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Sarcoplasmic reticulum calcium load regulates rat arterial smooth muscle calcium sparks and transient K_{Ca} currents

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The regulation of calcium (Ca^{2+}) sparks and transient calcium-sensitive K^+ (K_{Ca}) currents by acute changes in sarcoplasmic reticulum (SR) Ca^{2+} load ($[Ca^{2+}]_{SR}$) was investigated in rat cerebral artery smooth muscle cells using laser-scanning confocal microscopy in combination with patch clamp electrophysiology. $[Ca^{2+}]_{SR}$ was elevated by: (i) increasing the activity of the SR Ca^{2+}-ATPase with an anti-phospholamban monoclonal antibody, or (ii) blocking Ca^{2+} release from the SR with tetracaine, a membrane-permeant, reversible ryanodine-sensitive Ca^{2+} release (RyR) channel blocker. Alternatively, $[Ca^{2+}]_{SR}$ was progressively decreased over time with a low concentration of thapsigargin (20 nM), a SR Ca^{2+}-ATPase blocker. An elevation in $[Ca^{2+}]_{SR}$ increased Ca^{2+} spark and transient K_{Ca} current frequency, but did not alter the amplitude, decay or spatial spread of Ca^{2+} sparks or the coupling ratio or amplitude correlation between Ca^{2+} sparks and evoked transient K_{Ca} currents. Decreasing $[Ca^{2+}]_{SR}$ reduced Ca^{2+} spark frequency, amplitude and spatial spread and this reduced transient K_{Ca} current frequency and amplitude. However, even when mean Ca^{2+} spark amplitude and spread decreased by up to 47 and 56 % of control, respectively, the coupling ratio or amplitude correlation between Ca^{2+} sparks and transient K_{Ca} currents was not affected. These data demonstrate that acute changes in $[Ca^{2+}]_{SR}$ regulate Ca^{2+} sparks and transient K_{Ca} currents in arterial smooth muscle cells.

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Intracellular calcium (Ca^{2+}) regulates a diverse range of cellular functions, including contraction, secretion and gene transcription. However, recent studies have determined that the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of many cell types is not homogeneously distributed (for reviews see Clapham, 1995; Berridge, 1997). Several different types of intracellular Ca^{2+} elevations have been described to occur that differ in respect to temporal kinetics, spatial localization and physiological function (Bootman *et al.* 2001). One type of Ca^{2+} signalling modality that has been observed in cardiac (Cheng *et al.* 1993), skeletal (Tsugorka *et al.* 1995) and smooth muscle (Nelson *et al.* 1995) cells is termed a 'Ca^{2+} spark'. Ca^{2+} sparks are highly localized, cytosolic Ca^{2+} transients that occur due to the opening of a number of ryanodine-sensitive Ca^{2+} release (RyR) channels in the sarcoplasmic reticulum (SR; Jaggar *et al.* 2000).

In smooth muscle cells, Ca^{2+} sparks exhibit a rise time of ~20 ms, a half-time of decay of ~50 ms and a spatial spread of ~2–3 μm when imaged using a confocal microscope and fluorescent Ca^{2+} indicators such as fluo-3 (Nelson *et al.* 1995; Jaggar *et al.* 2000). Although Ca^{2+} sparks elevate intracellular Ca^{2+} in the immediate vicinity of the Ca^{2+} release site to micromolar concentrations (Perez *et al.* 2001), the impact of Ca^{2+} sparks on the global $[Ca^{2+}]_i$ is low, due to their transient and localized properties (Nelson *et al.* 1995; Jaggar *et al.* 2000). In arterial smooth muscle cells, most Ca^{2+} sparks occur in close proximity to the plasma membrane and activate a number of large-conductance Ca^{2+}-sensitive K^+ (K_{Ca}) channels to evoke a transient outward K^+ current (Nelson *et al.* 1995; Bolton & Gordienko, 1998; Perez *et al.* 1999), which has been termed a 'spontaneous transient outward current' or 'STOC' (Benham & Bolton, 1986). In arteries at physiological levels of pressure, inhibition of Ca^{2+} sparks or K_{Ca} channels leads to membrane depolarization, activation of voltage-dependent Ca^{2+} channels, an elevation in the arterial wall $[Ca^{2+}]_i$ and constriction (Nelson *et al.* 1995; Jaggar, 2001). An elevation in intravascular pressure activates voltage-dependent Ca^{2+} channels in arterial smooth muscle cells leading to an increase in the global $[Ca^{2+}]_i$ and activation of Ca^{2+} sparks (Jaggar, 2001). The resulting elevation in K_{Ca} channel activity opposes the pressure-induced constriction (Brayden & Nelson, 1992).

