Regulation of Calcium Sparks and Spontaneous Transient Outward Currents by RyR3 in Arterial Vascular Smooth Muscle Cells

Matthias Löhn, Wolfgang Jessner, Michael Fürstenau, Maren Wellner, Vincenzo Sorrentino, Hermann Haller, Friedrich C. Luft, Maik Gollasch

Abstract—Intracellular Ca\textsuperscript{2+} levels control both contraction and relaxation in vascular smooth muscle cells (VSMCs). Ca\textsuperscript{2+}-dependent relaxation is mediated by discretely localized Ca\textsuperscript{2+} release events through ryanodine receptor (RyR) channels in the sarcoplasmic reticulum (SR). These local increases in Ca\textsuperscript{2+} concentration, termed sparks, stimulate nearby Ca\textsuperscript{2+}-activated K\textsuperscript{+} (BK) channels causing BK currents (spontaneous transient outward currents or STOCs). STOCs are hyperpolarizing currents that oppose vasoconstriction. Several RyR isoforms are coexpressed in VSMCs; however, their role in Ca\textsuperscript{2+} spark generation is unknown. To provide molecular information on RyR cluster function and assembly, we examined Ca\textsuperscript{2+} sparks and STOCs in RyR3-deficient freshly isolated myocytes of resistance-sized cerebral arteries from knockout mice and compared them to Ca\textsuperscript{2+} sparks in cells from wild-type mice. We used RT-PCR to identify RyR1, RyR2, and RyR3 mRNA in cerebral arteries. Ca\textsuperscript{2+} sparks in RyR3-deficient cells were similar in peak amplitude (measured as F/F\textsubscript{0}), width at half-maximal amplitude, and duration compared with wild-type cell Ca\textsuperscript{2+} sparks. However, the frequency of STOCs (between −60 mV and −20 mV) was significantly higher in RyR3-deficient cells than in wild-type cells. Ca\textsuperscript{2+} sparks and STOCs in both RyR3-deficient and wild-type cells were inhibited by ryanodine (10 \textmu mol/L), external Ca\textsuperscript{2+} removal, and depletion of SR Ca\textsuperscript{2+} stores by caffeine (1 mmol/L). Isolated, pressurized cerebral arteries of RyR3-deficient mice developed reduced myogenic tone. Our results suggest that RyR3 plays a specific molecular role in the regulation of STOCs frequency in mouse cerebral artery VSMCs after decreased arterial tone. (Circ Res. 2001;89:667–675.)

Key Words: potassium currents ■ membrane potentials ■ caveolae ■ sarcoplasmic reticulum ■ ryanodine receptor calcium release channel

Several cellular functions are controlled by the generation of highly sophisticated Ca\textsuperscript{2+} signals characterized by a high degree of spatial and temporal complexity. Temporally, the Ca\textsuperscript{2+} signals can occur only once, transiently, or repetitively. Repetitive signals are responsible for the phenomenon of Ca\textsuperscript{2+} oscillations. Spatially, Ca\textsuperscript{2+} transients can be global, involving the entire cell, or they can be restricted to a limited area of the cytosol. Increased global intracellular Ca\textsuperscript{2+} levels, due to Ca\textsuperscript{2+} ions entering arterial vascular smooth muscle cells (VSMCs) through L-type voltage-dependent calcium channels, stimulate contraction by activating myosin light chain kinase. These activating Ca\textsuperscript{2+} signals, although not reaching very high [Ca\textsuperscript{2+}], levels (in the range of 100 to 300 nmol/L), are distributed throughout the entire cell (global) and last sufficiently long enough to permit Ca\textsuperscript{2+} ions to enter into equilibrium with myosin light chain kinase to regulate arterial contraction.

