Ontogeny of Local Sarcoplasmic Reticulum Ca\textsuperscript{2+} Signals in Cerebral Arteries
Ca\textsuperscript{2+} Sparks as Elementary Physiological Events

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Abstract—Ca\textsuperscript{2+} release through ryanodine receptors (RyRs) in the sarcoplasmic reticulum is a key element of excitation-contraction coupling in muscle. In arterial smooth muscle, Ca\textsuperscript{2+} release through RyRs activates Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} (K\textsubscript{Ca}) channels to oppose vasoconstriction. Local Ca\textsuperscript{2+} transients ("Ca\textsuperscript{2+} sparks"), apparently caused by opening of clustered RyRs, have been observed in smooth and striated muscle. We explored the fundamental issue of whether RyRs generate Ca\textsuperscript{2+} sparks to regulate arterial smooth muscle tone by examining the function of RyRs during ontogeny of arteries in the brain. In the present study, Ca\textsuperscript{2+} sparks were measured using the fluorescent Ca\textsuperscript{2+} indicator fluo-3 combined with laser scanning confocal microscopy. Diameter and arterial wall [Ca\textsuperscript{2+}] measurements obtained from isolated pressurized arteries were also used in this study to provide functional insights. Neonatal arteries (<1 day postnatal), although still proliferative, have the molecular components for excitation-contraction coupling, including functional voltage-dependent Ca\textsuperscript{2+} channels, RyRs, and K\textsubscript{Ca} channels and also constrict to elevations in intravascular pressure. Despite having functional RyRs, Ca\textsuperscript{2+} spark frequency in intact neonatal arteries was 1/100 of adult arteries. In marked contrast to adult arteries, neonatal arteries did not respond to inhibitors of RyRs and K\textsubscript{Ca} channels. These results support the hypothesis that RyRs organize during postnatal development to cause Ca\textsuperscript{2+} sparks, and RyRs must generate Ca\textsuperscript{2+} sparks to regulate the function of the intact tissue. (Circ Res. 1998;83:1104-1114.)

Key Words: Ca\textsuperscript{2+} spark ■ ryanodine receptor ■ K\textsuperscript{+} channel ■ vascular smooth muscle ■ development

Intracellular Ca\textsuperscript{2+} ions regulate a wide variety of cellular processes. It is a widely held belief that many of the actions of Ca\textsuperscript{2+} are not global in nature; rather, they occur in close proximity to Ca\textsuperscript{2+} release sites.\textsuperscript{1} Local Ca\textsuperscript{2+} transients ("Ca\textsuperscript{2+} sparks") are thought to be elementary Ca\textsuperscript{2+} signals in heart, skeletal and smooth muscle cells, and possibly neurons.\textsuperscript{2-6} Ca\textsuperscript{2+} sparks arise from the activation of ryanodine-sensitive Ca\textsuperscript{2+} release channels (ryanodine receptors [RyRs]) located in the membrane of the sarcoplasmic reticulum (SR).

The idea that Ca\textsuperscript{2+} sparks are "the" elementary Ca\textsuperscript{2+} signal of RyRs has been challenged.\textsuperscript{7-9} Lipp and Niggli\textsuperscript{7} found that flash photolysis of caged Ca\textsuperscript{2+} caused a homogenous release of Ca\textsuperscript{2+} from cardiac SR, without the detection of Ca\textsuperscript{2+} sparks. This observation suggests that SR Ca\textsuperscript{2+} release can occur via undetectable events, possibly representing the opening of single RyRs. Ca\textsuperscript{2+} sparks therefore represent the coordinated opening of a cluster of RyRs rather than the opening of a single RyR channel. Further, functional RyRs can be expressed in cultured cells; however, unlike native tissue, these cells do not exhibit Ca\textsuperscript{2+} sparks.\textsuperscript{10} This finding implies not only that the opening of multiple RyRs is responsible for Ca\textsuperscript{2+} sparks but that additional cellular factors are necessary to organize RyRs into functional Ca\textsuperscript{2+} spark sites.

This suggestion that Ca\textsuperscript{2+} release events are smaller (eg, through a single RyR) than Ca\textsuperscript{2+} sparks leads to a fundamental question: Do RyRs generate Ca\textsuperscript{2+} sparks to regulate the physiology of the tissue? In arterial smooth muscle, Ca\textsuperscript{2+} release through RyRs causes the activation of Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} (K\textsubscript{Ca}) channels in the sarcolemmal membrane, which appear to be involved in signaling vasodilation.\textsuperscript{5,11} For example, elevation of intravascular pressure to physiological levels (eg, 60 mm Hg) causes a graded smooth muscle cell membrane potential depolarization to ~40 mV, elevation in arterial wall Ca\textsuperscript{2+} to ~200 nmol/L, and constriction ("myogenic tone") of small cerebral arteries.\textsuperscript{12} Ca\textsuperscript{2+} release through RyRs increases in response to this elevation in Ca\textsuperscript{2+} entry, which in turn activates nearby K\textsubscript{Ca} channels in the sarcolemmal membrane to cause membrane potential hyperpolarization to oppose the pressure-induced depolarization. Recent evidence also suggests that frequency modulation and amplitude modulation of Ca\textsuperscript{2+} sparks and IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release events play a fundamental role in controlling cell function.\textsuperscript{13-16} However, it is unclear whether Ca\textsuperscript{2+} sparks are necessary for RyRs to have a significant effect on arterial diameter.