Several signalling elements regulate Ca^{2+} sparks in smooth muscle cells, including intracellular Ca^{2+} and protein kinases (Jaggar *et al.* 2000). Smooth muscle RyR channels that are incorporated into lipid bilayers are activated by cytosolic Ca^{2+} elevations (Herrmann-Frank *et al.* 1991; Xu *et al.* 1994), suggesting that an elevation in $[Ca^{2+}]_i$ may regulate Ca^{2+} sparks via an interaction with activation sites

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located on the cytosolic face of the RyR channel. However, an increase in cytosolic $[Ca^{2+}]_i$ or activation of the SR Ca^{2+}-ATPase may increase SR Ca^{2+} load ($[Ca^{2+}]_{SR}$), which could also regulate RyR channels. Cardiac (Sitsapesan & Williams, 1994; Gyorke & Gyorke, 1998; Xu & Meissner, 1998; Ching *et al.* 2000) and skeletal (Herrmann-Frank & Lehmann-Horn, 1996; Tripathy & Meissner, 1996) muscle RyR channels incorporated into lipid bilayers are activated by an elevation in luminal Ca^{2+} concentration. Furthermore, an elevation in $[Ca^{2+}]_{SR}$ activates Ca^{2+} sparks in cardiac myocytes (Santana *et al.* 1997; Satoh *et al.* 1997; Lukyanenko *et al.* 2001). Recent studies have also suggested that $[Ca^{2+}]_{SR}$ may regulate Ca^{2+} sparks in smooth muscle cells. Genetic ablation of phospholamban, an endogenous inhibitor of the SR Ca^{2+}-ATPase, leads to a chronic elevation in $[Ca^{2+}]_{SR}$ and Ca^{2+} spark frequency in arterial smooth muscle cells, when compared to wild type controls (Wellman *et al.* 2001). In *Bufo marinus* stomach smooth muscle cells, following partial depletion of the $[Ca^{2+}]_{SR}$ with caffeine, Ca^{2+} spark frequency and amplitude increase during $[Ca^{2+}]_{SR}$ refilling (ZhuGe *et al.* 1999). Vasodilators also stimulate Ca^{2+} sparks, in part, by elevating $[Ca^{2+}]_{SR}$ (Porter *et al.* 1998; Wellman *et al.* 2001). However, despite the important role that the $[Ca^{2+}]_{SR}$ may play in intracellular Ca^{2+} signalling in smooth muscle, the regulation of Ca^{2+} sparks and transient K_{Ca} currents by $[Ca^{2+}]_{SR}$ is poorly understood.

The goal of this study was to investigate the regulation of Ca^{2+} sparks and transient K_{Ca} currents by acute changes in $[Ca^{2+}]_{SR}$ in arterial smooth muscle cells. $[Ca^{2+}]_{SR}$ was elevated with a monoclonal antibody raised against phospholamban, or with tetracaine, a membrane-permeant reversible RyR channel blocker. Alternatively, $[Ca^{2+}]_{SR}$ was progressively decreased with a low concentration of thapsigargin, a selective inhibitor of the SR Ca^{2+}-ATPase. Data suggest that an elevation in $[Ca^{2+}]_{SR}$ increases Ca^{2+} spark and transient K_{Ca} current frequency, but does not alter the amplitude, decay or spatial spread of Ca^{2+} sparks or the coupling ratio or amplitude correlation between Ca^{2+} sparks and evoked transient K_{Ca} currents. In contrast, decreasing $[Ca^{2+}]_{SR}$ reduces Ca^{2+} spark frequency, amplitude and spatial spread, which decreases the frequency and amplitude of evoked transient K_{Ca} currents, although the coupling ratio and amplitude correlation between Ca^{2+} sparks and K_{Ca} channels does not change. These findings provide evidence that acute changes in $[Ca^{2+}]_{SR}$ directly alter the frequency and amplitude properties of Ca^{2+} sparks and evoked transient K_{Ca} currents.

METHODS

Tissue preparation

Sprague-Dawley rats (~250 g) of either sex were killed by peritoneal injection of a sodium pentobarbital overdose (150 mg kg^{-1}), in accordance with the Animal Care and Use Committee policies and procedures at the University of Tennessee. The brain was removed and placed into ice-cold (4 °C), oxygenated (95 % O_2–5 % CO_2), physiological saline solution (PSS) containing (mM): 119 NaCl, 4.7 KCl, 24 $NaHCO_3$, 1.2 KH_2PO_4, 1.6 $CaCl_2$, 1.2 $MgSO_4$, 0.023 EDTA and 11 glucose (adjusted to pH 7.4 with NaOH). Posterior cerebral and cerebellar arteries (50–200 μm diameter) were removed, cleaned of basolateral connective tissue and maintained in ice-cold PSS. Where appropriate, the endothelium was removed by allowing an air bubble to remain in the lumen of the artery for 2 min, followed by a 30 s wash with H_2O (Jaggar & Nelson, 2000). Individual smooth muscle cells were enzymatically dissociated from cerebral arteries using a procedure similar to that described in Jaggar (2001).

