RYR2 Proteins Contribute to the Formation of Ca\(^{2+}\) Sparks in Smooth Muscle

GUANGJU JI,1 MORRIS E. FELDMAN,1 KAI SU GREENE,1 VINCENZO SORRENTINO,2 HONG-BO XIN,1 and MICHAEL I. KOTLIKOFF1

1Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853
2Molecular Medicine Section Department of Neuroscience, University of Siena, Siena, Italy 53100

ABSTRACT Calcium release through ryanodine receptors (RYR) activates calcium-dependent membrane conductances and plays an important role in excitation-contraction coupling in smooth muscle. The specific RYR isoforms associated with this release in smooth muscle, and the role of RYR-associated proteins such as FK506 binding proteins (FKBPs), has not been clearly established, however. FKBP12.6 proteins interact with RYR2 Ca\(^{2+}\) release channels and the absence of these proteins predictably alters the amplitude and kinetics of RYR2 unitary Ca\(^{2+}\) release events (Ca\(^{2+}\) sparks). To evaluate the role of specific RYR2 and FBKP12.6 proteins in Ca\(^{2+}\) release processes in smooth muscle, we compared spontaneous transient outward currents (STOCs), Ca\(^{2+}\) sparks, Ca\(^{2+}\)-induced Ca\(^{2+}\) release, and Ca\(^{2+}\) waves in smooth muscle cells freshly isolated from wild-type, FKBP12.6\(^{-/-}\), and RYR3\(^{-/-}\) mouse bladders. Consistent with a role of FKBP12.6 and RYR2 proteins in spontaneous Ca\(^{2+}\) sparks, we show that the frequency, amplitude, and kinetics of spontaneous, transient outward currents (STOCs) and spontaneous Ca\(^{2+}\) sparks are altered in FKBP12.6 deficient myocytes relative to wild-type and RYR3 null cells, which were not significantly different from each other. Ca\(^{2+}\)-induced Ca\(^{2+}\) release was similarly augmented in FKBP12.6\(^{-/-}\), but not in RYR3 null cells relative to wild-type. Finally, Ca\(^{2+}\) wave speed evoked by CICR was not different in RYR3 cells relative to control, indicating that these proteins are not necessary for normal Ca\(^{2+}\) wave propagation. The effect of FKBP12.6 deletion on the frequency, amplitude, and kinetics of spontaneous and evoked Ca\(^{2+}\) sparks in smooth muscle, and the finding of normal Ca\(^{2+}\) sparks and CICR in RYR3 null mice, indicate that Ca\(^{2+}\) release through RYR2 molecules contributes to the formation of spontaneous and evoked Ca\(^{2+}\) sparks, and associated STOCs, in smooth muscle.

KEY WORDS: Ca\(^{2+}\)-induced Ca\(^{2+}\) release • ryanodine receptor • FKBP12.6 • RYR3 • knockout mouse

INTRODUCTION

RYR participate in Ca\(^{2+}\) mobilization in smooth muscle through the generation of spontaneous Ca\(^{2+}\) sparks (Nelson et al., 1995), calcium-induced calcium release (CICR) (Imaizumi et al., 1998; Collier et al., 2000), and stretch-induced calcium release (SICR) (Ji et al., 2002). Whether spontaneous, induced by I\(_{Ca}\), or by stretch, Ca\(^{2+}\) sparks repeatedly arise from a few specialized regions within the myocyte, termed frequent discharge sites (Gordienko et al., 1998). As smooth muscle cells express multiple RYR genes (Marks et al., 1989; Hakamaeta al., 1992; Ledbetter et al., 1994; Neylon et al., 1995), the ascription of discreet functional roles to these genes has been problematic (Kotlikoff, 2003).