More recently, local [Ca\textsuperscript{2+}], transients (Ca\textsuperscript{2+} sparks) have been visualized in arterial VSMCs by use of fluorescent, calcium-sensitive indicators and laser scanning confocal microscopy. Ca\textsuperscript{2+} sparks are caused by the opening of small ryanodine-sensitive Ca\textsuperscript{2+} release channel (RyR) clusters localized in the sarcoplasmic reticulum (SR). A single spark is capable of producing a very high (10 to 100 \textmu mol/L) local ([Ca\textsuperscript{2+}]\textsubscript{local}) increase in [Ca\textsuperscript{2+}], while increasing the global [Ca\textsuperscript{2+}], only by <2 nmol/L. Ca\textsuperscript{2+} sparks, in close proximity to the cell membrane, can stimulate Ca\textsuperscript{2+}-activated K\textsuperscript{+} (BK) channels. BK channels have in fact a 10\textsuperscript{4}-fold increase in their open probability in the presence of high [Ca\textsuperscript{2+}], as during a spark, but are otherwise...
not responsive to the much lower global [Ca\(^{2+}\)] (ie, 100 to 300 nmol/L).\(^7\) BK currents caused by Ca\(^{2+}\) sparks were originally termed spontaneous transient outward currents or STOCs.\(^6\) In arterial VSMCs, STOCs cause a global hyperpolarization of the cell membrane and close voltage-dependent Ca\(^{2+}\) channels. STOCs, therefore, feed back to decrease global [Ca\(^{2+}\)], and arterial contraction.\(^2\) In support of this mechanism, Ca\(^{2+}\) spark inhibitors of BK channels nondi

depolarize the cell membrane potential by \(\approx 10\) mV and increase global [Ca\(^{2+}\)], by \(\approx 50\) nmol/L. This increase leads to a 30% arterial vasoconstriction.\(^4,7\) Two recent studies showed that in mice lacking the \(\beta_1\) subunit of BK, Ca\(^{2+}\) sparks were unable to activate BK channels.\(^8,10\) The uncoupling between RyRs and BK channels was associated with an elevated systemic blood pressure.

Three molecularly distinct subtypes of RyR channels (RyR1, RyR2, and RyR3) have been identified and cloned to date. Each exists as a homotetramer in the SR membrane.\(^11\) RyR1 is found primarily in skeletal muscle, whereas RyR2 and RyR3 are predominantly found in heart tissue and brain, respectively.\(^12,13\) In skeletal muscle, both RyR3 and RyR1 isoforms produce Ca\(^{2+}\) sparks with nearly identical properties.\(^14,15\) In cardiac muscle, RyR2 appears to be responsible for Ca\(^{2+}\) sparks.\(^16,17\) Although mRNA transcripts for RyR1, RyR2, and RyR3 have been found in VSMCs of different tissues,\(^18\) RyR3 probably represents the prominent RyR isoform in smooth muscle.\(^19\) We therefore tested the hypothesis that RyR3 contributes to Ca\(^{2+}\) spark formation in arterial VSMCs using RyR3-deficient knockout mice.\(^20\)

Materials and Methods

Adult +/- RyR3 and +/+ mice (both SV 129/C57BL, 4 to 8 weeks; 20 to 40 g) were used.\(^20\) VSMCs from basilar arteries were isolated.\(^7,10\) In order to measure Ca\(^{2+}\) sparks, VSMCs were seeded onto glass coverslips and incubated with the Ca\(^{2+}\) indicator fluo-3/AM for 30 minutes at room temperature in Ca\(^{2+}\)-free Hanks solution.\(^7\) Single smooth muscle cells were imaged using a BioRad laser scanning confocal microscope attached to a Nikon Diaphot microscope.\(^5\) Whole-cell K\(^{+}\) currents were measured by the perforated patch technique.\(^21,22\) The diameter of cerebral arteries was measured using a video-microscopic system (Nikon Diaphot) and connected to a personal computer with appropriate software (TSE) for detection of changes of vessel diameter.

Quantitative and RT-PCR on Isolated Mouse Cerebral Arteries

RNA was prepared from basilar arteries of an adult mouse. The vessel was frozen in liquid nitrogen, pulverized by mortar and pestle, dissolved in 350 \(\mu\)L buffer RLT containing 1% b-mercaptoethanol, homogenized by QIAshredder, and loaded onto a RNeasy mini spin column (Qiagen). After washing and centrifugation, the RNA was eluted in 30 \(\mu\)L RNase-free water. RNA samples were used as templates in random-hexamers-primed reverse transcription. PCR reagents were supplied by PE-Applied Biosystems. The cycling conditions were 1 minute at 95°C followed by 35 cycles for 1 minute 94°C, 1 minute 58°C, and 1 minute 72°C in a PE 9600 PCR machine. All PCR reagents were supplied by PE-Applied Biosystems. The samples were analyzed on a 2% agarose gel.