Confocal calcium imaging

Arterial segments (1–2 mm in length) or isolated smooth muscle cells were placed into Hepes-buffered PSS of composition (mM): 134 NaCl, 6 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 Hepes and 10 glucose (pH 7.4, NaOH) containing 10 μM fluo-4 AM and 0.05 % pluronic F-127 for 60 min or 15 min, respectively, at 22 °C. To allow indicator de-esterification, arteries or isolated cells were subsequently placed into Hepes-buffered PSS for 30 min at 22 °C. Smooth muscle cells were imaged using a Noran Oz laser scanning confocal microscope (Noran Instruments, Middleton, WI, USA) and a × 60 water immersion objective (NA = 1.2) attached to a Nikon TE300 microscope by illuminating with a krypton–argon laser at 488 nm. Planar images (56.3 μm × 52.8 μm) were recorded every 16.7 ms, i.e. 60 images s^{-1}. To compare confocal Ca^{2+} imaging data with electrophysiological recordings performed in this study, Ca^{2+} sparks in smooth muscle cells of arterial segments were measured in an extracellular solution containing 30 mM K^+, which depolarizes smooth muscle cells to ~−40 mV, and is similar to the membrane potential of cerebral arteries pressurized to 60 mmHg (see Jaggar *et al.* 1998; Jaggar & Nelson, 2000; Jaggar, 2001 for similar procedure). The 30 mM K^+ bath solution contained (mM): NaCl 110; KCl 30; Hepes 10; $CaCl_2$ 2; $MgCl_2$ 1; and glucose 10 (pH 7.4, NaOH). For imaging smooth muscle cells in arteries, at least two different representative areas (56.3 μm × 52.8 μm) of the same segment were each scanned for at least 10 s under each condition. The same area of artery was scanned only once to avoid any laser-induced changes in Ca^{2+} signalling, and the effects of drugs were measured in paired experiments. Where appropriate, diltiazem (50 μM) was applied for 15 min to ensure a complete and steady state decrease in Ca^{2+} sparks and global Ca^{2+} fluorescence prior to tetracaine application. Tetracaine was then added in the presence of diltiazem, and further Ca^{2+} imaging measurements were made 5 min later. Time-matched control experiments confirmed that diltiazem induced steady-state block of Ca^{2+} sparks prior to tetracaine application. Ca^{2+} spark frequency in smooth muscle cells was not different after 15 (0.32 ± 0.09 Hz) or 20 min (0.26 ± 0.09 Hz) in the continued presence of diltiazem (50 μM, $P > 0.05$, $n = 5$ arteries). In experiments where confocal microscopy was employed in combination with patch clamp electrophysiology, current and fluorescence measurements were synchronized using a light emitting diode positioned above the recording chamber that was triggered during acquisition. Each single smooth muscle cell was imaged for at least 10 s under each condition. Ca^{2+} sparks were detected in smooth muscle cells using custom analysis software written using IDL 5.0.2 (Research Systems Inc., Boulder, CO, USA) kindly provided by Drs M. T. Nelson and A. D. Bonev (University of Vermont, VT, USA). Automated and manual detection of Ca^{2+} sparks was performed by dividing an area 1.54 μm (7 pixels) × 1.54 μm (7 pixels) (i.e. 2.37 μm^2) in each

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image (F) by a baseline (F_0) which was determined by averaging 10 images without Ca^{2+} spark activity. The entire area of each image was analysed to detect Ca^{2+} sparks. A Ca^{2+} spark was defined as a localized increase in F/F_0 that was greater than 1.2. In intact artery experiments, Ca^{2+} spark frequency (Hz) in each condition was calculated from at least two different areas of each artery wall. Ca^{2+} spark spatial spread was calculated as the full width at half-maximal amplitude (FWHM). Global Ca^{2+} fluorescence was calculated from the same images used for Ca^{2+} spark analysis and was the mean pixel value of 100 different images acquired over 10 s.

Ratiometric calcium measurements

Cerebral artery segments (1–2 mm in length) were incubated with the ratiometric fluorescent Ca^{2+} indicator fura-2 AM (2 μM) and 0.05 % pluronic F-127 for 20 min, followed by a 15 min wash. Experiments were performed using a 6 mM potassium Hepes-buffered PSS (composition described above). Arteries were alternately excited at 340 or 380 nm using a PC driven Hyperswitch (Ionoptix, Milton, MA, USA), and background corrected ratios were collected at every 0.2 s at 510 nm using a photomultiplier tube. $[Ca^{2+}]_{SR}$ was determined by rapidly applying a high concentration of caffeine (20 mM), a RyR channel activator, wherein the amplitude of the $[Ca^{2+}]_i$ transient would be related to the $[Ca^{2+}]_{SR}$. $[Ca^{2+}]_i$ concentrations were calculated using the following equation (Grynkiewicz *et al.* 1985):