To address the role of specific RYR isoforms in RYR-mediated Ca\(^{2+}\) release in smooth muscle, we have examined Ca\(^{2+}\) sparks and spontaneous, transient outward currents (STOCs), which are the local membrane response to Ca\(^{2+}\) sparks (Benham and Bolton, 1986; Nelson et al., 1995; Perez et al., 1999) in urinary bladder smooth muscle cells dissociated from RYR3 and FKBP12.6 null mice. RYR3 null mice demonstrate some impaired responses in neonatal skeletal muscles (Berlocchi et al., 1997), but do not display a prominent smooth muscle phenotype. It has been reported that RYR3 proteins are not involved in Ca\(^{2+}\) sparks or global Ca\(^{2+}\) release in vascular myocytes (but that RYR1 and RYR2 are required) (Coussin et al., 2000); however, recent studies of Ca\(^{2+}\) sparks and STOCs in cerebral myocytes from RYR3 null mice reported an increase in the frequency of Ca\(^{2+}\) sparks and a decreased myogenic tone in these mice (Lohn et al., 2001). Further complicating this analysis, Jiang et al. (2003) recently identified a highly expressed deletion splice variant in smooth muscle that acts as an inhibitory subunit.

As FKBP12.6 proteins are known to selectively associate with RYR2 channels (Timerman et al., 1996; Xin et al., 1999), and inactivation of the FKBP12.6 gene in mice results in a characteristic lengthening of the Ca\(^{2+}\)...
sparks in heart cells (Xin et al., 2002), we reasoned that FKBP12.6 null mice could be used to determine whether Ca$^{2+}$ sparks in smooth muscle arise from RYR2 molecules. We report here that spontaneous Ca$^{2+}$ sparks and STOCs in myocytes from FKBP12.6 null mice are markedly altered, whereas RYR3-deficient mice show no observable alterations in spark amplitude, kinetics, or frequency. Similar results were obtained for Ca$^{2+}$ sparks evoked by voltage-clamp steps to activate $I_{Ca}$ and CICR, confirming the essential similarity of these processes. Finally, Ca$^{2+}$ wave speed was equivalent in WT and RYR3 null mice, suggesting that RYR3 molecules are not essential for wave propagation. These data strongly argue for a central functional role of RYR2 in smooth muscle Ca$^{2+}$ release.

**Materials and Methods**

**Cell Preparation**

Mice were anesthetized and killed in accordance with an approved laboratory animal protocol and bladder myocytes prepared as previously described (Ji et al., 2002). Briefly, the urinary bladder was dissected in ice-cold oxygenated Ca$^{2+}$-free solution containing (mM): 80 Na-glutamate, 55 NaCl, 6 KCl, 2 MgCl$_2$, 10 HEPES, and 10 glucose. The detrusor muscle was minced and incubated for 20 min at 37°C in dissociation solution containing 1 mg/ml dithioerythritol, 1 mg/ml papain, and 1 mg/ml bovine serum albumin (Sigma-Aldrich), and the partially digested tissue was then transferred to a solution containing 1 mg/ml collagenase type II (Worthington Biochemical), 1 mg/ml bovine serum albumin, and 100 μM Ca$^{2+}$. After incubation for 10 min, the digested tissue was washed and gently triturated in dissociation solution to yield single smooth muscle cells.

**Patch-Clamp Recording**

Membrane currents recorded at room temperature using whole-cell voltage clamp methods. To examine STOCs, cells were clamped at −20 mV, or stepped from −50 to −20 mV in 10-mV increments to examine voltage dependence. The intracellular solution was (mM): 130 KCl, 1.8 MgCl$_2$, 1.0 Na$_2$ATP, 0.05 CaCl$_2$, 0.1 EGTA, pH 7.3, and the extracellular solution was: 137 NaCl, 5.4 KCl, 1.5 CaCl$_2$, 1.0 MgCl$_2$, 10 glucose, 10 HEPES, pH 7.4. Currents were filtered at 500 Hz and digitized at 2 kHz. In some cases voltage-clamped cells were dialedized with 30 μM Fluo-4 and simultaneously scanned to measure Ca$^{2+}$ sparks (see below). Current clamp measurements of membrane potential were performed by adjusting the holding current to produce membrane voltages of approximately −20 mV. The pipette solution was: 50 KCl, 80 KAsp, 1 MgCl$_2$, 0.1 EGTA, 3 MgATP, and 10 HEPES (pH adjusted to 7.2 with KOH).