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\text{Ryr-1F: 5'-GCTTACGCTAGGCTCAGCTGG-3'} \\
\text{Ryr-1R: 5'-AGGGGTTGACAGCAGTTTAT-3'} \\
\text{Ryr-2F: 5'-GAATACGCATGTGACATCC-3'} \\
\text{Ryr-2R: 5'-GTCATGACATTTATCCCTGCA-3'} \\
\text{Ryr-3F: 5'-CTCTGATTCCACGAAAGCTT-3'} \\
\text{Ryr-3R: 5'-TAGCTGCTTAAAAGCTTCCAGC-3'} \\
\text{GAPDH F: 5'-ACCAACATCTACGGAATC-3'} \\
\text{GAPDH R: 5'-TCCACACCTTTGTGGCAGA-3'}
\]

Quantitative RT-PCR (TaqMan) was done using a TaqMan 5700. Total RNA was reverse transcribed into cDNA and amplified using a TaqMan EZ kit from Perkin Elmer. An optimal PCR curve could be observed within 45 cycles. The following primers and probes were designed using the Primer Express program (Perkin Elmer).

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\text{Ryr1-1524F: 5'-CCACCTCAAGTGCCTTCC-3'} \\
\text{Ryr1-1588R: 5'-GTGACGCTACCTGCTCCA-3'} \\
\text{Ryr1-1546F (Tamra): 5'-TGCGAGCTCCCTCCAGGA-3'} \\
\text{Ryr2-672F: 5'-CTGAGACGTCCTCCAGAA-3'} \\
\text{Ryr2-748R: 5'-GTGTGACTGTCGTTGGG-3'} \\
\text{Ryr2-695 (Tamra): 5'-TCTGAGACGTCCTCCAGAA-3'}
\]

Materials

Fluo-3/AM was purchased from Molecular Probes. Stock solutions (0.25 mmol/L) of fluo-3/AM were made using DMSO as the solvent. Ryanodine was obtained from Calbiochem. All salts and other drugs were obtained from Sigma-Aldrich or Merck. High external potassium solutions were made by isosmotic substitution of NaCl with KCl in the PSS.

Statistics

All values are given as mean \pm SEM. Data were compared with Student’s t test (\(P<0.05\)). The number of cells or arteries tested is represented by n.

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

Results

RyRs Isoforms in Cerebral Artery

RyR isoform expression in VSMCs varies depending on the tissue of origin.\(^*\) We therefore performed RT-PCR on RNA prepared from isolated wild-type mouse cerebral arteries using oligonucleotides capable of detecting RyR1, RyR2, and RyR3 mRNA. Figure 1 shows that all three RyR mRNAs were present in these cells. Quantitative RT-PCR using Taqman technology showed similar expression of RyR1 and RyR2 in VSMCs of +/- wild-type and +/- RyR3-deficient mice. The mean mRNA expression of RyR1 and RyR2 was not significantly increased in VSMCs of +/- wild-type compared with VSMCs of +/- RyR3-deficient mice by factor 1.42 \pm 0.17 and 1.39 \pm 0.21, respectively (\(n=4, P>0.05\)).

Ca\(^{2+}\) Sparks in +/- Cerebral Artery Smooth Muscle Cells

Figure 2A shows a confocal line-scan image of two Ca\(^{2+}\) sparks and the time course in a fluo-3–loaded VSMCs from a normal control +/- RyR3 mouse. The Ca\(^{2+}\) transient time course was determined over the line indicated by the two
arrows. Each line-scan image is a plot of fluorescence along a scanned line (position) on the ordinate versus time (abscissa). The line scan image duration was 1 second whereas each line was 2 ms. On average, the peak [Ca$^{2+}$]$_i$ amplitude of Ca$^{2+}$ sparks (measured as F/F$_o$) and the width at half-maximal amplitude were 2.23$\pm$0.2 (n=29 sparks and n=21 cells) and 2.25$\pm$0.2 $\mu$m (n=29 sparks and n=20 cells), respectively. The rise to peak time was $\approx$20 ms. The Ca$^{2+}$ transient decay half-life was $\approx$150 ms. The local Ca$^{2+}$ transient duration at half-maximal amplitude was 55.3$\pm$2.9 ms (n=26 sparks and n=20 cells). Ca$^{2+}$ sparks were observed only at line-scan fluorescence images in close proximity to the cell membrane. Ca$^{2+}$ sparks were not observed in more central or perinuclear cell regions (ie, along scanned lines with distances $>\approx$2 $\mu$m from the cell membrane). Some sites occurred much more frequently than others within a given cell did. This finding indicates that Ca$^{2+}$ spark generation might occur at repetitive firing sites.$^{6,23}$ Ca$^{2+}$ spark occurrence was increased by the L-type Ca$^{2+}$ channel agonist BayK8644 (1 $\mu$mol/L, n=7), by membrane depolarization (60 mmol/L external K$^+$, n=5