$$[Ca^{2+}] = K_d \frac{(R - R_{min})}{(R_{max} - R)} \frac{S_{f2}}{S_{b2}},$$

where R is the 340/380 nm ratio, R_{min} and R_{max} are the minimum and maximum ratios determined in Ca^{2+}-free and saturating Ca^{2+} solutions, respectively, S_{f2}/S_{b2} is the Ca^{2+}-free/Ca^{2+}-replete ratio of emissions at 380 nm excitation, and K_d is the dissociation constant for fura-2. R_{min}, R_{max}, S_{f2} and S_{b2} were determined at the end of each experiment and in separate experiments by increasing the Ca^{2+} permeability of smooth muscle cells with ionomycin (10 μM), and perfusing cells with a high Ca^{2+} (50 mM) or Ca^{2+}-free (10 mM EGTA) solution. The *in situ* apparent dissociation constant (K_d) for fura-2 used in this study was 282 nM (Knot & Nelson, 1998).

Patch clamp electrophysiology

Isolated cells were allowed to adhere to a glass coverslip in the bottom of a chamber for 10 min prior to experimentation. K^+ currents were measured using either the conventional whole-cell or perforated patch configuration (Horn & Marty, 1988) of the patch clamp technique (Hamill *et al.* 1981) with an Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA). Bath solution was 6 mM K^+ Hepes-buffered PSS (composition described above). For perforated patch experiments, the pipette solution contained (mM): 110 potassium aspartate, 30 KCl, 10 NaCl, 1 $MgCl_2$, 10 Hepes, 0.05 EGTA (pH 7.2 with KOH). For conventional whole-cell experiments, the pipette (i.e. intracellular) solution contained (mM): 140 KCl, 1.9 $MgCl_2$, 0.037 $CaCl_2$, 0.1 EGTA, 10 Hepes, 1 Na_2ATP (pH 7.2 with KOH); the calculated free Ca^{2+} and free Mg^{2+} concentrations of this solution are 100 nM and 1 mM, respectively (WEBMAXC, Stanford University, CA, USA). The conventional whole-cell configuration was used in experiments with anti-phospholamban antibody. Elsewhere in this study, the perforated patch configuration was used. All patch clamp experiments were performed with a holding potential of −40 mV. Membrane currents were recorded with a sample rate of 2.5 kHz and filtered at 1 kHz. Transient K_{Ca} current analysis was performed off-line using a custom analysis program provided by Drs M. T. Nelson and A. D. Bonev (University of Vermont). A transient K_{Ca} current was defined as the simultaneous opening of three or more K_{Ca} channels. In the presence of Ca^{2+} spark blockers, the simultaneous opening of three single K_{Ca} channels was not observed at −40 mV (Nelson *et al.* 1995; Bonev *et al.* 1997; Porter *et al.* 1998). For experiments performed in the absence or presence of anti-phospholamban antibody, transient K_{Ca} current properties were compared between 5 and 10 min after whole-cell patch formation. Where appropriate, anti-phospholamban antibody was heat-inactivated by incubation at 94 °C for 15 min.

Statistical analysis

Values are expressed as means ± standard error of the mean. Student's t test or Student-Newman-Keuls tests were used for comparing paired or multiple data sets, respectively. First-order polynomial linear fits were used to calculate statistical correlation between the amplitude of Ca^{2+} sparks and evoked transient K_{Ca} currents (Origin, OriginLab Corp., Northampton, MA, USA). ANCOVA of first-order polynomial best fits were used to compare amplitude correlation data sets (Prism, GraphPad Software, Inc., San Diego, CA, USA). $P < 0.05$ was considered significant.

Chemicals

Unless stated otherwise, all chemicals used in this study were obtained from Sigma Chemical Company (St Louis, MO, USA). Papain was purchased from Worthington Biochemical Co. (Lakewood, NJ, USA), fluo-4 AM and pluronic F-127 from Molecular Probes (Eugene, OR, USA), and anti-phospholamban mouse IgG1 clone A1 from Upstate Biotechnology (Lake Placid, NY, USA).

RESULTS

A monoclonal antibody targeted against phospholamban elevates transient K_{Ca} current frequency in cerebral artery smooth muscle cells

To investigate the regulation of transient K_{Ca} currents by an acute elevation in $[Ca^{2+}]_{SR}$, we used a commercially available monoclonal antibody (mouse IgG1) raised against phospholamban, an endogenous inhibitor of the SR Ca^{2+}-ATPase. This antibody has previously been demonstrated to elevate $[Ca^{2+}]_{SR}$ in cardiac myocytes (Suzuki & Wang, 1986; Sham *et al.* 1991; Lukyanenko *et al.* 2001). Transient K_{Ca} current properties were compared between 5 and 10 min after conventional whole-cell formation in cells in which antibody (4 μg ml^{-1}) or heat-inactivated antibody (4 μg ml^{-1}) was included in the pipette solution, or antibody was absent.

