transient and contraction [2].

The work of the heart would be futile if it were not for arteries, which deliver the blood pumped by the left ventricle to each organ in the body. Blood flow through the vasculature depends on the pressure generated by the contraction of the ventricles and the systemic resistance, which is determined by blood vessel diameter. Ultimately, the contractile state of the smooth muscle lining the walls of arteries controls arterial caliber and hence blood flow [3]. Like cardiac myocytes, arterial myocyte contraction is controlled by changes in membrane potential and Ca²⁺ influx via Ca_v1.2 channels.

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Disclosures

None.

Although $\text{Ca}_v1.2$ channels play a critical role in cardiac and smooth muscle EC coupling [4, 5], the mechanisms by which their activation increases global $[\text{Ca}^{2+}]_i$ and initiates contraction are fundamentally different. In cardiac cells, activation of $\text{Ca}_v1.2$ channels during the AP allows a small amount of Ca^{2+} to enter the cytosol [4]. These Ca^{2+} influx events, which we are calling a “ $\text{Ca}_v1.2$ sparklet” to distinguish them from sparklets produced by other Ca^{2+} -permeable channels (e.g. TRPV4, AChRs, and $\text{Ca}_v2.2$ and $\text{Ca}_v1.3$ Ca^{2+} channels), activate closely apposed ryanodine receptors (RyRs) located in the junctional sarcoplasmic reticulum (jSR) via the mechanism of Ca^{2+} -induced Ca^{2+} release (CICR) [4, 6]. Accordingly, CICR amplifies the initial Ca^{2+} entry event, causing a global elevation in $[\text{Ca}^{2+}]_i$ that triggers contraction.

Unlike cardiac myocytes, an AP is not required for the activation of $\text{Ca}_v1.2$ channels in vascular smooth muscle. Rather, increases in intravascular pressure cause a graded depolarization of the sarcolemma of vascular smooth muscle that activate $\text{Ca}_v1.2$ sparklets [7, 8]. In these cells, $\text{Ca}_v1.2$ sparklets are directly responsible for cell-wide increases in global $[\text{Ca}^{2+}]_i$ that activates the contractile machinery during EC coupling [8].

Optical recording of $\text{Ca}_v1.2$ sparklets have revealed interesting features about the channels underlying these events in cardiac and vascular smooth muscle cells [4, 9, 10]. *First*, the activity of $\text{Ca}_v1.2$ channels varies throughout the sarcolemma of cardiac and vascular smooth muscle. *Second*, the $\text{Ca}_v1.2$ channels form clusters and can gate coordinately along the sarcolemma of these cells [10–13]. In this review, we discuss the mechanisms underlying heterogeneous $\text{Ca}_v1.2$ channel activity, their cooperative activation, and the functional implications of these observations on EC coupling strength and stability under physiological and pathological conditions in cardiac and vascular smooth muscle.

Molecular composition and spatial organization of $\text{Ca}_v1.2$ channels in cardiac and vascular smooth muscle

$\text{Ca}_v1.2$ channels are heteromeric proteins composed of a transmembrane, pore-forming α_{1C} ($\text{Ca}_v1.2$) and accessory β , $\alpha_2\delta$ -1 and, in many cells, γ subunits [14]. The expression of the auxiliary $\beta(1-3)$ subunit has been associated with enhanced channel trafficking and expression. This subunit also regulates the voltage dependencies of $\text{Ca}_v1.2$ channels [15]. β_2 and β_3 subunits are the predominant β subunits expressed in ventricular and vascular smooth muscle cells, respectively. Recently, the transmembrane $\alpha_2\delta(1)$ was identified as being critical for functional trafficking of $\text{Ca}_v1.2$ α_1 subunits to the plasma membrane in vascular smooth muscle [16].

The α_{1C} subunit contains four highly conserved repeat regions with variable length N terminus and a relatively long intracellular C terminus. Three α_{1C} isoforms have been identified: cardiac, $\text{Ca}_v1.2a$; smooth muscle, $\text{Ca}_v1.2b$; and neuronal, $\text{Ca}_v1.2c$. Although $\text{Ca}_v1.2a-c$ display significant sequence differences in their N and C termini, they all physically interact with the anchoring protein AKAP150/79 via their C-termini and are regulated by intracellular kinases and phosphatases to control Ca^{2+} entry and excitability [14].