Figure 2. Ca$^{2+}$ sparks in wild-type and +/- RyR3-deficient single smooth muscle cells from mouse cerebral arteries. A, Confocal line-scan image of a fluo-3-loaded wild-type cell with the time course of Ca$^{2+}$ sparks indicated below. The fluorescence time course of the Ca$^{2+}$ sparks was determined over the line indicated by the two arrows. Each line-scan image is a plot of fluorescence along a scanned line (ordinate) versus time (abscissa). The line scan image duration was 1 second, and each line was 2 ms. B, Confocal line-scan image of a fluo-3-loaded +/- RyR3 cell, with the time course of a Ca$^{2+}$ spark indicated below. C, Comparison of spatial-temporal characteristics of Ca$^{2+}$ sparks in wild-type and +/- RyR3 cells. Amplitudes of Ca$^{2+}$ sparks were measured as local fractional fluorescence increases (F/F$_o$). Ca$^{2+}$ spark width and duration were determined at half-maximal amplitude. Frequency, amplitude, width, and spark duration were analyzed using randomly selected cells and scanned lines. Each cell was scanned for a total time of 30 seconds. The cell number examined is indicated in the text.
the advantages that Ca2+ sparks can be examined (1) at controlled membrane potentials and (2) for relatively long recording times (usually >5 minutes), providing greater statistical power.

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**Ca2+ Sparks in −/− RyR3 Cells**

Figure 2B shows 4 Ca2+ sparks in a −/− RyR3 cell. The averaged peak [Ca2+]i, Ca2+ spark amplitude (measured as F/F0) in −/− RyR3 (2.38 ± 0.2, n = 70 sparks and n = 38 cells) and in +/+ cells (2.23 ± 0.2, n = 29 sparks and n = 21 cells) were not different (P > 0.05; Figure 2C). The spatial Ca2+ spark size in −/− RyR3 cells was not significantly different from that in wild-type cells (width at half-maximal amplitude, 2.25 ± 0.19 μm, n = 29 and n = 20 cells versus 2.34 ± 0.12 μm, n = 69 sparks and n = 36 cells; P > 0.05; for +/+ wild-type versus −/− RyR3 cells, respectively). Moreover, Ca2+ spark duration in −/− RyR3 (duration at half-maximal amplitude, 47.83 ± 2.2 ms, n = 69 sparks and n = 38 cells) and in wild-type cells (55.3 ± 2.9 ms, n = 26 sparks and n = 20 cells) were not different (P > 0.05). Figure 2B shows that Ca2+ sparks in RyR3-deficient cells occurred in repetitively firing sites, as observed in wild-type cells (cf. Figure 2A). Ca2+ sparks in −/− RyR3 cells were completely inhibited by ryanodine (10 μmol/L, n = 22 cells). Moreover, the Ca2+ spark occurrence was increased by the L-type Ca2+ channel agonist BayK8644 (1 μmol/L), by membrane depolarization (using 60 mM/L external K+), or by the RyR activator caffeine (300 μmol/L), as observed for Ca2+ sparks in wild-type cells (data not shown). Thus, we conclude that there is no substantial difference in Ca2+ spark cycle between RyR3-deficient and wild-type cells, and RyR3 deficiency does not lead to a loss of the sensitivity of the remaining RyR clusters to Ca2+ and other ligands.

Figure 2C shows that there was a trend toward a higher basal Ca2+ spark frequency in −/− RyR3 cells (0.086 ± 0.02 events/μm second, n = 104 and n = 54 cells) than in wild-type cells (0.042 ± 0.008 events/μm second, n = 52 and n = 38 cells, P = 0.19). The statistical power of analyzing Ca2+ spark frequencies is very low because Ca2+ sparks occurred at low rates and recording times were only 30 seconds per cell (see Materials and Methods). We were unable to increase the statistical power by increasing the recording time because longer recordings resulted in fluo-3 bleaching and cell damage (data not shown). Therefore, we recorded Ca2+ sparks—caused BK currents (STOCs) by means of the perforated patch clamp technique with amphotericin. This technique has the advantages that Ca2+ sparks can be examined (1) at controlled membrane potentials and (2) for relatively long recording times (usually >5 minutes), providing greater statistical power.