Mean transient K_{Ca} current frequency was ~2.7-fold higher in cells in which anti-phospholamban antibody was included in the pipette solution ($n = 11$), when compared with cells in which heat-inactivated antibody was included ($n = 6$ cells), or antibody was absent ($n = 11$, Fig. 1). In contrast, the anti-phospholamban antibody did not change mean transient K_{Ca} current amplitude (Fig. 1). These data suggest that acute activation of the SR Ca^{2+}-ATPase with an anti-phospholamban antibody increases the frequency, but does not alter the amplitude, of transient K_{Ca} currents in arterial smooth muscle cells.

Tetracaine inhibits Ca^{2+} sparks in cerebral artery smooth muscle cells

We sought to measure the regulation of Ca^{2+} sparks and transient K_{Ca} currents before and after an elevation in $[Ca^{2+}]_{SR}$ in the same cell. We hypothesized that RyR channel blockers would increase $[Ca^{2+}]_{SR}$ by preventing Ca^{2+} release from the SR under conditions where the SR Ca^{2+}-ATPase would continue to sequester Ca^{2+} from the cytosol. To examine this possibility we used tetracaine, a membrane-permeant, reversible blocker of cardiac (Gyorke *et al.* 1997; Lukyanenko *et al.* 2001) and skeletal (Xu *et al.* 1993) muscle RyR channels. To determine if tetracaine blocks RyR channels in smooth muscle cells, the regulation of Ca^{2+} sparks by tetracaine (50 μM) was measured in the smooth muscle cells of endothelium-denuded cerebral artery segments. To compare confocal Ca^{2+} imaging data with electrophysiological data performed in this study, Ca^{2+} sparks were measured in a bath solution containing 30 mM K^+, which depolarizes smooth muscle cells from ~−60 to −40 mV, a membrane potential similar to that of cerebral arteries pressurized to 60 mmHg (see Jaggar *et al.* 1998; Jaggar & Nelson, 2000; Jaggar, 2001 for similar procedure). To prevent any potential indirect regulation of Ca^{2+} sparks due to non-specific effects on voltage-dependent Ca^{2+} channels (Sugiyama & Muteki, 1994), tetracaine was applied in the continued presence of diltiazem (50 μM), a voltage-dependent Ca^{2+} channel blocker.

Diltiazem reduced mean global Ca^{2+} fluorescence in smooth muscle cells (F/F_0) to 53 ± 3 % of control, and decreased mean Ca^{2+} spark frequency to ~20 % of control, although mean Ca^{2+} spark amplitude (F/F_0) did not change ($n = 6$ arteries, Fig. 2). In the same arteries in the continued presence of diltiazem, tetracaine reduced mean Ca^{2+} spark frequency to ~3 % of control, but did not change mean Ca^{2+} spark amplitude (Fig. 2). Tetracaine did not alter mean global F/F_0 (98 % ± 2 % of diltiazem), suggesting that Ca^{2+} sparks were not blocked via a reduction in global $[Ca^{2+}]_i$ (Fig. 2). These data demonstrate that tetracaine, a RyR channel blocker, inhibits Ca^{2+} sparks and does not alter global $[Ca^{2+}]_i$ in cerebral artery smooth muscle cells, when applied in the continued presence of diltiazem.