In this study, we explored the nature and fundamental functional roles of RyRs by examining their elementary
properties in smooth muscle cells of neonatal and adult cerebral arteries of rat. To gain insights into these issues, we examined the properties of RyRs from the elementary level of Ca\(^{2+}\) sparks to their functional effects in intact pressurized arteries. We found that the elementary behavior of RyRs changes during postnatal development of cerebral arteries. Smooth muscle cells in neonatal cerebral arteries, although incompletely differentiated, express functional RyRs, K\(_{Ca}\) channels, contractile proteins, and voltage-dependent Ca\(^{2+}\) channels. Neonatal arteries constrict to increases in transmural pressure, like adult arteries. However, our results indicate that Ca\(^{2+}\) sparks develop late in differentiation and that Ca\(^{2+}\) sparks are required for RyRs to activate K\(_{Ca}\) channels and serve as a "brake" on vasoconstriction.

**Materials and Methods**

Adult male and female Sprague-Dawley rats (12 to 14 weeks; \(~228\) g) were euthanized under deep pentobarbital anesthesia (intraperitoneal; 150 mg/kg body weight). Neonatal male and female Sprague-Dawley rats (1 to 2 days old) were euthanized under deep inhalation anesthesia with methoxyflurane. After decapitation, the brain was removed and quickly transferred to cold (4°C), oxygenated (95% O\(_2\); 5% CO\(_2\)) PSS of the following composition (in mmol/L): NaCl 119, KCl 4.7, NaHCO\(_3\) 24, KH\(_2\)PO\(_4\) 1.2, CaCl\(_2\) 1.6, MgSO\(_4\) 1.2, EDTA 0.023, and glucose 11. All experiments were conducted in accordance with the guidelines for the care and use of laboratory animals (NIH publication No. 85-23, 1985) and followed protocols approved by the Institutional Animal Use and Care Committee of the University of Vermont. Animals were supplied by Charles River Laboratories, Inc, St. Constant, Quebec, Canada.

**Staining of Arteries for Proliferating Cell Nuclear Antigen**

Isolated cerebral arteries were fixed in freshly depolymerized paraformaldehyde (2%) in 0.1 mol/L PBS for 2 minutes at room temperature (20°C to 22°C) and then were washed twice in PBS for 5 minutes. Immunoreactivity of proliferating cell nuclear antigen (PCNA) was determined using an anti-PCNA antibody (Clone PC10, DAKO, Carpinteria, Calif) that was coupled to horseradish peroxidase. For immunodetection of PCNA, arteries were treated with 3% H\(_2\)O\(_2\) for 5 minutes and then incubated with the antibody to PCNA for 60 minutes at room temperature.

**Immunofluorescence Staining**

Smooth muscle cells from cerebral (basilar) arteries of adult and neonatal rats were isolated as previously described. Freshly isolated adult and neonatal cells were incubated with the antibody to PCNA and a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson Laboratories, West Grove, Pa) for 2 hours at room temperature. Cover slips mounted onto slides were viewed with a laser scanning confocal microscope (BioRad MRC 1000). Excitation light of 488 nm for FITC and 647 nm for Cy5-conjugated secondary antibodies was used and emissions measured at 515 to 565 nm (FITC) and 670 to 810 nm (Cy5). Negative control experiments were performed with labeling buffer instead of the primary antibodies; in each case, no specific staining was observed (data not shown).

**Ca\(^{2+}\) Spark Measurements**

Freshly isolated adult and neonatal cells were incubated with the Ca\(^{2+}\) indicator fluo-3-AM (5 \(\mu\)mol/L) and pluronic acid (0.005%; wt/vol) for 30 minutes at room temperature in Ca\(^{2+}\)-free Hanks’ solution. To examine Ca\(^{2+}\) sparks in intact artery segments, basilar arteries (diameter, \(~100\) \(\mu\)m) from adult rats were slipped over rectangular glass cannulas (220 \(\mu\)m \(\times\) 40 \(\mu\)m \(\times\) 10 mm) in a manner similar to that previously described. Arteries on glass cannulas then were placed into HEPES-PSS containing 10 \(\mu\)mol/L fluo-3-AM and 0.05% pluronic acid and incubated at 22°C for 60 minutes. After loading with fluo-3-AM, tissues were washed with HEPES-PSS for 30 to 40 minutes at 22°C. The HEPES-PSS had the following composition (in mmol/L): NaCl 135, KCl 5.4, CaCl\(_2\) 1.8, MgCl\(_2\) 1, HEPES 10, and glucose 10 (pH 7.4 with NaOH).