Electrophysiological studies indicate that approximately 10,000–16,000 functional $\text{Ca}_v1.2$ channels are expressed in a typical ventricular myocyte. Similar approaches suggest that vascular smooth muscle cells express fewer $\text{Ca}_v1.2$ channels than ventricular myocytes (5,000–10,000) [5]. However, due to differences in cell size, the average density of $\text{Ca}_v1.2$ channels in cardiac and smooth muscle cells is similar (i.e., 5–15 channels/ μm^2) [5, 17, 18].

Immunofluorescence and confocal imaging approaches have been used to determine the spatial organization of $\text{Ca}_v1.2$ channels in cardiac and vascular smooth muscle. These data suggest that $\text{Ca}_v1.2$ channels are differentially distributed throughout the sarcolemma of these cells. In cardiac myocytes, however, $\text{Ca}_v1.2$ channels are preferentially expressed in the transverse tubules (T-tubules) [19]. In vascular smooth muscle, $\text{Ca}_v1.2$ channels seem to be broadly distributed along the sarcolemma of these cells [10, 20, 21].

Using stimulated emission depletion (STED) ultra-resolution microscopy, Enrico Stefani's group was able to image small clusters of variable size of $\text{Ca}_v1.2$ channels in the T-tubules of ventricular myocytes [22]. This is consistent with a recent study in which raster imaging correlation spectroscopy (RICS) and **n**umber & **b**rightness (N&B) analyses were employed to determine the rate of diffusion and cluster size of fluorescently tagged $\text{Ca}_v1.2$ channels in heterologous cells and cardiac myocytes [11]. Using RICS, the diffusion co-efficient of wild type and $\text{Ca}_v1.2$ channels carrying a mutation linked to long QT syndrome 8 (LQT8 or Timothy syndrome) was estimated to be about $2.5 \mu\text{m}^2/\text{s}$ in these cells. N&B analysis — a moment analysis that can be used to determine the fluorescence of diffusing monomeric fluorescent molecules and to estimate the number of molecules and brightness within a volume — suggested that while most $\text{Ca}_v1.2$ (WT and LQT8) channels diffuse in solitude, clusters of 2 to 5 channels can diffuse throughout the surface membrane of tsA-201 cells expressing $\text{Ca}_v1.2$ channels fused to the tag red fluorescence protein ($\text{Ca}_v1.2$ -tRFP channels) and in ventricular myocytes expressing $\text{Ca}_v1.2$ -LQT8-tRFP channels (Figure 4A). Although similar studies have not been performed in vascular smooth muscle, the observation of $\text{Ca}_v1.2$ clusters in tsA-201 and ventricular myocytes suggest this may be a general feature of these channels in excitable cells. Future studies should examine this issue in detail and also investigate the molecular mechanisms determining the size of $\text{Ca}_v1.2$ clusters in cardiac and vascular smooth muscle.

$\text{Ca}_v1.2$ sparklets in cardiac and vascular smooth muscle

Heping Cheng's team coined the term Ca^{2+} sparklet to name the small, local Ca^{2+} signals produced by the opening of L-type $\text{Ca}_v1.2$ channels in ventricular myocytes [4]. As mentioned above, we propose the use of the word sparklet preceded by the name of the gene encoding the Ca^{2+} -permeable channel producing the local Ca^{2+} signal to link the local Ca^{2+} to the channel that produced it. For example, we would call $\text{Ca}_v1.2$, $\text{Ca}_v1.3$, $\text{Ca}_v2.2$, AChRs and TRPV4 sparklets local Ca^{2+} signals produced by $\text{Ca}_v1.2$, $\text{Ca}_v1.3$, $\text{Ca}_v2.2$, AChRs and TRPV4 channels, respectively [10, 23–26].

Since Cheng's original report, we and others, have implemented ultra fast 2-dimensional confocal and total internal reflection fluorescence (TIRF) imaging to monitor with high temporal and spatial resolution the activity of $\text{Ca}_v1.2$ sparklets in tsA201 expressing these channels, in isolated cardiac and vascular smooth muscle and in intact arteries (Figure 1A) [9, 10]. Readers interested in learning more about the strategies for the recording, detection, and analysis of sparklets will find detailed descriptions in the following publications: [12, 26–30].