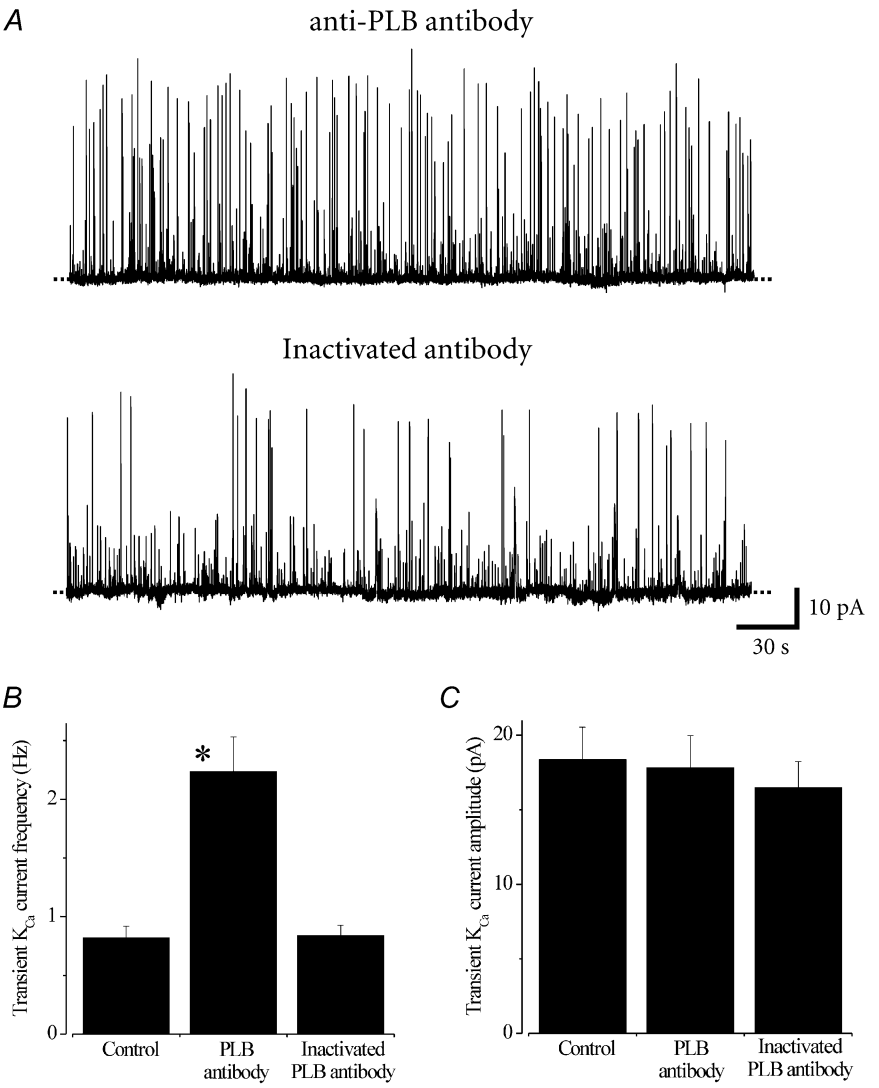

Figure 1. A monoclonal antibody raised against phospholamban (anti-PLB antibody) elevates transient K_{Ca} current frequency, but not amplitude, in cerebral artery smooth muscle cells

A, original traces illustrating typical transient K_{Ca} current activity at −40 mV in cells in which anti-PLB antibody (4 μg ml^{-1}, upper panel) or heat-inactivated anti-PLB antibody (4 μg ml^{-1}, lower panel) was included in the pipette solution. Average transient K_{Ca} current frequency (*B*) and amplitude (*C*) in cells in which no antibody (control), anti-PLB antibody, or heat-inactivated antibody was included in the pipette solution. * $P < 0.05$; Student's unpaired *t* test.

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Tetracaine elevates sarcoplasmic reticulum Ca^{2+} load in cerebral arteries

Blocking Ca^{2+} release from the SR with tetracaine should lead to an increase in $[Ca^{2+}]_{SR}$. To examine this hypothesis, the effect of tetracaine (50 μM) on the amplitude of caffeine (20 mM)-induced $[Ca^{2+}]_i$ transients was investigated in endothelium-denuded cerebral artery segments using the ratiometric Ca^{2+} indicator fura-2. Experiments were performed in a bath solution containing 6 mM K^+.

Diltiazem did not change mean arterial wall $[Ca^{2+}]_i$ (control, 99 ± 5 nM *vs.* diltiazem, 100 ± 5 nM) or the amplitude of caffeine-induced $[Ca^{2+}]_i$ transients (Fig. 3, $n = 6$ arteries). In the same arteries, although tetracaine did not change mean arterial wall $[Ca^{2+}]_i$ (104 ± 4 nM), mean caffeine-induced $[Ca^{2+}]_i$ transients increased ~1.4-fold after a 7 min application. Five minutes after tetracaine washout, caffeine-induced $[Ca^{2+}]_i$ transients returned to pre-tetracaine levels (Fig. 3). These data suggest that tetracaine, a RyR channel blocker, reversibly elevates $[Ca^{2+}]_{SR}$ in cerebral artery smooth muscle cells, presumably by blocking Ca^{2+} release from the SR (Fig. 2).

Regulation of transient K_{Ca} currents by tetracaine

If tetracaine inhibits Ca^{2+} sparks, it should also block transient K_{Ca} currents. Furthermore, if an increase in $[Ca^{2+}]_{SR}$ activates Ca^{2+} sparks, transient K_{Ca} currents should be elevated immediately after washout of tetracaine, when compared with transient K_{Ca} currents prior to tetracaine application. To investigate this hypothesis, the regulation of transient K_{Ca} currents by tetracaine was measured in voltage-clamped (−40 mV) cerebral artery smooth muscle cells. Tetracaine was applied, and washed out, in the continued presence of Cd^{2+} (250 μM), a voltage-dependent Ca^{2+} channel blocker.