**Measurement of Ca$^{2+}$ Fluorescence**

Myocytes were incubated with 10 μM Fluo-4 a.m. (Molecular Probes) for 10 min at room temperature and transferred into a recording chamber mounted on an inverted microscope (TE300; Nikon). Cells were allowed to adhere to the bottom of the recording chamber for 15 min and then perfused with extracellular solution (see above) for 40 min. For CICR experiments, 200 ms voltage steps from −70 to −20 mV were imposed and fluo-4 fluorescence recorded using a laser scanning confocal head (Radiance 2000, Bio-Rad Laboratories) coupled to an inverted microscope (TE-300; Nikon) equipped with a plan-apo 60x water immersion objective (1.2 n.a.; Nikon). Cells were excited with 488 nm light from a krypton/argon laser and linescan images recorded using Lasersharp software (Bio-Rad Laboratories).

**Knockout Mice and RTPCR**

Mice homozygous for the inactive RYR3 allele (Bertocchini et al., 1997) were obtained and maintained as heterozygotes, continuously backcrossed onto a C57Bl/6 background. FKBP12.6 null mice (Xin et al., 2002), maintained on a C57Bl/6 background, were aged matched with RYR3 and wild-type (WT) C57Bl/6 mice. RT-PCR was performed by routine methods on detrusor muscle dissected free of other tissue layers, using the following specific primers (forward and reverse, respectively): RYR1-GCA-CACGTGTCAGGAGTGTGATG, GGGTGATGCACAGGATTITAT; RYR2-GAATTCATCATCACGATACTCTACGC, GTCATGCACTATCCTTGCCAT; RYR3-CTTGAAGTTTACGACAACTCAG, TAGCTGCTAAAAGCTTTTCAAGC.

**Data Analysis**

Ca$^{2+}$ sparks were analyzed using custom software written in Matlab. To display the linescan images in a consistent way, $F_0$ was obtained by averaging the fluorescence for each pixel (x dimension) for a period preceding activation of a Ca$^{2+}$ spark, and the fluorescence of all pixels ($F$) was divided on a pixel by pixel basis by $F_0$. Linescans were then displayed with a colormap ranging from 0.5 to 3 $F/F_0$. Profiles were constructed by averaging the pixels bisecting a Ca$^{2+}$ spark for each time point in the scan. Calcium sparks were fit to a function with six free parameters ($F_0$, start time, rise time, peak $F/F_0$, half time decay and final offset) using a Levenberg-Marquardt nonlinear least squares fitting routine. STOC decay kinetics were measured by fitting the current recordings to a single exponential decay function using Clamplt. STOC rise time and peak current were measured by hand from the raw current recordings. Results are expressed as mean ± SE where applicable. Data from the three groups (WT, RYR3$^{-/-}$, and FKBP12.6$^{-/-}$) were compared by one-way, repeated measures of ANOVA and significant differences between groups determined by the Student-Newman-Keuls (SNK) test for pairwise comparisons. Ca$^{2+}$ wave speed was determined by constructing multiple pseudolinescans along the axis of Ca$^{2+}$ wave propagation; for each repeatedly imaged pixel, the frame in which Ca$^{2+}$ fluorescence increased from the previous average of 5 frames by 20% or greater were identified and a best fit line through these pixels was used to determine the slope (wave velocity). For each experiment three or four lines were used and the slopes averaged to obtain the Ca$^{2+}$ wave speed. Measurements in WT and RYR3$^{-/-}$ myocytes were compared by the Student’s t test.