Smooth muscle cells were visualized using a Nikon Diaphot microscope. Images were obtained by illuminating with a krypton/argon laser at 488 nm and recording all emitted light >500 nm. The sampling rate was 60 Hz (1 image every 16.7 ms). Unless indicated, Ca\(^{2+}\) sparks were measured in HEPES-PSS at room temperature (20°C to 22°C).

For single-cell analysis, fluorescence records were normalized by dividing each image by the average of 8 images obtained during the prestimulus period. Normalized images were filtered subsequently with a 3\(\times\)3 median filter using Noran software. Ca\(^{2+}\) sparks recorded from 56.3\(\times\)52.8 \(\mu\)m\(^2\) areas of intact artery segments were analyzed using custom software written by Dr Adrian Bonev in our laboratory (using IDL 5.0.2; Research Systems, Inc). Baseline fluorescence (F\(_0\)) was determined by averaging 10 images without Ca\(^{2+}\) spark activity. Fractional fluorescence increases (F/F\(_0\)) were determined by dividing an area (1.54\(\times\)1.54 \(\mu\)m\(^2\)), where a Ca\(^{2+}\) spark was present, by F\(_0\). Ca\(^{2+}\) sparks were defined as local F/F\(_0\) >1.3.

**K\(^{+}\) Current Recordings**

Whole-cell K\(^{+}\) currents in freshly isolated cerebral artery myocytes from neonatal rats were measured using the perforated patch configuration of the patch-clamp technique at room temperature (20°C to 24°C). The external solution contained (in mmol/L): NaCl 134, KCl 6, MgCl\(_2\) 1, CaCl\(_2\) 2, glucose 10, and HEPES 10 (pH 7.4). Patch pipettes (resistance, 3 to 5 M\Omega) were filled with a solution containing (in mmol/L): K\(_2\)SO\(_4\) 110, KCl 30, NaCl 10, MgCl\(_2\) 1, HEPES 10, and EGTA 0.05 (pH 7.2). Amphotericin B (Sigma) was dissolved in DMSO and diluted into the pipette solution to give a final concentration of 200 \(\mu\)g/mL. K\(_{Ca}\) channel activity (NP; N indicates number of functional channels; P\(_o\); open probability) was calculated over 2- to 5-minute intervals as

\[
\frac{\sum_{j=1}^{N} t_j}{T}
\]

where \(t_j\) is the time spent with \(j=1,2,\ldots, N\) channels open, \(N\) is the maximum number of channels observed, and \(T\) is the duration of the recording.

**Arterial Wall [Ca\(^{2+}\)] and Diameter**

Arterial wall [Ca\(^{2+}\)] and diameter were measured as previously described. Intact isolated distal posterior cerebral arteries of adult rats and intact isolated basilar arteries of neonatal rats were loaded with the ratiometric Ca\(^{2+}\)-sensitive fluorescent dye Fura-2/AM (2 \(\mu\)mol/L) at room temperature (20°C to 22°C) for 45 minutes. Fura-2-loaded arteries then were mounted in an arteriograph with continuous superfusion (3 to 6 mL/min) of oxygenated PSS at 37°C. After a 20-minute equilibration period, intravascular pressure was...
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increased gradually from 2 mm Hg to either 40 mm Hg (neonatal arteries) or 60 mm Hg (adult arteries). Ratio images were obtained at a rate of 0.2 Hz from background-corrected 4-frame–averaged images of the 510±40-nm emission from the arteries alternately excited at 340 and 380 nm using the Image-1/FL quantitative ratio imaging software (Universal Imaging Corp). Arterial wall \([\text{Ca}^{2+}]\) was calculated using the following equation (from Grynkiewicz et al\(^{20}\)):

\[
[\text{Ca}^{2+}] = K_s \times (R - R_{\text{min}})/(R_{\text{max}} - R).
\]

At the end of every experiment, \(R_{\text{max}}\) and \(R_{\text{min}}\) were measured from ionomycin-treated arteries,\(^{12}\) and \(b\) was determined. An apparent \(K_s\) of 282 nmol/ of Fura-2 for \([\text{Ca}^{2+}]\) was determined previously in this preparation.\(^{12}\)

Materials

Iberiotoxin (IbTx) was obtained from Peptides International. Fura-2/AM, Fluo-3-AM, and pluronic acid were purchased from Molecular Probes. Sodium nitroprusside, dibutyryl-cAMP (db-cAMP), forskolin, and rapamycin were from Sigma. Stock (1 mmol/L) solutions of fluo-3-AM, rapamycin, and Fura-2/AM were made using DMSO as the solvent. Caffeine was from Serva. Ryanodine was obtained from Calbiochem. Nisoldipine was a gift from Dr A. Scriabine of Miles (Bayer) Laboratories (West Haven, Conn). High external K\(^+\) solutions were made by isosmotic substitution of NaCl with KCl in the PSS. The monoclonal mouse anti-RyR2 (Clone 3-33, IgG,) and antiproliferating cell nuclear antigen (Clone PC10) antibodies were from Affinity Bioreagents and DAKO, respectively. All other salts and drugs were obtained from Sigma Chemical Co. All values are given as mean±SEM. The term “n” represents the number of arteries or cells tested. Differences were considered statistically significant at \(P<0.05\) (unpaired Student t test).