Implementation of these approaches revealed multiple interesting features of $\text{Ca}_v1.2$ channels in cardiac and vascular smooth muscle [4, 8, 10]. For example, the amplitude of $\text{Ca}_v1.2$ sparklets is quantal. In 20 mM external Ca^{2+} , the quantal unit of Ca^{2+} influx via these channels is about 40 nM (Figure 1B). In physiological 2 mM external Ca^{2+} , the amplitude of most $\text{Ca}_v1.2$ sparklets were <20 nM or undetectable, even at very negative potentials (−70 mV), where the driving force for Ca^{2+} influx is high.

Interestingly, $\text{Ca}_v1.2$ sparklet activity is bimodal, with most of the sarcolemma being optically silent, while other regions display either low or high $\text{Ca}_v1.2$ sparklet activity [8]. $\text{Ca}_v1.2$ channel agonists increase Ca^{2+} influx by increasing $\text{Ca}_v1.2$ sparklet activity in previously silent sites and low activity sites. A $\text{Ca}_v1.2$ sparklet is always associated with an inward Ca^{2+} current (Figure 1C). Accordingly, the signal mass of the fluorescence signal follows a linear relationship when compared to the Ca^{2+} flux through $\text{Ca}_v1.2$ channels in vascular smooth muscle. An intriguing observation made in these studies is that while the majority of $\text{Ca}_v1.2$ sparklets seem to be produced by the stochastic opening of $\text{Ca}_v1.2$ channels, a subpopulation of $\text{Ca}_v1.2$ sparklets seems to be produced by the coordinated opening of small clusters of $\text{Ca}_v1.2$ channels (Figure 3).

Mark Nelson's group recently recorded sparklets resulting from Ca^{2+} influx via TRPV4 channels in endothelial cells [24]. Although TRPV4 sparklets are not the focus of this review, we discuss some of the conclusions of this study here for three important reasons. (1) In conjunction with the $\text{Ca}_v1.2$ sparklet work discussed above, this study suggests that optical approaches can be used to image elementary TRPV4 and $\text{Ca}_v1.2$ sparklets events. This work is particularly impressive because TRPV4 sparklets were recorded from endothelial cells in resistance arteries exposed to physiological extracellular Ca^{2+} . Indeed, it was found that the amplitude of quantal TRPV4 sparklets is larger than that of $\text{Ca}_v1.2$ sparklets, suggesting that Ca^{2+} flux is larger via TRPV4 than $\text{Ca}_v1.2$ channels. (2) An important observation by Sonkusare et al [24] is that, like $\text{Ca}_v1.2$ sparklets, TRPV4 sparklet activity varies throughout the surface membrane of endothelial cells, suggesting that a relatively small number of TRPV4 channels can have important functional consequences in these cells. (3) Finally, it was also discovered that, like $\text{Ca}_v1.2$ channels, small clusters of TRPV4 channels could gate coordinately to produce large TRPV4 sparklets in endothelial cells. Collectively, these findings suggest that heterogeneous and cooperative channel gating could be a common feature of TRPV4 and $\text{Ca}_v1.2$ channels.

Mechanisms underlying regional variations in $\text{Ca}_v1.2$ sparklet activity in cardiac and vascular smooth muscle

The mechanisms underlying heterogeneous $\text{Ca}_v1.2$ sparklet activity have been the subject of intense investigation during the last several years. Based on this work, a model has been proposed in which the anchoring protein A-kinase anchoring protein 150 (AKAP150) plays a central role in the formation of a macromolecular signaling complex that regulates $\text{Ca}_v1.2$ channels and hence $\text{Ca}_v1.2$ sparklet activity in cardiac and vascular smooth muscle (Figure 2A). AKAP150 is responsible for targeting protein kinase A (PKA), protein kinase C (PKC), and the protein phosphatase calcineurin, to specific regions of the sarcolemma where they can differentially regulate $\text{Ca}_v1.2$ channel activity (Figure 2). $\text{Ca}_v1.2$ channels that are associated with this macromolecular complex are capable of operating in a high activity mode. An important implication of this model is that the level of sparklet activity depends on the relative activities of AKAP150-associated kinases (e.g., PKA or PKC) and the phosphatase (e.g., calcineurin) on $\text{Ca}_v1.2$ channels (Figure 2B and 2C).