**Results**

STOCs in RYR3 and FKBP12.6 Null Urinary Bladder Myocytes

We first sought to confirm the presence of RYR isoforms in mouse urinary bladder smooth muscle, as studies had not reported the RYR expression pattern in the mouse. The detrusor muscle was carefully dissected free from the mucosal and fibrosal layers and RNA prepared for RT-PCR. As shown in Fig. 1 A, all three isoforms were detected in experiments using primers spe-
specific for the respective transcripts, whereas no bands were obtained in the absence of reverse transcriptase. The relative intensity of individual bands, even from the same pool of RNA, should not be interpreted quantitatively, as numerous cell types beside muscle are present at low levels within smooth muscle tissues and the efficiency of amplification may be quite variable. Moreover, such results are of limited use in the determination of functionally relevant isoforms and highlight the necessity of using other methods to resolve the specific isoform(s) contributing to smooth muscle Ca\(^{2+}\)/calmodulin release.

Previous data indicate that FKBP12.6 plays an important role in regulating Ca\(^{2+}\)/calmodulin release in cardiac myocytes (Xin et al., 2002), a process that is mediated by RYR2. The selective interaction of FKBP12.6 with RYR2 (Timmerman et al., 1996; Xin et al., 1999) allowed us to use FKBP12.6-deficient mice to determine whether Ca\(^{2+}\)/calmodulin release events were altered in a manner consistent with that observed for cardiac Ca\(^{2+}\) sparks (Xin et al., 2002).

As STOCs are a convenient indicator of spontaneous SR Ca\(^{2+}\) release events (Walsh and Singer, 1983; Benjamin and Bolton, 1986; Trieschmann and Isenberg, 1989; Strehno-Bittel and Sturek, 1992; Nelson et al., 1995; Wang et al., 1997), we first measured the amplitude, frequency, and kinetics of STOCs in urinary bladder myocytes from wild-type, FKBP12.6/−/−, and RYR3/−/− mice.
mice. Cells were progressively depolarized from a holding potential of \(-50\) mV to increase the frequency of spontaneous events. As shown in Fig. 1, the frequency and amplitude of spontaneous Ca\(^{2+}\) release events, reflected by the frequency of STOCs, was markedly higher in FKBP12.6 null mice than in WT or RYR3-deficient myocytes, at all potentials examined. In a series of experiments identical voltage-clamp protocols were imposed on cells dissociated from each group (cells were used from at least four separate mice in each group) and the STOC frequency determined at each voltage. At holding voltages from \(-60\) to \(-20\) mV (10-mV intervals) the STOC frequency was significantly higher in FKBP12.6 null mice than in WT or RYR3-deficient myocytes, at all potentials examined. 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important role in the regulation of STOCs in mouse bladder myocytes, consistent with the notion that these currents arise from a close coupling of sarcoplasmic maxiK channels with RYR2 in the SR membrane, and likely reflect the underlying Ca$^{2+}$ release kinetics and amplitude.

**FKBP12.6 Regulates Ca$^{2+}$ Sparks in Smooth Muscle**

To further test the assumption that altered STOCs reflect changes in the underlying Ca$^{2+}$ release events, we measured Ca$^{2+}$ sparks in voltage-clamped urinary bladder myocytes from WT, RYR3$^{-/-}$, and FKBP12.6$^{-/-}$ mice. Linescan images were obtained to resolve the kinetics of these events and STOCs activated by the spontaneous Ca$^{2+}$ sparks were simultaneously recorded. Fig. 3 shows typical recordings from the three cell types as well as a plot of normalized fluorescence intensity within the area of the spark. Ca$^{2+}$ sparks in wild-type cells displayed typical kinetics, which extended beyond the duration of the related STOC, as has been previously reported (Nelson et al., 1995; ZhuGe et al., 2000). Events in RYR3 null cells were quite similar in frequency and amplitude as WT cells. In contrast, FKBP12.6$^{-/-}$ cells displayed a higher frequency of spontaneous Ca$^{2+}$ sparks and these events were of higher amplitude and spread than in wild-type or RYR3 null myocytes, often forming propagated Ca$^{2+}$ waves, with correspondingly large transient outward currents. Examples of individual Ca$^{2+}$ sparks at higher time resolution, and a summary of the mean amplitude, spread (full width, half-maximum), and decay for the different cell types are shown in Fig. 4. Ca$^{2+}$ sparks in FKBP12.6$^{-/-}$ null myocytes were of significantly greater amplitude and spread, and the decay time significantly increased, relative to the other two groups, which were not significantly different from each other.