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**−/− RyR3 Ca2+ Sparks Cause STOCs**

In sharp contrast to +/+ cells (Figure 3A), −/− RyR3 cells showed a 10- to 50-fold higher STOC frequency over a wide range of membrane potentials between −50 mV and −20 mV (Figure 3B). At −40 mV, which typically occurs in pressurized cerebral arteries,2 STOC frequency in −/− RyR3-deficient cells was 9.6-fold higher than in wild-type cells (7.7 ± 1.2 STOCs/second, n = 20, versus 0.8 ± 0.2 STOCs/second, n = 20, respectively, P < 0.05; Figure 3C). These findings are consistent with a shift in the voltage-dependency of basal Ca2+ spark frequency to more negative membrane potentials. Figure 3B shows STOC recordings in a −/− RyR3 cell. These cells generated individual STOCs with several amplitudes and stable duration (46.5 ± 5.4 ms at −40 mV, n = 5 cells, versus 43.0 ± 3.1 ms at −40 mV, n = 7 control cells) at all ranges of physiological potentials from −60 to −20 mV. STOC amplitude and frequency increased with increasing membrane potential, consistent with a strong voltage-dependency of Ca2+ spark frequency and BK channel currents.25,26 At potentials positive to −40 mV, individual STOCs were often complex with an asymmetrical bell shape, a fast upstroke, and a decay phase that declined mono- or bi-exponentially. At potentials positive to −30 mV, several STOCs of the same or different amplitudes overlapped and formed complex STOCs with different shapes.

Figure 4A shows that STOCs in −/− RyR3 cells were completely blocked by 10 μmol/L ryanodine within 10 minutes (n = 15 cells, P < 0.05). A ryanodine washout for...
more than 20 minutes never produced recovery of STOC firing frequency. The BK channel blocker, tetraethylammonium chloride (TEA; 1 mmol/L), blocked STOCs in −/− RyR3 cells within 3 minutes (n=8 cells, P＜0.05) (Figure 4B). TEA washout produced a complete STOC recovery (not shown). These data indicate that STOCs were generated by the opening of large-conductance BK channels with each STOC activated by an individual Ca2+ spark as observed in wild-type cells of rat and mouse cerebral arteries.4,24

Figure 4. Inhibition of STOCs in −/− RyR3 cells by ryanodine and tetraethylammonium (TEA). A, The trace shows an example of STOCs at a holding potential of −40 mV. STOCs were completely blocked by the Ca2+ spark inhibitor, ryanodine (10 μmol/L, 7 minutes). B, STOCs were completely inhibited by the BK channel blocker, TEA (1 mmol/L). Holding potential −40 mV.

−/− RyR3 Ca2+ Sparks, Ca2+ Influx, and Ca2+ Store Filling

Ca2+ sparks are triggered by external Ca2+ influx.22,23,26 Figure 5A shows that Ca2+ removal from the external solution inhibited STOCs in −/− RyR3 cells within seconds suggesting that Ca2+ influx is required to trigger Ca2+ sparks both in the absence and presence of RyR3. We next examined a potential role for internal Ca2+ stores in STOC generation in more detail using caffeine (1 mmol/L). Caffeine (n=6) transiently increased steady state current (hump) in −/− RyR3 cells followed by a temporary STOC inhibition (Figure 5B). STOCs were recovered to control values within 2 minutes after caffeine was removed. After the internal Ca2+ stores were repleted and STOCs were recovered, caffeine again stimulated the steady state current (hump). Caffeine (1 mmol/L) was not able to induce a transient stimulatory effect on steady state current (hump) during caffeine-induced temporary STOC inhibition (not shown).

Figure 5. Effects of external Ca2+ and caffeine on STOCs) in −/− RyR3-deficient cells. A, The trace shows an example of STOCs at a holding potential of −40 mV. Removal of external Ca2+ immediately abolished STOCs. Adding 2 mmol/L Ca2+ to the external solution induced STOC recovery. STOCs were completely blocked by the Ca2+ spark inhibitor, ryanodine (10 μmol/L). B, Caffeine (1 mmol/L) transiently increased steady state current (hump) followed by a temporary inhibition of STOCs. After removing of caffeine from the bath, STOCs were recovered to control values within 2 minutes.