Results

Neonatal Myocytes Are Proliferative and Have Voltage-Dependent Ca\(^{2+}\) Channels and Ryanodine Receptors

It is well known that smooth muscle cells in neonatal arteries are still proliferative and incompletely differentiated,\(^{27}\) as indicated by the presence of PCNA (n=6 arteries; Figure 1A). PCNA is an indicator for cells in S-phase,\(^{28,29}\) and as expected, smooth muscle cells in intact adult arteries did not stain positive for PCNA (n=6 arteries; data not shown). Smooth muscle cells isolated from neonatal (<1 day postnatal) arteries are smaller (membrane capacitance: adult, 10.8 pF; neonate, 3.8±0.2 pF; n=6 cells), and less elongated than adult cells. However, 2 key components of excitation-contraction (E-C) coupling in muscle, voltage-dependent Ca\(^{2+}\) channels and RyRs, appear to be present in smooth muscle cells isolated from both neonatal (<1 day postnatal) and adult arteries. The cell membranes of both adult and neonatal myocytes stained positive for the alpha 1C subunit of the voltage-dependent (L-type) Ca\(^{2+}\) channel (Figure 1B; also see Figures 2 and 3 for functional evidence of Ca\(^{2+}\) channels in neonatal arteries).\(^{22,30}\) To provide qualitative evidence for RyRs, isolated smooth muscle cells were stained with a monoclonal anti-RyR2 antibody (Figure 1C). Adult cells had distinct staining along the cell membrane, whereas the neonatal cells had much more diffuse staining (n=30 for both adult and neonatal cells). These results support the existence of RyRs in both adult and neonatal cells and are consistent with the subplasma membrane location of RyRs,\(^{31}\) and hence Ca\(^{2+}\) sparks, in adult cells. The presence of RyRs in neonatal myocytes suggested the possibility that these cells should also exhibit Ca\(^{2+}\) sparks.

Isolated Smooth Muscle Cells From Neonatal Arteries Do Not Exhibit Ca\(^{2+}\) Sparks

In adult myocytes, Ca\(^{2+}\) sparks can be observed readily and occur near the cell membrane (Figure 4A), consistent with the proposed activation of nearby plasmalemmal K\(_{\text{Ca}}\) channels.\(^{5}\) Although neonatal myocytes have RyRs (Figure 1C), Ca\(^{2+}\) sparks were not observed in smooth muscle cells isolated from neonatal cerebral arteries bathed in PSS (Figure 4B; n=55 cells; 10 s of scanning per cell). In an attempt to enhance the probability of visualizing Ca\(^{2+}\) sparks in neonatal cells, several agents were used that previously have been shown to increase Ca\(^{2+}\) spark frequency in isolated adult myocytes. For example, activators of adenyl cyclase (eg, forskolin, db-cAMP) or sodium nitroprusside (SNP), a nitrovasodilator that increases cGMP levels, have been shown to increase Ca\(^{2+}\) spark frequency in adult cells by ≈3-fold.\(^{16}\) However, Ca\(^{2+}\) sparks were not observed in neonatal cells bathed in forskolin (10 \(\mu\text{mol/L}\), db-cAMP (250 \(\mu\text{mol/L}\)), or SNP (10 \(\mu\text{mol/L}\)) (n=52 cells). Increasing Ca\(^{2+}\) influx through Ca\(^{2+}\) channels by either membrane depolarization (by elevating external K\(^+\) to 60 mmol/L) or application of the Ca\(^{2+}\) channel agonist, Bay K 8644 (1 \(\mu\text{mol/L}\)), which significantly increases Ca\(^{2+}\) spark frequency in adult myocytes,\(^{5}\) did not cause Ca\(^{2+}\) sparks in neonatal cells (n=34 cells). In addition, Ca\(^{2+}\) sparks were not observed in neonatal cells bathed in high (60 mmol/L) K\(^+\) in the presence of forskolin (n=18 cells). Rapamycin (10 \(\mu\text{mol/L}\)), which affects the FK-506 binding protein and prolongs cardiac muscle Ca\(^{2+}\) sparks,\(^{32}\) also did not cause Ca\(^{2+}\) sparks in neonatal cells (n=25 cells). Because protein kinase C activators decrease Ca\(^{2+}\) spark frequency in adult myocytes,\(^{18}\) the effects of an inhibitor of PKC (calphostin C, 300 mmol/L) were examined on cells from neonatal arteries, with no Ca\(^{2+}\) sparks being observed (n=21 cells). To summarize, a total of 336 smooth muscle cells from neonatal cerebral arteries were examined, without 1 spark being detected. In contrast, Ca\(^{2+}\) sparks could be observed in the majority of smooth muscle cells isolated from adult arteries during 10 s of scanning, as has been shown previously\(^{16,18,19}\) (Figure 4), with an apparent frequency of 0.12±0.03 Hz per cell (n=60). Based on the Ca\(^{2+}\) spark frequency in adult myocytes and taking into account cell size differences, at least 200 Ca\(^{2+}\) sparks should have been observed in neonatal cells over the observation period. These results suggest the possibility that RyRs in neonatal myocytes are incapable of generating Ca\(^{2+}\) sparks.