Evidence in support of this model is compelling. *First*, AKAP150 binds PKA, PKC, calcineurin and $\text{Ca}_v1.2$ channels in the sarcolemma of neurons, and cardiac and vascular smooth muscle [19, 20, 31]. Accordingly, AKAP150, PKA and PKC co-localize to specific regions within the sarcolemma of cardiac and vascular smooth muscle [19, 20, 31]. *Second*, ablation of AKAP150 prevents the induction of high-activity $\text{Ca}_v1.2$ sparklet events (Figure 2B) [19, 27]. *Third*, the actions of PKA and PKC on $\text{Ca}_v1.2$ channel activity are opposed by calcineurin (Figure 2C) [27, 32, 33]. *Fourth*, only a subpopulation of $\text{Ca}_v1.2$ channels is associated with AKAP150 [20].

Several aspects of this AKAP-Ca_v1.2 sparklet model compel further investigation, however. For example, future studies should determine whether AKAP150 is involved in PKA- and ROS-dependent regulation of Ca_v1.2 sparklets, as suggested by recent studies [32, 34, 35]. It is also necessary to determine whether AKAP150 tethers PKA in vascular smooth muscle as suggested by a recent report [32, 34, 35], or whether AKAP150 and PKC are co-localized to similar subcellular regions in cardiac cells. Furthermore, it will be interesting to investigate whether these or similar regulatory mechanisms underlie heterogeneous activity in other cell types (e.g., neurons) or other ion channels (e.g., TRPV4 channels).

Coupled gating of Ca_v1.2 sparklets in cardiac and vascular smooth muscle

One of the most intriguing observations in sparklet studies is that subsets of Ca_v1.2 channels (and also TRPV4 channels) expressed in tsA-201 cells, as well as cardiac and vascular smooth muscle can open synchronously, generating large sparklets (Figure 3) [12]. However, coupling between Ca_v1.2 channels seems to occur with a higher probability in regions of the myocyte where these channels co-exist with AKAP150. This imposes technical challenges for the recording and analysis of the molecular mechanisms underlying coupled activation of Ca_v1.2 channels. For example, since only a subpopulation of Ca_v1.2 channels could undergo coupled gating, the use of patch-clamp approaches to record currents from relatively small areas of the membrane is likely to yield limited results in terms of the number of coupled gating events per trial. Accordingly, optical recordings of Ca_v1.2 channels from relatively large portions of the surface membrane may represent a more efficient approach to study coupled gating between these channels. This approach has also proved to be fruitful for the recording of TRPV4 sparklets resulting from the cooperative gating among 4 of these channels [24].

Identification and analysis of Ca_v1.2 sparklets and currents resulting from the coordinated opening of Ca_v1.2 channels requires a multi-pronged approach [12, 36]. For Ca_v1.2 channels, this analysis has included an assessment of whether the frequency of observed multi-channel events exceeds the probability of simultaneous openings and closing of the number of channels involved (Figure 3A–D). For example, the open probability (P_o) of a single Ca_v1.2 channel is only ≈ 0.3 at +50 mV [5]. Thus, the probability of independently gating channels opening simultaneously at this potential should be very low: ≈ 0.09 , 0.03 , and 0.01 for the random, coincidental opening of 2, 3, or 4 channels. Accordingly, if L-type Ca²⁺ channels open randomly, the probability of observing Ca_v1.2 sparklets resulting from simultaneous openings of these channels should be progressively lower as the number of participating channels increases, which should result in a histogram with peaks of decreasing amplitudes as multichannel events become less apparent (Figure 3).

A complementary approach used by our group is the implementation of a coupled Markov chain model [37] to determine the coupling coefficient or strength (κ) among Ca_v1.2 channels from optical and electrical recordings [9, 12]. This model consists of a simplified Markov chain model with only 3 free parameters that takes the form of a set of binary chains that are interdependent according to a lumped coupling coefficient parameter (κ). The other two parameters (ρ and ζ) describe the intrinsic characteristics of the binary chains. The κ value could range from 0 for independently gating channels to 1 for “tightly” coupled channels that open and close simultaneously at all times. Analysis of Ca_v1.2 sparklets and currents using this model suggested a median κ value of 0.21, suggesting that the channels in these clusters seem to gate independently most of the time. This is consistent with a model in which functional coupling between Ca_v1.2 channels is sporadic [12].