**Figure 4. Augmented spontaneous Ca$^{2+}$ sparks in FKBP12.6 null myocytes.** (A) Confocal linescans of representative Ca$^{2+}$ sparks from the three mouse groups indicate the increase in amplitude, spread, and decay time in FKBP12.6 null myocytes. The characteristic lengthened decay of Ca$^{2+}$ sparks is seen in the example shown. (B) Mean properties of Ca$^{2+}$ sparks from the three groups (23/16, 14/8, and 16/10 for the cells/mice used in WT, RYR3$^{-/-}$, and FKBP12.6$^{-/-}$ mice, respectively). The amplitude (F/Fo), size (full width at half maximal amplitude, FWHM), and exponential decay half time were all significantly greater in FKBP12.6 null myocytes than in WT cells. * and ** indicate P < 0.05 and 0.01, respectively by ANOVA using the Student-Newman-Keuls test. Ca$^{2+}$ sparks in FKBP12.6$^{-/-}$ null mice were of significantly greater amplitude and spread, and the decay time significantly increased, relative to the other two groups, which were not significantly different from each other.
distributions were generated for F/F₀, FWHM, and decay. The distribution of Ca²⁺ spark frequency, amplitude, size, and kinetics was markedly similar between control and RYR3 null mice, whereas sparks from FKBP12.6 null mice were markedly skewed toward more frequent, larger, and longer events. Thus, for example over 85% Ca²⁺ sparks from WT cells were of amplitude between 1.25–2 F/F₀, FWHM between 1.5–3 μm, and decay of between 0–250 ms; the corresponding ranges for FKBP12.6 null cells were 1.25–2.75 F/F₀, 3.5–6.5 μm, and 50–350 ms.

FKBP12.6 Regulates Transient Hyperpolarizations

A major consequence of spontaneous Ca²⁺ release in smooth muscle is the regulation of membrane potential through the activation of calcium-dependent sarcolemmal ion channels (Nelson et al., 1995). Spontaneous Ca²⁺ sparks and hyperpolarizations have not been simultaneously measured, however. To examine the relationship between spontaneous changes in membrane potential and the Ca²⁺ sparks that generate them, and to determine whether the alterations in Ca²⁺ sparks associated with targeted deletion of FKBP12.6 result in altered spontaneous depolarization, we simultaneously measured Ca²⁺ sparks and myocyte membrane potential in current-clamped myocytes. To increase the frequency of spontaneous Ca²⁺ sparks, cells were clamped at current injection levels producing a membrane potential of approximately −20 mV. Typical recordings in WT and transgenic myocytes are shown in Fig. 5. Ca²⁺ sparks produced prominent hyperpolarizations, as seen by voltage excursions temporally linked to Ca²⁺ release events. In WT cells, hyperpolarizations averaged ~12 mV and were always observed with larger Ca²⁺ sparks. Consistent with voltage clamp experiments, Ca²⁺ sparks in FKBP12.6 null cells induced larger and longer hyperpolarizations than observed in WT cells. Peak hyperpolarizations were 12.7 ± 1.3 mV (n = 54) in WT, 9.9 ± 1.3 mV (n = 16) in RYR3⁻/⁻, and 18.2 ± 2.0 mV (n = 37) in FKBP12.6 null cells, respectively (P < 0.05).
Calcium-induced Calcium Release in Wild-type, RYR3−/−, and FKBP12.6−/− Myocytes