Ca\(^{2+}\) Spark Frequency Is Very Low in Intact Neonatal Arteries

The single cell results suggest that RyRs may organize during development to form “Ca\(^{2+}\)” spark sites. If so, it may be possible to detect a small number of spark sites, if a larger sample of neonatal cells were examined. We recently have developed a method to measure Ca\(^{2+}\) sparks in intact cerebral arteries.\(^{24}\) With this approach, a significant number of cells can be examined simultaneously in an intact arterial wall. Measurement of Ca\(^{2+}\) sparks in intact tissue also minimizes concerns about the condition of isolated cells after exposure to digestive enzymes.\(^{24,33}\) Approximately 1200 cells were examined in 6 different neonatal arteries over ≈1800 s. A very small number of Ca\(^{2+}\) sparks (4)
Figure 1. Staining of smooth muscle cells from neonatal arteries for PCNA, L-type Ca^{2+} channels, and RyRs. A, Many smooth muscle cells in neonatal cerebral arteries stain positive (darkly stained nuclei) for PCNA. Immunoreactivity of PCNA was determined in cross-sections of neonatal arteries using an anti-PCNA antibody coupled with horseradish peroxidase. B, Single smooth muscle cells from adult and neonatal arteries stained for L-type voltage-dependent Ca^{2+} channels. Isolated vascular smooth muscle cells were incubated with a polyclonal rabbit anti-alpha 1C antibody generated against the amino acid sequence 799 to 817 (EEEEKERKKLARTASPEKK) of the cytoplasmic linker between repeat II and III of the alpha 1C subunit of the L-type voltage-dependent Ca^{2+} channel. C, Adult and neonatal cells stained with a monoclonal anti-RyR2 antibody. Adult cells had distinct staining along the cell membrane, whereas staining in neonatal cells was more diffuse.
were detected in these arteries bathed in PSS. In the same 6 arteries, membrane depolarization by elevating the $K^+$ concentration in the PSS from 6 to 30 mmol/L increased the number of Ca$^{2+}$ sparks observed from 4 to 23. In sharp contrast, 2000 sparks would have been observed in smooth muscle cells in 6 intact adult cerebral arteries bathed in 30 mmol/L $K^+$ over the same scan duration and scan areas (based on a Ca$^{2+}$ spark frequency of 1.20±0.28 Hz in a 56.3×52.8-μm area; n=6; Figure 5B; see also Jaggar et al24). These results suggest a ~100-fold difference in Ca$^{2+}$ spark frequency between neonatal and adult arteries. Ca$^{2+}$ spark frequency increased to adult levels over the first 3 weeks of postnatal development (spark frequency at 1 week, 0.51±1.2 Hz; at 3 weeks, 1.30±2.6 Hz; 30 mmol/L extracellular $K^+$; n=4). The fractional increase in fluorescence during a Ca$^{2+}$ spark and $t_{1/2}$ of decay, however, were similar ($P>0.05$) in intact neonatal and adult arteries in 30 mmol/L $K^+$ [F/Fo: neonatal, 1.62±0.06 (n=35 sparks); adult, 1.69±0.02 (n=135 sparks); $t_{1/2}$: neonatal, 58.4±14.5 ms, (n=6 sparks); adult, 46.7±4.3 ms (n=14 sparks)]. These single cell and intact artery data indicate that Ca$^{2+}$ sparks are very rare in neonatal (<1 day postnatal) arteries, even under conditions that would greatly increase their frequency (eg, membrane depolarization).

Caffeine Causes Ca$^{2+}$ Transients in Neonatal and Adult Myocytes

The existence of Ca$^{2+}$ sparks, albeit at very low frequency, as well as that of RyR staining suggests that RyRs are present in neonatal arterial myocytes. A common feature of RyR1 and RyR2 is their ability to be activated by caffeine, which increases their open-state probability.34 To

Figure 2. Ryanodine increases arterial wall Ca$^{2+}$ and causes vasoconstriction in adult but not neonatal cerebral arteries. Original recordings of the simultaneous measurement of arterial wall [Ca$^{2+}$] and diameter in isolated pressurized adult (A) and neonatal (B) cerebral arteries loaded with Fura-2/AM. The stable levels of arterial wall [Ca$^{2+}$] and constriction at elevated intravascular pressure are indicated by horizontal dotted lines. In arteries isolated from adult animals, ryanodine (RYA: 10 μmol/L) increased arterial wall [Ca$^{2+}$] and caused vasoconstriction. The effects of ryanodine and IbTx (100 nmol/L) on diameter and Ca$^{2+}$ levels were not additive in arteries from adults. In arteries isolated from neonatal animals, ryanodine had no effect. Increased extracellular K$^+$ (60 mmol/L) increased arterial wall [Ca$^{2+}$] and constricted both adult and neonatal cerebral arteries, whereas inhibitors of L-type Ca$^{2+}$ channels [nimodipine (Nim; 10 nmol/L) or nisoldipine (Nisol; 100 nmol/L)] decreased arterial wall [Ca$^{2+}$] and maximally dilated both adult and neonatal cerebral arteries. Lower panel represents intravascular pressure ($P$, mm Hg).