Regulation of Myogenic Tone in −/− RyR3 Arteries

To understand the physiological consequences of the increased Ca2+ sparks/STOCs frequency in −/− RyR3 VSMCs, we evaluated the effect of pressure on arterial diameter of intact, isolated cerebral arteries. Wild-type +/+ RyR3 arteries were significantly more constricted at a given pressure (>40 mm Hg) than were −/− RyR3 arteries (Figures 6A through 6C). At a physiological pressure of 60 mm Hg, the vessel diameters of +/+ wild-type and −/− RyR3 arteries were 126.2±3.7 μm (n=11) and 133.3±5.8 μm (n=6), respectively (P＜0.05). The contribution of RyR3 to the regulation of arterial tone can be evaluated by examining the effects of the BK channel blocker iberiotoxin on arterial tone. Iberiotoxin (100 nmol/L) caused a vessel constriction of 11.5 μm in the control (Figure 6D and 6F). In contrast, iberiotoxin induced a stronger constriction in −/− RyR3 arteries (Figure 6E and 6F). In arteries of wild type and RyR3-deficient mice, 60 mmol/L KCl evoked similar constrictions (Figure 6D and 6E). These results indicate that increased activity of BK channels in −/− RyR3 arteries contributes to the regulation of arterial tone and that RyR3 limits arterial tone by an inhibition of basal BK channel/STOCs activity.

Discussion

In VSMCs, RyRs are responsible for highly localized Ca2+ release events (sparks), which activate BK currents (STOCs). STOCs in turn inhibit voltage-dependent Ca2+ channel activ-
ity, decrease global [Ca\textsuperscript{2+}], and diminish arterial contraction leading to decreased blood pressure. Multiple RyR isoforms are coexpressed in VSMCs, but whether they serve specialized functions or merely contribute to spark activity is not known. Work in neonatal skeletal muscle cells indicated that RyR1 and RyR3 serve distinct functions in the signaling pathway controlling skeletal muscle contraction.\textsuperscript{20,27} To understand the contribution of RyR isoforms to arterial contraction, we tested the molecular role of RyR3 in Ca\textsuperscript{2+} spark regulation and STOCs generation in arterial VSMCs. Ca\textsuperscript{2+} sparks in RyR3-deficient cells were similar in peak amplitude, width at half-maximal amplitude, and wild-type cell Ca\textsuperscript{2+} spark duration. Interestingly, analysis of BK current activation in arterial VSMCs from RyR3 knockout mice indicated that RyR3 abrogation resulted in a significant increase in the STOCs frequency. From these results it would appear that RyR3 channels have a specific function in setting the frequency of Ca\textsuperscript{2+} sparks/STOCs in arterial VSMCs and thereby influence arterial myogenic tone. Thus, RyR3 channels seems to contribute to Ca\textsuperscript{2+} sparks by a mechanism controlling the basal Ca\textsuperscript{2+} spark frequency, whereas the remaining RyRs (ie, RyR1 and RyR2) mainly contribute to the Ca\textsuperscript{2+} release underlying a single spark. This RyR isoform-dependent regulation of Ca\textsuperscript{2+} sparks differs from that reported in skeletal muscle, where Ca\textsuperscript{2+} sparks are produced independently by two RyR isoforms (type 1 or type 3).\textsuperscript{14,15} Consistent with the latter idea, expression of RyR3 in dyspedic 1B5 myotubes resulted in production of Ca\textsuperscript{2+} sparks similar to those of sparks recorded in permeabilized frog skeletal muscle fibers.\textsuperscript{28}