The processes that underlie spontaneous Ca²⁺ sparks are likely quite similar to those associated with CICR, as CICR often manifests as the triggering of individual Ca²⁺ sparks from the same sites from which spontaneous Ca²⁺ sparks originate (Imaizumi et al., 1998; Collier et al., 2000; Ji et al., 2002). If equivalent Ca²⁺ release mechanisms underlie both processes, deletion of specific components of the release system by gene targeting should result in similar alterations in both spontaneous and evoked Ca²⁺ sparks. To determine the phenotype of Ca²⁺ sparks evoked by CICR in FKBP12.6 and RYR3 null mice, voltage-clamped myocytes were depolarized from −70 to −30 mV to activate small I_{Ca} and isolated Ca²⁺ sparks, and simultaneously imaged by confocal scanning. As shown in Fig. 6, Ca²⁺ sparks triggered by depolarizations to evoke I_{Ca} were increased in amplitude, duration, and size in FKBP12.6-deleted mice, whereas sparks triggered by I_{Ca} in RYR3 null myocytes were not significantly different from those of WT mice. Moreover, the degree of augmentation of Ca²⁺ release events triggered by I_{Ca} was roughly similar as that observed with spontaneous events (compare Figs. 4 B and 6 B). The extended tail of Ca²⁺ sparks in FKBP12.6 null myocytes was significantly greater than in the other two genotypes and no significant difference was observed between WT and RYR3 null mice. Experiments from five, four, and six WT, RYR3 null, and FKBP12.6 null mice, respectively. ** indicates P < 0.01 for FKBP12.6 null mice, versus the other two groups, and lack of significance of any other comparison.

Figure 6. Augmented calcium-induced calcium release in FKBP12.6 null myocytes. (A) Representative linescan images of Ca²⁺ sparks evoked by a voltage-clamp depolarization to −30 mV to evoke single Ca²⁺ sparks. Note the extended decay (tail) of the Ca²⁺ spark in the FKBP12.6 myocyte and its earlier occurrence after the voltage step. Linescan calibration bar is 10 μm. (B) Mean characteristics of the evoked Ca²⁺ sparks from the experiments shown. The amplitude, size, and decay of Ca²⁺ sparks in FKBP12.6 null myocytes was significantly greater than in the other two genotypes and no significant difference was observed between WT and RYR3 null mice. Experiments from five, four, and six WT, RYR3 null, and FKBP12.6 null mice, respectively. ** indicates P < 0.01 for FKBP12.6 null mice, versus the other two groups, and lack of significance of any other comparison.
As shown in Fig. 7, Ca$^{2+}$ waves are propagated in voltage-clamped RYR3 null myocytes after induction of CICR by ramp depolarizations. Wave speed was determined in rapid confocal imaging experiments (20 or 30 frames/s) in a series of experiments in which myocytes were depolarized from $-60$ to $-10$ mV, by constructing pseudolinescans along three to four directions of wave propagation and averaging these values for a given experiment (Fig. 7 B). As shown in Fig. 7 C, a series of such experiments in WT and RYR3 null myocytes demonstrated equivalent Ca$^{2+}$ wave speed, suggesting that RYR3 channels do not play an essential role in the propagation of Ca$^{2+}$ waves in urinary bladder myocytes.

**DISCUSSION**

We show here that FKBP12.6 proteins play an important functional role in RYR-mediated Ca$^{2+}$ release in urinary bladder smooth muscle, altering the characteristics of unitary Ca$^{2+}$ release events and associated Ca$^{2+}$-activated currents. FKBP12.6 proteins have been shown to selectively associate with cardiac (RYR2) SR Ca$^{2+}$ channels (Timerman et al., 1996; Xin et al., 1999, 2002) and recent work from our laboratory has demonstrated a specific association between FKBP12.6 and RYR2, but not RYR1 or RYR3 proteins, in smooth muscle (Wang et al., 2004). These results imply an important functional role for RYR2 channels in Ca$^{2+}$ spark generation, as the effect of FKBP12.6 deletion on the frequency, amplitude, and kinetics of potassium currents activated by Ca$^{2+}$ sparks, the properties of spontaneous Ca$^{2+}$ sparks, and the properties of Ca$^{2+}$ sparks triggered by CICR in urinary bladder myocytes is consistent with the effect of this deletion on Ca$^{2+}$ release in cardiac myocytes (Marx et al., 2000, 2001; Xin et al., 2002).