Figure 3. IbTx increases arterial wall Ca$^{2+}$ and causes vasoconstriction in adult but not in neonatal cerebral arteries. Original recordings of the simultaneous measurement of arterial wall [Ca$^{2+}$] and diameter in isolated pressurized adult (A) and neonatal (B) cerebral arteries loaded with Fura-2/AM. The stable levels of arterial wall [Ca$^{2+}$] and constriction at elevated intravascular pressure are indicated by horizontal dotted lines. In arteries isolated from adult animals, IbTx (100 nmol/L) increased arterial wall [Ca$^{2+}$] and caused vasoconstriction. The effects of IbTx and ryanodine (10 μmol/L) on diameter and Ca$^{2+}$ levels were not additive in arteries from adults. In arteries isolated from neonatal animals, IbTx had no effect. Increased extracellular K$^+$ (60 mmol/L) increased arterial wall [Ca$^{2+}$] and constricted both adult and neonatal cerebral arteries, whereas inhibitors of L-type Ca$^{2+}$ channels (Nim, 10 nmol/L or Nisol, 100 nmol/L) decreased arterial wall [Ca$^{2+}$] and maximally dilated both adult and neonatal cerebral arteries. See Figure 2 legend for expansions to abbreviations.
explore the functionality of RyRs in neonatal myocytes, the effects of caffeine were examined. Ca\(^{2+}\) sparks were not observed in isolated neonatal cells, even in the continued presence of 0.3 mmol/L (n=45 cells) or 1.0 mmol/L (n=12 cells) caffeine. In contrast, superfusion of caffeine at 0.3 mmol/L increased the frequency of Ca\(^{2+}\) sparks in single myocytes isolated from adult arteries ~4-fold, from 0.12±0.03 Hz to 0.54±0.11 Hz, respectively (n=60 cells).
To increase further the activation of RyRs, caffeine was applied rapidly at a higher concentration (10 mmol/L). This concentration of caffeine, as previously has been shown, causes a typical whole-cell global Ca$^2+$ transient in adult myocytes because of the rapid activation of a significant number of RyRs (Figure 6). Such caffeine-induced Ca$^2+$ transients have been used routinely as a measure of SR Ca$^2+$ content in muscle cells. Rapid application of caffeine (10 mmol/L) to neonatal myocytes caused a global Ca$^2+$ transient, with an amplitude similar ($P>0.05$) to adult myocytes ($F/F_o=7.7\pm0.5$ in adult versus $8.3\pm0.8$ in neonatal cells; $n=12$; Figure 6). These results indicate the presence of functional, caffeine-sensitive RyRs in neonatal cells. Furthermore, this result and the similar Ca$^2+$ spark amplitudes in neonatal and adult arteries also suggest that differences in SR Ca$^2+$ load are not responsible for the observed differences in Ca$^2+$ spark frequency.

Ca$^2+$ Sparks Are Required for the Regulation of Arterial Wall Ca$^2+$ and Diameter by Ryanodine Receptors

The existence of functional RyRs in (adult) cells with Ca$^2+$ sparks and in (neonatal) cells with a very low spark frequency provided the opportunity to probe the physiological role of Ca$^2+$ sparks. In other words, is RyR activity in the absence of Ca$^2+$ sparks sufficient to regulate tissue function? Elevation of intravascular pressure (eg, from 10 to 40 or 60 mm Hg) has been shown to cause an elevation of global Ca$^2+$ (from 120 to 200 nmol/L) and constriction (by 30%) of small cerebral arteries from adult animals (Figures 2A and 3A). Elevation of intravascular pressure from 10 to 40 mm Hg also increased arterial wall Ca$^2+$ from $121\pm18$ nmol/L ($n=3$) to $195\pm19$ nmol/L ($n=15$) and constricted neonatal arteries from $211\pm10$ to $168\pm8$ nmol/L ($n=14$; Figures 2B and 3B). In both cases, the elevation in arterial wall Ca$^2+$ and the vasoconstriction were reversed by inhibitors of L-type, voltage-dependent Ca$^2+$ channels such as nisoldipine (100 nmol/L; Figures 2 and 3). These results suggest that the mechanisms that cause pressure-induced elevations in Ca$^2+$ and vasoconstriction, including the contractile proteins, are present and functional in neonatal myocytes. Furthermore, L-type Ca$^2+$ channels are present (see also Figure 1B) and function to regulate the diameter of neonatal arteries.