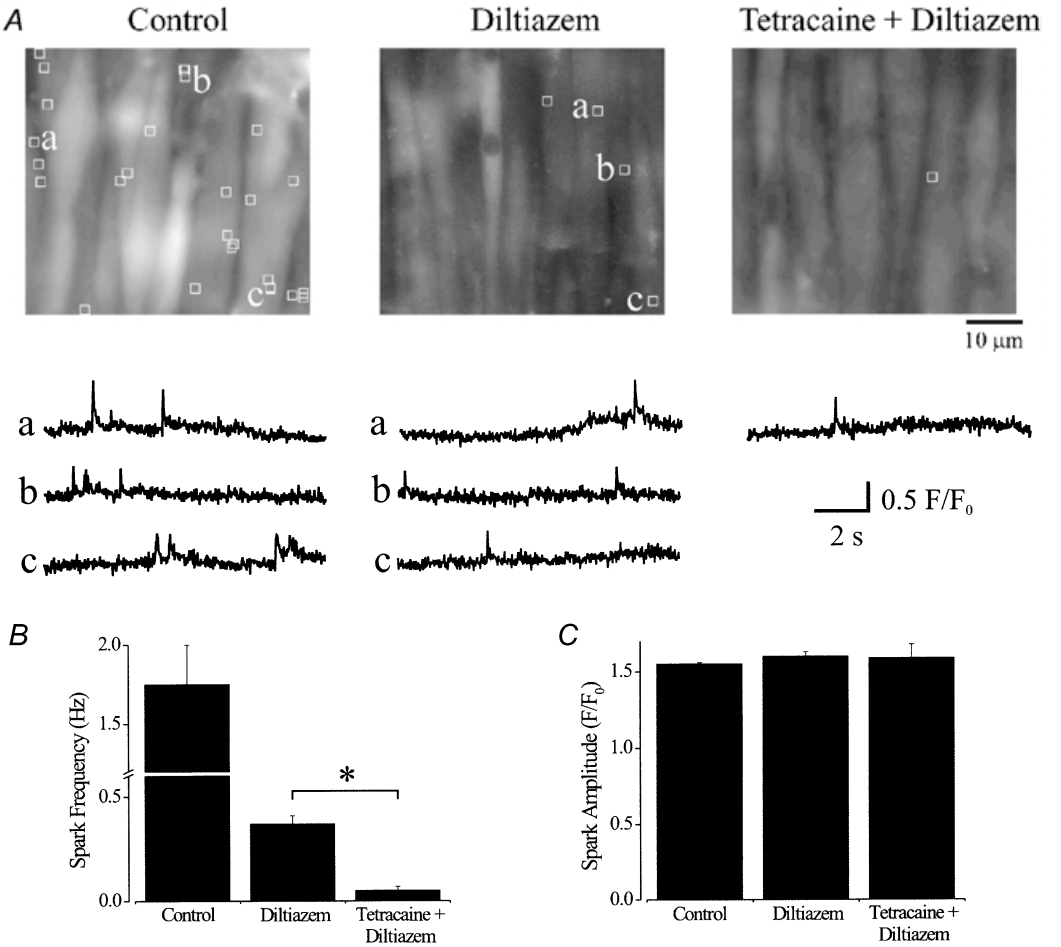

Figure 2. Tetracaine blocks Ca^{2+} sparks in smooth muscle cells of intact cerebral arteries

A, average fluorescence (100 of 600 images) over 10 s of three different 56.3 μm × 52.8 μm areas of the same cerebral artery in control (30 mM K^+), diltiazem (50 μM) and tetracaine (50 μM) + diltiazem (50 μM). The locations of Ca^{2+} sparks that occurred during 10 s are indicated by white boxes (1.54 μm × 1.54 μm) and representative localized F/F_0 changes with time are illustrated below respective images and labelled accordingly. In this artery over 10 s, 27 sparks occurred in control, five sparks in diltiazem and one spark in tetracaine + diltiazem. Average effects on Ca^{2+} spark frequency (*B*) and amplitude (*C*). * $P < 0.05$; Student-Newman-Keuls test.

Cd^{2+} reduced mean transient K_{Ca} current frequency to ~54 % of control, but did not change mean transient K_{Ca} current amplitude (Fig. 4, $n = 6$ cells). In the continued presence of Cd^{2+}, tetracaine (50 μM, 7 min application) further reduced mean transient K_{Ca} current frequency, but did not alter mean amplitude. Washout of tetracaine was rapid and led to a significant increase in mean transient K_{Ca} current frequency. Between 1 and 3 min after tetracaine washout, mean transient K_{Ca} current frequency was ~3.1-fold higher than that prior to tetracaine application, although mean transient K_{Ca} current amplitude was not significantly different (Fig. 4). Approximately 5 min after tetracaine washout, but in the continued presence of Cd^{2+}, transient K_{Ca} current frequency returned to pre-tetracaine levels (Fig. 4).