Although the mechanisms underlying coupled gating of Ca_v1.2 channels in cardiac and vascular smooth muscle are not completely understood, multiple lines of evidence suggest

that $\text{Ca}_v1.2$ channels coordinate their openings via protein-to-protein interactions (Figure 4) [11, 12]. This would require that $\text{Ca}_v1.2$ channels cluster, that the distance between adjacent channels is small enough to allow physical interactions, that neighboring channels can form stable physical interactions, and finally, that physical linkage between $\text{Ca}_v1.2$ channels is sufficient to increase channel activity and coupling strength.

Consistent with this, the N&B analysis [38] suggest that $\text{Ca}_v1.2$ channels are sufficiently close to form small clusters of variable sizes that diffuse throughout the sarcolemma of ventricular myocytes (Figure 4A) [11]. $\text{Ca}_v1.2$ channels lacking most of their carboxy terminal do not undergo coupled gating [12], suggesting this segment of the channel is necessary for coupled gating. FRET analysis suggested that the carboxy terminal of adjacent $\text{Ca}_v1.2$ channels comes into close proximity under conditions that increase the probability of coupled gating (Figure 4B) [12]. Furthermore, light-induced fusion of the carboxy terminal of $\text{Ca}_v1.2$ channels increased activity and coupling strength between adjoined channels (Figure 4C) [11]. In native cells, physical interactions between $\text{Ca}_v1.2$ channels that translates into an increase in channel activity and coupling seem to be facilitated, and likely stabilized, by AKAP150, as coupled gating is eliminated by ablation of this anchoring protein. Collectively, these results strongly suggest that coupled gating of $\text{Ca}_v1.2$ sparklets involves transient interactions between the carboxy terminals of a subpopulation of $\text{Ca}_v1.2$ channels associated with AKAP150 in cardiac and vascular smooth muscle.

$\text{Ca}_v1.2$ sparklets in cardiac EC coupling

Having discussed the biophysical properties of $\text{Ca}_v1.2$ sparklets, we now turn our attention to their role in cardiac and smooth muscle. We focus first on cardiac EC coupling (Figure 5). In heart, every AP triggers a contraction. Indeed, under basal physiological conditions, the coefficient of variation (COV = standard deviation/mean) of the $[\text{Ca}^{2+}]_i$ transient that activates contraction is about 0.1, indicating a low level of trial-to-trial variation during EC [39]. The $[\text{Ca}^{2+}]_i$ transient is presumably initiated by the opening of multiple, independently gating $\text{Ca}_v1.2$ channels during an AP. The stochastic lifetimes of these channels are exponentially distributed so that the standard deviation of their lifetimes is equal to the mean, which should result in a COV = 1. This suggests that the level of variability in the open times of $\text{Ca}_v1.2$ channel could be as much as 10-fold higher than that of the $[\text{Ca}^{2+}]_i$ transient.

An important factor determining EC coupling is the amplitude of elementary $\text{Ca}_v1.2$ channel currents. At +50 mV, a membrane potential similar to the plateau of the AP, and in the presence of 20 mM Ca^{2+} with the dihydropyridine agonist FPL64176, $\text{Ca}_v1.2$ channels have a unitary conductance of about 0.5 pA. Under these experimental conditions, a $\text{Ca}_v1.2$ sparklet produced by the opening of a single $\text{Ca}_v1.2$ channel can activate a Ca^{2+} spark [4]. However, in 2 mM external Ca^{2+} , the current produced by the opening of a single $\text{Ca}_v1.2$ channel is estimated to be about 0.01 pA [40]. With this low unitary current, > 10 $\text{Ca}_v1.2$ channels must open simultaneously to reliably activate a Ca^{2+} spark and account for a $P_{\text{spark}} \approx 1$ during the plateau of the cardiac AP [40]. If the maximum open probability of $\text{Ca}_v1.2$ channels gating independently is 0.3, how can $P_{\text{spark}} \approx 1$ during the plateau of the AP when the probability of coincident activation of 10 $\text{Ca}_v1.2$ channels could be as low as 0.3^{10} ? Furthermore, how does the AP-evoked $[\text{Ca}^{2+}]_i$ transient achieves a high level of reproducibility given these constraints?

During the ventricular AP, membrane depolarization activates $\text{Ca}_v1.2$ sparklets. These Ca^{2+} signals activate small clusters of ryanodine receptors (RyRs) located in nearby jSR, initiating Ca^{2+} release from this organelle [41]. The functional unit composed of $\text{Ca}_v1.2$ channels and associated RyRs is called a “couplon” [42]. The coupling strength between

these two channels is proportional to the amount of Ca^{2+} flux via $\text{Ca}_v1.2$ channels and local $[\text{Ca}^{2+}]_i$ [6]. Synchronous activation of multiple couplons generates cell-wide increases in cytosolic $[\text{Ca}^{2+}]_i$ that triggers contraction in cardiac muscle [4].