In sharp contrast to adult arteries, neonatal arteries did not respond to inhibitors of Ca$^2+$ sparks (ryanodine) and inhibitors of K$_{Ca}$ channels (IbTx), which cause a profound elevation of arterial wall Ca$^2+$ and vasoconstriction of adult arteries (Figures 2, 3, and 7) (see also Nelson et al and Knot et al for more data on adult arteries). Ryanodine (10 mmol/L),
IbTx (100 nmol/L), and the combination had no effect on arterial wall Ca$^{2+}$ and diameter of pressurized neonatal arteries with tone (n = 11), whereas these agents increased arterial wall Ca$^{2+}$ by 50 nmol/L and constricted adult arteries by 90 nm. Membrane depolarization with high K$^+$ or activation of RyRs with caffeine (10 mmol/L) caused a similar elevation of arterial wall Ca$^{2+}$ and constriction in neonatal and adult arteries (Figure 7). Ryanodine (10 μmol/L) blocked the effects of 10 mmol/L caffeine in arteries from both adults and neonates confirming the functional presence of RyRs in both tissue types. Therefore, it appears that the presence of caffeine-sensitive RyRs in neonatal myocytes is not sufficient for regulation of KCa channels and arterial diameter, even when the 2 major activators of RyRs (cytoplasmic and SR Ca$^{2+}$) are similar in neonatal and adult myocytes. This result is consistent with the idea that RyRs generate Ca$^{2+}$ sparks to regulate KCa channels, and ultimately arterial diameter.

Smooth Muscle Cells From Neonatal Arteries Have KCa Channels

The lack of effect of ryanodine or IbTx on arterial wall Ca$^{2+}$ and diameter may reflect a lack (or low frequency) of Ca$^{2+}$ sparks and/or the absence of KCa channels. To examine KCa channels, K$^+$ currents were measured by the whole cell perforated patch-clamp technique in single cells isolated from neonatal arteries. Membrane capacitance of these cells (3.8±0.2 pF; n=6) was lower than that of adult cells (≈11 pF), consistent with a smaller surface area. Currents through single KCa channels were clearly discernible in these isolated neonatal cells (Figure 8A). The channels had the characteristic single channel conductance (120±4 pS, −20 to 20 mV; n=6; Figure 8B) and voltage-dependence of KCa channels as well as sensitivity to block by IbTx (NP$_o$=0.0219±0.006, control versus NP$_o$=0.0024±0.006 in the presence of 100 nmol/L IbTx; holding potential (V$_h$)=30 mV; n=6). KCa channel currents [spontaneous transient outward currents (STOCs)] caused by Ca$^{2+}$ sparks were not observed in neonatal cells (V$_h$=−40 to −20 mV), consistent with a lack of Ca$^{2+}$ sparks in these cells. These results indicate that neonatal arterial myocytes have functional KCa channels. However, these results do not address the issue of whether the density or location of KCa channels is appropriate for STOC generation, if Ca$^{2+}$ sparks were to occur.

Discussion

Developmental Changes in Ryanodine Receptors That Cause Ca$^{2+}$ Sparks: Implications for the Nature of Ca$^{2+}$ Sparks

At birth, cerebral arteries possess voltage-dependent Ca$^{2+}$ channels, RyRs, and KCa channels as well as the ability to constrict to pressure. Smooth muscle cells from both neonatal and adult arteries have RyRs (Figure 1C) and respond robustly to the RyR-activator, caffeine (Figures 6 and 7). Yet, Ca$^{2+}$ spark frequency was extremely low in
neonatal myocytes, even under conditions to enhance RyR open probability (eg, membrane depolarization and caffeine). Based on caffeine-induced Ca$^{2+}$ transients, SR Ca$^{2+}$ load seemed similar in neonatal and adult myocytes; therefore, Ca$^{2+}$ spark amplitude should be similar, as observed. There are several possible explanations for the observed low frequency of Ca$^{2+}$ sparks in neonatal arteries: (1) If cytoplasmic Ca$^{2+}$, which activates RyRs, was much lower in neonatal myocytes, this might explain some of the difference in Ca$^{2+}$ spark frequency. However, cytoplasmic Ca$^{2+}$ was similar in smooth muscle cells of neonatal and adult arteries (Figures 2 and 3), arguing against this possibility. (2) If RyR open time is much shorter in the neonatal than adult myocytes, then amplitudes of many Ca$^{2+}$ sparks might fall below the detection limit. However, the amplitudes of the Ca$^{2+}$ sparks observed in the neonatal arteries were no different from those in adult arteries. (3) If RyR density were much lower (<1/100) in neonatal myocytes than in adult myocytes, then Ca$^{2+}$ spark frequency also would be much lower. However, neonatal myocytes stained positively for an antibody to RyRs, and the rise time and amplitudes of the caffeine-induced Ca$^{2+}$ transients were similar in neonatal and adult myocytes. (4) If the coordinated opening of several tightly clustered RyRs is required to cause a Ca$^{2+}$ spark, then it is possible that RyRs in neonatal myocytes are not clustered in a manner to permit Ca$^{2+}$ sparks to occur (Figure 9). In this case, the unitary efflux of Ca$^{2+}$ through a single RyR would be too low to be detected, and the coordinated opening of a tightly clustered group of RyRs causes a Ca$^{2+}$ spark. There are several lines of evidence from both cardiac and skeletal muscle of a Ca$^{2+}$ spark (“the elementary event”) being made up of several smaller events (“quarks” or “fundamental events”). Figure 9 illustrates a model for our results based on the latter hypothesis. Specifically, we propose that during development (>1 day postnatal), RyRs cluster in terminal SR plaques to cause a Ca$^{2+}$ spark to activate K$_{Ca}$ channels. The appearance of Ca$^{2+}$ sparks occurs in development after the appearance of RyRs, Ca$^{2+}$ channels, and K$_{Ca}$ channels. It appears as if the molecular components for E-C coupling are first expressed, and then spatially organized to perform their specific functions.