Figure 6. Diameter measurements of isolated, pressurized +/+ wild type, and −/− RyR3-deficient cerebral arteries. Original recordings of +/+ wild type (A) and −/− RyR3-deficient (B) cerebral arteries. Intravasal pressure was stepwise increased from 10 mm Hg to 60 mm Hg. At 40 to 60 mm Hg, +/+ arteries were more constricted than −/− RyR3 arteries (C). Iberiotoxin (100 nmol/L) induced a stronger constriction in −/− RyR3 arteries (E and F) than +/+ wild type arteries (D and F). External 60 mmol/L KCl evoked in arteries of wild type and RyR3-deficient mice comparable constrictions (D and E).
We present data indicating that the spatial-temporal characteristics of individual Ca$^{2+}$ sparks in −/− RyR3 cells did not differ from those in +/+ RyR3 cells. We obtained evidence that RyR3-deficient sparks are localized in close proximity to the cell membrane and cause STOCs. This finding suggests functional coupling of RyRs other than RyR3 to nearby large-conductance, plasma membrane BK channels. Based on STOC recordings, we provide strong evidence that the frequency of Ca$^{2+}$ sparks in the absence of RyR3 is significantly higher than in cells containing RyR3. Thus, we suggest that RyR3 is part of the SR Ca$^{2+}$ spark release unit and feeds back on RyR1 and RyR2 to decrease Ca$^{2+}$ spark frequency. Our results support the notion that Ca$^{2+}$ spark triggering and arterial tone are fine-tuned by RyR3.

We used ryanodine and caffeine to further elucidate the role of internal Ca$^{2+}$ stores in STOC generation in our cell preparations.28,30 Ryanodine caused a decrease in STOCs firing frequency in both −/− RyR3 and +/+ RyR3 cells.6,10,24 Caffeine led to a temporary arrest in STOCs in our cells. After STOC recovery, caffeine again transiently increased the steady state current (iump), indicating that the internal Ca$^{2+}$ stores had been repleted. This finding suggests that STOCs are merely dependent on the refilling state of the internal Ca$^{2+}$ stores. Similar results were observed in wild-type cells.22,29 Taken together, these results indicate that lack of RyR3 does not lead to a loss of sparking RyR cluster sensitivity to Ca$^{2+}$ store filling, Ca$^{2+}$, and other ligands. These data are also consistent with the findings of Coussin et al31 who reported that antisense oligonucleotides against RyR3 had no effect on amplitude, width, and duration of Ca$^{2+}$ sparks in VSMCs isolated from the rat portal vein. However, these findings should be interpreted with caution because the antisense approach may not inhibit mRNA and protein expression completely, and there might exist differences between arteries.

Ca$^{2+}$ sparks in arterial smooth muscle cells depend on Ca$^{2+}$ influx through voltage-gated Ca$^{2+}$ channels in the plasma membrane.2,25,32 Thus, when extracellular Ca$^{2+}$ is removed from the bath solution, a decrease in STOC firing frequency would be expected. We observed that Ca$^{2+}$ sparks and STOCs in RyR3-deficient cells were immediately abolished after removal of Ca$^{2+}$ from the bath solution. We conclude that the lack of RyR3 does not lead to a loss of the sensitivity of sparking RyR clusters to Ca$^{2+}$ influx trigger mechanisms. Instead, Ca$^{2+}$ influx seems to activate RyRs that are intimately involved in formation of the Ca$^{2+}$ spark cycle, ie, RyR1 and/or RyR2. That increased Ca$^{2+}$ spark/STOC frequency in −/− RyR3 cells results from overexpression of RyR1 and/or RyR2 is unlikely. Our TaqMan analyses revealed no difference in expression of RyR1 and RyR2 in cerebral artery VSMCs of +/+ wild-type and −/− RyR3 mice. Consistent with the latter finding, expression of RyR1/2 was not affected by deletion of RyR3.33

Our data can be explained by a model in which BK channels are linked to a SR Ca$^{2+}$ release compartment containing multiple RyR channels, including RyR1, RyR2, and RyR3, as schematically shown in Figure 7. We suggest that the simultaneous opening of multiple RyR1 and RyR2 primarily determines the Ca$^{2+}$ spark cycle. Once the cycle is triggered, individual RyR3 channels create an inhibitory mechanism that controls the basal Ca$^{2+}$ spark frequency. The mechanism for the RyR3 inhibiting Ca$^{2+}$ spark frequency is not clear. Because RyR3 channels have been proposed to be less sensitive to Ca$^{2+}$-dependent inactivation than other RyR channels,34,35 we speculate that RyR3 could interfere with the modulation of the Ca$^{2+}$ spark frequency by one of three mechanisms. First, prolonged Ca$^{2+}$ release through RyR3 may inhibit basal Ca$^{2+}$ spark activity. Second, prolonged Ca$^{2+}$ release through RyR3 may inhibit Ca$^{2+}$ influx necessary to trigger sparks, for example by inducing Ca$^{2+}$-dependent inactivation of voltage-dependent plasma membrane Ca$^{2+}$ channels. Third, prolonged Ca$^{2+}$ release through RyR3 may empty the Ca$^{2+}$ store from which Ca$^{2+}$ sparks are operated and, thereby, prevent Ca$^{2+}$ spark generation. However, the latter mechanism seems unlikely because Ca$^{2+}$ spark amplitudes were not smaller in RyR3-deficient cells compared to wild-type cells even at similar Ca$^{2+}$ spark intervals. Nevertheless, all three mechanisms would definitely result in a reduction of basal Ca$^{2+}$ spark frequency (negative feedback). The contribution of each