A potential answer to question of how a $P_{\text{spark}} \approx 1$ is achieved at positive potentials, is that coupling of $\text{Ca}_v1.2$ channels increases the probability of synchronous openings of these channels and thus the probability of Ca^{2+} spark activation during EC coupling [43]. Although this hypothesis has not been tested yet, recent findings from our group are consistent with it. For example, loss of AKAP150, which decreases the probability of coupled gating between $\text{Ca}_v1.2$ channels, diminished the amplitude of the AP-evoked $[\text{Ca}^{2+}]_i$ transient [9]. Furthermore, light-induced fusion of wild type (WT) $\text{Ca}_v1.2$ channels increased Ca^{2+} influx by increasing the activity and coupling strength of adjacent $\text{Ca}_v1.2$ channels [11]. This culminated in an increase in whole-cell $\text{Ca}_v1.2$ currents, diastolic and systolic $[\text{Ca}^{2+}]_i$ levels, and contractility of cardiac cells (Figure 4D) [11].

Coupling between $\text{Ca}_v1.2$ channels, even if transient could have profound pathological implications in cardiac cells. Indeed, a recently discovered point mutation (G406R) in the $\text{Ca}_v1.2$ channels has been linked to Timothy Syndrome (TS) and arrhythmias [44]. LQT8 channels inactivate at a slower rate than WT channels [44–46]. Although phosphorylation of the channel by the Ca^{2+} /CaM-dependent kinase II (CAMKII) has been suggested as a potential culprit for abnormal TS channel function [47], the mechanisms underlying larger Ca^{2+} currents by these channels are incompletely understood.

Recent data from our team shed some light on this issue, however. Using optogenetic approaches, it was found that light-induced fusion between WT and TS channels induced the WT channel to take on the biophysical properties of the “dominant” TS channel. Fusion of these channels conferred TS properties to the linked WT channel, thereby increasing Ca^{2+} currents, $[\text{Ca}^{2+}]_i$ transient, contractility and arrhythmogenic Ca^{2+} fluctuations in cardiac cells [11]. Data suggested that the channel with the higher activity (e.g. TS channels) is likely to determine the level of activity of the linked channel. This observation has profound functional implications, especially in the context of the TS disease, where only about 23% of $\text{Ca}_v1.2$ channels in the heart carry the mutation [44]. Thus, the expression of a small number of TS channels could amplify Ca^{2+} influx in cardiac cells by interacting and modulating the activity of neighboring WT channels.

An important observation is that AKAP150 plays a critical role in the chain of events shaping abnormal TS channel function in cardiac cells that is independent of CAMKII activity or its ability to target PKA [9]. In the presence of the TS mutation, AKAP150 functions like a subunit of TS channels. AKAP150 most likely interacts with TS channels via leucine zipper domains located in the carboxy terminal of both proteins [31]. The formation of this aberrant channel-anchoring protein complex serves to stabilize the open conformation and increase the frequency of coupled events in TS channels, thus leading to cardiac hypertrophy and arrhythmias. Data in support of this model is compelling. *First*, using a transgenic mouse that expresses the TS mutation solely in the heart, it was found that ablation of AKAP150 protects against cardiac hypertrophy during TS. *Second*, loss of AKAP150 was sufficient to restore inactivation rates of whole-cell Ca^{2+} currents, single-channel activity, frequency of coupled events, EC coupling, action potential waveform, and cardiac rhythm to WT levels in cardiac cells expressing the TS mutation. Thus, it is proposed that AKAP150 interacts with TS channels via the carboxy terminal of both proteins to function as an allosteric modulator of the channel with detrimental physiological consequences. Future studies should determine the molecular mechanisms governing the interaction and regulation of AKAP150 in WT and TS channels.