Figure 8. K$_{Ca}$ channels are present in myocytes from cerebral arteries of neonatal animals. A, Consecutive records of single K$_{Ca}$ channel openings before (control) and after application of IbTx (100 nmol/L; 10 minutes) recorded using the whole-cell perforated patch configuration of the patch-clamp technique ($V_h$=30 mV). STOCs were not observed in neonatal cells (n=6). B, Relationship between single K$_{Ca}$ channel current amplitude and voltage (n=6). [K$^+$_o]=6 mmol/L, [K$^+$_i]=140 mmol/L.

Figure 9. Proposed model illustrating the organization of RyR in neonatal and adult arteries. This model proposes that during development (>1 day postnatal), RyRs cluster in terminal SR plaques to cause a Ca$^{2+}$ spark to activate K$_{Ca}$ channels. The appearance of Ca$^{2+}$ sparks occurs in development after the appearance of RyRs, Ca$^{2+}$ channels, and K$_{Ca}$ channels, to cause a membrane potential ($V_m$) hyperpolarization, which closes voltage-dependent calcium channels (VDCCs).
Open Probability of K$_{Ca}$ Channels Is Very Low in the Absence of Ca$^{2+}$ Sparks

K$_{Ca}$ channels are both voltage- and Ca$^{2+}$-sensitive. Our approach (whole-cell perforated patch-clamp technique) enabled the measurement of the whole-cell activity (NP$_o$) of single K$_{Ca}$ channels. In the absence of Ca$^{2+}$ sparks, whole-cell K$_{Ca}$ channel NP$_o$ was extremely low in both adult myocytes (0.005; 0 mV) and neonatal (0.02; 30 mV; Figure 8) myocytes. Given their Ca$^{2+}$ dependence and voltage dependence, whole-cell K$_{Ca}$ channel NP$_o$ in both adult and neonatal myocytes would be in the order of $10^{-2}$ to $10^{-2}$ under physiological conditions ($\sim 40$ mV; 200 nmol/L global Ca$^{2+}$; see Porter et al$^{16}$ for a detailed discussion of this issue). However, the frequency of Ca$^{2+}$ sparks ($\sim 1$ spark/s per cell), measured at physiological membrane potentials and arterial wall Ca$^{2+}$, elevate the whole-cell NP$_o$ to between 0.1 and 1.0, which would contribute significantly to the cell’s membrane potential, given the input resistance of smooth muscle cells and the relatively large single K$_{Ca}$ channel conductance.\textsuperscript{16,37,40}

Regardless of the uncertainties, K$_{Ca}$ channels would not contribute significantly to the membrane conductance of arterial myocytes without a Ca$^{2+}$ spark frequency of $>10^{-1}$ Hz/cell. These considerations also support the idea that the apparent Ca$^{2+}$ spark frequency observed in neonatal myocytes ($<10^{-2}$ Hz/cell) would not cause sufficient K$_{Ca}$ channel activity to regulate the membrane potential of smooth muscle cells in intact neonatal arteries.

Ca$^{2+}$ Sparks Are Elementary Physiological Events

Neonatal arteries possess the molecular components for E-C coupling and its negative feedback regulation (ie, L-type, voltage-dependent Ca$^{2+}$ channels, RyRs, K$_{Ca}$ channels). As expected, blockers of L-type Ca$^{2+}$ channels lower arterial wall Ca$^{2+}$ and dilate neonatal arteries (Figures 2 and 3). Contrary to expectation, blockers of RyRs (ryanodine) and K$_{Ca}$ channels (bTx) had no effect on arterial wall Ca$^{2+}$ and diameter of pressurized neonatal arteries with tone, even though these arteries have RyRs and K$_{Ca}$ channels. The missing feature of E-C coupling in neonatal arteries is Ca$^{2+}$ sparks. In contrast, adult arteries have a much higher spark frequency (>100-fold) than neonatal arteries and respond robustly to inhibitors of RyRs and K$_{Ca}$ channels.\textsuperscript{3,11,38} Therefore, these results provide unique support for the idea (Figure 9) that RyRs must generate Ca$^{2+}$ sparks to regulate arterial function.

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References


Ontogeny of Local Sarcoplasmic Reticulum Ca$^{2+}$ Signals in Cerebral Arteries: Ca$^{2+}$ Sparks as Elementary Physiological Events

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