Figure 7. Proposed model of ryanodine receptor organization in small resistance-sized arteries to cause Ca$^{2+}$ sparks and STOCs. This model proposes that ryanodine receptors (RyR) cluster in terminal sarcoplasmic reticular plaques in close proximity to BK channels in the plasma membrane. Ca$^{2+}$ sparks arise from the synchronized opening of multiple RyR1 and/or RyR2 acting in concert. In contrast, RyR3 do not release significant Ca$^{2+}$. Because RyR3 activity shows less inactivation than RyR1 and RyR2, prolonged Ca$^{2+}$ release through RyR3 may induce Ca$^{2+}$-dependent RyR inactivation. Alternatively, prolonged Ca$^{2+}$ release may inhibit Ca$^{2+}$ influx thereby triggering sparks resulting in Ca$^{2+}$-dependent voltage-gated Ca$^{2+}$ channel inactivation. All three mechanisms could result in a basal Ca$^{2+}$ spark frequency reduction (negative feedback). RyR3 does not have significant impact on Ca$^{2+}$ spark properties in arterial VSMCs but increased the Ca$^{2+}$ spark and STOC frequency, with the β1 subunit (BKβ1) of the Ca$^{2+}$-activated (BK) K$^{+}$ channel being the molecular sensor coupling Ca$^{2+}$ sparks to BK channel activity and the BK$\alpha$ subunit forming the channel pore.
particular mechanism remains to be studied in further experiments, and we cannot exclude the possibility that the inhibitory mechanism emerging after deletion of the RyR3 gene may result from a combination of more than one of the three proposals.

Our results support the concept that RyR3 is required to specifically tune the release of Ca²⁺ sparks to the needs of an arterial VSMC. We showed that although Ca²⁺ sparks at physiological low frequencies, thereby enabling BK channel regulation of arterial tone. Our data indicate that RyR3 regulates arterial myogenic tone and that deletion of RyR3 causes increased BK channel activity (STOCs). Increased STOC activity in turn leads to membrane hyperpolarization and reduced arterial tone.

In summary, we demonstrated that RyR3 plays a central molecular role in Ca²⁺ release from the SR in arterial VSMCs. We showed that although Ca²⁺ sparks are not affected by deletion of RyR3 gene, the Ca²⁺ spark frequency, as visualized by STOC measurements, is strongly increased in RyR3 knockout mice, as if the frequency of sparks was negatively regulated by RyR3 channels. The data also indicate that the Ca²⁺ spark cycle can be determined only by the other two isoforms, namely RyR1 and/or RyR2. Furthermore, absence of RyR3 does not prevent functional coupling between RyRs and BK channels to produce STOCs.

Acknowledgments
This work was supported by the Deutsche Forschungsgemeinschaft (to M.G.) and Telethon 1151 and MURST (to V.S.). M.G. is a recipient of a Helmholtz fellowship. M.F. and M.L. were supported by the Boehringer Ingelheim Fonds. M.G. and B. Gollasch designed the cover page. We thank Jana Czychi for excellent technical assistance and Birgit Lauterbach for help in cell isolation and immunostaining.

References
Regulation of Calcium Sparks and Spontaneous Transient Outward Currents by RyR3 in Arterial Vascular Smooth Muscle Cells
Matthias Löhn, Wolfgang Jessner, Michael Fürstenau, Maren Wellner, Vincenzo Sorrentino, Hermann Haller, Friedrich C. Luft and Maik Gollasch

Circ Res. published online October 18, 2001;

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2001/10/18/hh2301.100250.citation

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