Conversely, the properties of STOCs and spontaneous or evoked Ca$^{2+}$ sparks were equivalent in RYR3-deficient and WT myocytes. These results are in contrast to a previous report (Lohn et al., 2001), which demonstrated a marked increase in STOC frequency (10–50-fold increase over the voltage range reported here) in cerebral arterial myocytes. Consistent with that report, however, no alteration in the amplitude, width, or duration of Ca$^{2+}$ sparks was observed in RYR3 null cells compared with WT. Thus, both our results and those of Lohn et al. (2001) argue against an essential role of RYR3 proteins in the generation of Ca$^{2+}$ sparks in smooth muscle. Recent studies have indicated that RYR3 proteins may play a secondary, regulatory role associated with the formation of heterotetramers or dominant negative effects (Jiang et al., 2003). The marked difference in our findings and those of Lohn et al. (2001) regarding the frequency of STOCs may therefore result from different levels of expression of RYR3 splice variants in different smooth muscles, or may derive from a more general role of these channels in the regulation of [Ca$^{2+}$], unrelated to CICR. Thus, RYR3 deletion may alter the basal level of [Ca$^{2+}$], resulting in an increase in Ca$^{2+}$ spark frequency in some myocytes, without an alteration in the fundamental properties (e.g., channel open time) of the release event. While we are not able to definitively exclude a role of RYR1 in smooth muscle Ca$^{2+}$ release, it seems quite unlikely that FKBP12.6 deletion results in an alteration in RYR1 function, as RYR1 proteins do not associate with FKBP12.6 proteins (Timerman et al., 1996). A functional role for RYR1 proteins in the formation of Ca$^{2+}$ sparks and a role for RYR3 proteins in global Ca$^{2+}$ release has been reported in vascular myocytes using antisense approaches in cultured cells (Coussin et al., 2000; Mironneau et al., 2001).
We also examined Ca$^{2+}$ waves in RYR3 null myocytes, as these proteins could be involved in transmitting unitary Ca$^{2+}$ release events through myocytes, although they do not appear to be primarily involved in the initial Ca$^{2+}$ spark formation. Our finding of equivalent wave speed in both cell types indicates that these channels are not required for the propagation of locally generated events. This finding is also consistent with the observation of similar Ca$^{2+}$ spark width in RYR3$^{-/-}$ and WT myocytes, as one would predict a decrease in the spread of Ca$^{2+}$ sparks, which appear to consist of a continuum of events from locally confined Ca$^{2+}$ sparks to globally propagated waves. While these findings do not exclude a role for RYR3 channels in the amplification of Ca$^{2+}$ release events in smooth muscle cells, they establish the sufficiency of the other expressed isoforms to produce the major features of ryanodine receptor–mediated Ca$^{2+}$ release and unitary release from discrete sites and amplification to generate propagated waves.

Finally, the equivalent effect of FKBP12.6 deletion on spontaneous Ca$^{2+}$ sparks and on CICR evoked by depolarizations to activate I$_{Ca}$ suggests a fundamental similarity between these processes and argues for a critical role of RYR2 proteins. Spontaneous Ca$^{2+}$ release events occur repeatedly from the same frequent discharge sites (Nelson et al., 1995; Bolton and Gordienko, 1998) and CICR and SICR events occur from the same frequent discharge sites (Collier et al., 2000; Ji et al., 2002). Thus, our results suggest that these areas contain RYR2 and FKBP12.6 proteins; SR release is likely initiated by gating of these molecules, which display gating kinetics that shape the resulting Ca$^{2+}$ sparks. Preferential initiation of Ca$^{2+}$ sparks in a few subcellular locations could arise due to a association between the sarcolemma and SR containing RYR2, or due to specialized SR in which a higher concentration of these molecules are inserted. Further proof of this hypothesis will require the selective inactivation of RYR2 channels in smooth muscle.

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