Ca_v1.2 sparklets in vascular smooth muscle EC coupling

Unlike ventricular myocytes, Ca_v1.2 channels and RyRs are not functionally coupled in vascular smooth muscle [48–50]. Rather, activation of Ca_v1.2 sparklets contributes to a global cytosolic Ca²⁺ pool from which the SR can draw to accelerate store refilling and/or directly activates the contractile machinery in vascular smooth muscle (Figure 5) [48]. Ca²⁺ entering via persistent Ca²⁺ sparklets with coupled events accounted for ~50% of the total dihydropyridine-sensitive Ca²⁺ influx that regulates steady-state global [Ca²⁺]_i [8]. Thus, an increase in the frequency of persistent Ca²⁺ sparklets with coupled events could have profound effects on vascular smooth muscle excitability.

Ca_v1.2 sparklet activity and frequency of coupled events are significantly elevated in vascular smooth muscle during hypertension and in type II diabetes [32, 51], thus increasing arterial tone during these pathological conditions. The mechanisms underlying this increased in sparklet activity and frequency of coupled events depend on the pathological condition, however. Although AKAP is required for the generation of high-activity Ca_v1.2 sparklets and induction of coupled events in both cases, increases in sparklet activity and coupled gating in type II diabetes results from local activation of an AKAP-targeted PKA [32]. On the other hand, increased activity and coupled gating of Ca_v1.2 sparklets during hypertension is the result of an increase in Ca_v1.2 α1 subunit expression [52], AKAP150-targeted PKCα activity [51], and possibly PKCα-dependent reactive oxygen species (ROS) signaling [34, 35]. Regardless of the mechanism, data indicate that an increase in Ca_v1.2 sparklet activity and frequency of coupled events could translate into enhanced Ca²⁺ influx, global [Ca²⁺]_i, and thus contraction, that may contribute to the development of vascular dysfunction during type II diabetes and hypertension.

Ca_v1.2 sparklets in ET coupling

Ca_v1.2 sparklets could also regulate gene expression in cardiac and vascular smooth muscle. Ca²⁺ influx through Ca_v1.2 channels is required for activation of the transcription factor NFATc3 via calcineurin after myocardial infarction and during angiotensin II-induced hypertension [51, 53–56]. Activation of this pathway results in the down-regulation of a number of K⁺ channels that contribute to excitability of cardiac and vascular smooth muscle (Figure 5) [53–55].

During physiological conditions, calcineurin and NFATc3 activity are relatively low because of low levels of persistent Ca_v1.2 sparklets and frequency of coupled events [12, 20], and high nuclear NFATc3 export rates [57]. However, an increase in the activity of AKAP-targeted PKA and/or AKAP-targeted PKC during myocardial infarction and type II diabetes, or hypertension, respectively, induces high-activity Ca_v1.2 sparklets and coupled gating behavior. This will produce an increase in local Ca²⁺ influx that can be sense by the AKAP-targeted Ca²⁺/CaM phosphatase calcineurin. Once activated, calcineurin can dephosphorylate NFATc3, thus inducing its translocation into the nucleus where it can modify the expression of Kv and BK channel subunits in cardiac and vascular smooth muscle. Downregulation of K⁺ channel subunits decreases Ca²⁺-sensitive and voltage-dependent K⁺ currents, thus contributing to vascular smooth muscle depolarization during hypertension and possibly type II diabetes. In the heart, downregulation of K⁺ channel subunits decreases K⁺ currents leading to action potential prolongation. In both cases, the end result is an increase in global [Ca²⁺]_i that ultimately perpetuates the pathological condition in cardiac and vascular smooth muscle. Consistent with this model, ablation of AKAP150, which prevents the induction of persistent Ca_v1.2 sparklets and coupled events, protects against the development of angiotensin II-induced hypertension [12, 20]. Future studies should determine whether loss of AKAP150, or disruption of the interaction between AKAP150 and any of its binding partners (PKA, PKC or calcineurin) prevents the activation

of NFATc3, and thus the remodeling of K^+ channels function during hypertension, vascular dysfunction during type II diabetes or myocardial infarction.

Conclusions

The application of novel imaging techniques and stringent analytical approaches over the last decade has allowed the study of the functional organization of $Ca_v1.2$ channels with high spatial and temporal resolution. These optical recordings and analytical approaches are applicable to the study of any Ca^{2+} permeable ion channel in excitable and non-excitable cells. Using these approaches, four major fundamental features about $Ca_v1.2$ channels in cardiac and vascular smooth muscle have been revealed. *First*, the activity and spatial organization of functional channels is variable throughout the sarcolemma. *Second*, a subpopulation of these channels can become functionally coupled to promote Ca^{2+} signal amplification. *Third*, the scaffolding protein AKAP150 can function as an auxiliary subunit of $Ca_v1.2$ channels to promote coupled gating between adjacent channels. Consistent with this, heterogeneous $Ca_v1.2$ sparklet activity and coupled gating seems to be dependent on a signaling module formed by a subpopulation of $Ca_v1.2$ channels in association with AKAP150, PKA, PKC, and calcineurin in specific foci of the sarcolemma. *Fourth*, in a cluster with coupled $Ca_v1.2$ channels, the channel with the higher open probability (i.e. P_O) is likely to determine the level of activity of the linked channel, thus effectively amplifying calcium influx. Evidently, the formation of this signaling module has profound implications for EC and ET coupling during physiological and pathological conditions in cardiac and vascular smooth muscle.

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Figure 1. Cav1.2 sparklets

A) Total internal reflection fluorescence and swept-field confocal images of Cav1.2 sparklets recorded from representative tsA201 cells expressing Cav1.2 channels, isolated vascular smooth muscle, neonatal cardiac cells or smooth muscle cells in an intact artery. The traces to the right of each image show the time-course of $[\text{Ca}^{2+}]_i$ in the sites highlighted by the green circle (square) in the images. B) Amplitude histograms of Cav1.2 sparklets recorded from tsA201 cells expressing Cav1.2 channels or vascular smooth muscle cells. The solid black lines in the histograms are the best fit to the data following a multicomponent Gaussian function with a quantal unit of Ca^{2+} influx of 38 nM Ca^{2+} . C) Plot of the relationship between signal mass as a function of Ca^{2+} flux in vascular smooth muscle. Parts of this figure have been adapted from Navedo et al [10, 27], and Amberg et al [8].

Figure 2. Mechanisms of heterogeneous $\text{Ca}_v1.2$ sparklet activity

A) Schematic diagram of the interaction of AKAP150 with a subpopulation of $\text{Ca}_v1.2$ channels. B) Representative time-course of $[\text{Ca}^{2+}]_i$ traces illustrating that activation of PKC with PDBu increases $\text{Ca}_v1.2$ sparklet activity in vascular smooth muscle. Right panel shows fluorescence traces indicating that AKAP150 is required for increased $\text{Ca}_v1.2$ sparklet activity. C) Time-course of $[\text{Ca}^{2+}]_i$ demonstrating that inhibition of calcineurin with cyclosporine A increases $\text{Ca}_v1.2$ sparklet activity. Parts of this figure have been adapted from Navedo et al [20, 27].

Figure 3. Coupled gating of $\text{Ca}_v1.2$ channels

A) Representative optical recordings of single $\text{Ca}_v1.2$ channels in neonatal cardiac cells (top), tsA201 cells (middle), and vascular smooth muscle cells (bottom). B) All-points histogram of $\text{Ca}_v1.2$ sparklet traces in A. C) Fluorescence time-course of a $\text{Ca}_v1.2$ sparklet site with coupled channels. D) All-points histogram of $\text{Ca}_v1.2$ sparklet traces in D. E) Representative fluorescence traces of stochastic (left panel) and coupled $\text{Ca}_v1.2$ sparklets in tsA201 cells, vascular smooth muscle and cardiac cells. Parts of this figure have been adapted from Navedo et al [12].

Figure 4. Ca²⁺ signaling amplification by oligomerization of Ca_v1.2 channels

A) Bar plot of the number & brightness analysis showing the relative percentage of mobile pixels in tsA201 cells or cardiac cells expressing wild type (wt) or Timothy syndrome (TS) Ca_v1.2 channels tagged with tRFP. B) Bar plot of the mean FRET efficiency between wt Ca_v1.2 channels or TS Ca_v1.2 channels. C) Representative recordings of Ca_v1.2 currents (upper panel) and sparklets (lower panel) obtained from tsA201 cells transfected with Ca_v1.2 channels tagged with a light-activated fusion system [58] before and after induction of channels fusion. D) Evoked Ca²⁺ transients and simultaneous cell length changes in untransfected cardiac cells (upper panel) and cells expressing Ca_v1.2 channels tagged with the light-activated fusion system (lower panel) before and after channels fusion. Adapted from Dixon et al [11].

Figure 5. Proposed model for the role of Cav1.2 sparklet activity and coupled gating events on EC and ET coupling in cardiac and vascular smooth muscle
See main text for details.