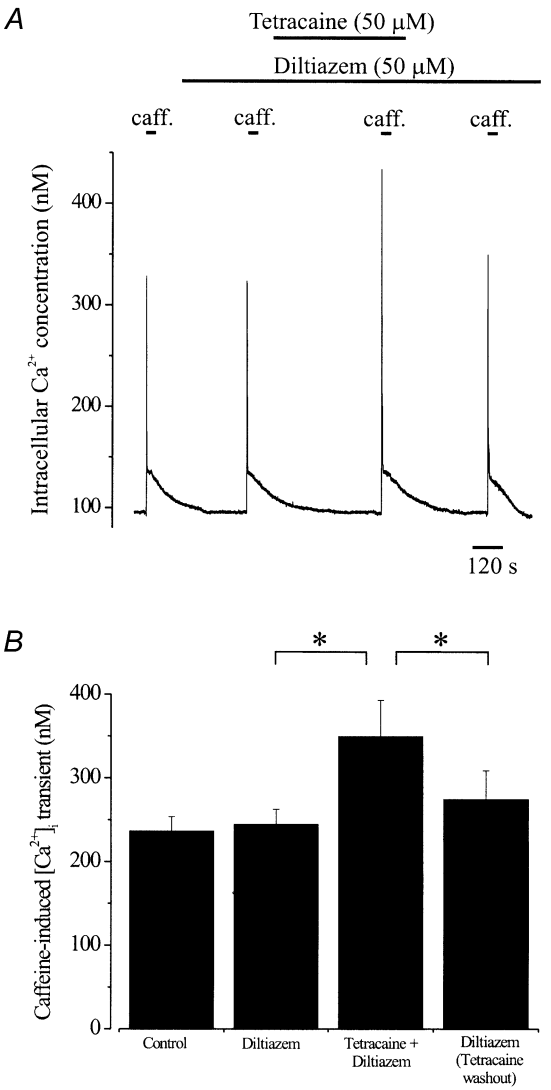

Figure 3. Tetracaine elevates $[Ca^{2+}]_{SR}$ in cerebral artery segments

A, intracellular Ca^{2+} concentration and caffeine (20 mM)-induced $[Ca^{2+}]_i$ transients in an endothelium-denuded cerebral artery segment. Diltiazem (50 μM) did not change arterial wall $[Ca^{2+}]_i$ or caffeine-induced $[Ca^{2+}]_i$ transients. Tetracaine (50 μM, 7 min application) did not change arterial wall $[Ca^{2+}]_i$, but significantly increased the amplitude of the caffeine-induced $[Ca^{2+}]_i$ transient. Five minutes after washout of tetracaine, the caffeine-induced $[Ca^{2+}]_i$ transient was of similar amplitude to control. *B*, average caffeine-induced $[Ca^{2+}]_i$ transients in control, diltiazem, tetracaine + diltiazem, and 5 min after tetracaine washout + diltiazem. * $P < 0.05$; Student-Newman-Keuls test.

These data demonstrate that tetracaine blocks transient K_{Ca} currents. After washout of tetracaine, when $[Ca^{2+}]_{SR}$ would have increased (Fig. 3), transient K_{Ca} current frequency was significantly higher than prior to tetracaine application, although transient K_{Ca} current amplitude was not different. Because tetracaine washout does not alter cytosolic $[Ca^{2+}]_i$ (Fig. 3), these data suggest that an acute elevation in $[Ca^{2+}]_{SR}$ increases transient K_{Ca} current frequency, but not amplitude. Furthermore, the elevation in transient K_{Ca} current frequency was short lived after tetracaine washout, suggesting that elevated Ca^{2+} release from the SR leads to a return of $[Ca^{2+}]_{SR}$ and transient K_{Ca} current frequency to pre-tetracaine levels.

Tetracaine washout elevates Ca^{2+} spark frequency, but not amplitude

We sought to investigate the effect of an acute elevation in $[Ca^{2+}]_{SR}$ on Ca^{2+} sparks and transient K_{Ca} currents. Essentially 100 % of Ca^{2+} sparks induce a transient K_{Ca} current in rat cerebral artery smooth muscle cells (Perez *et al.* 1999). Therefore, our finding that an elevation in $[Ca^{2+}]_{SR}$ elevates transient K_{Ca} current frequency suggests that this occurs via an increase in Ca^{2+} spark frequency. However, an acute increase in $[Ca^{2+}]_{SR}$ could conceivably elevate Ca^{2+} spark amplitude via activation of RyR channels (Herrmann-Frank & Lehmann-Horn, 1996; Tripathy & Meissner, 1996; Gyorke & Gyorke, 1998; Xu & Meissner, 1998; Ching *et al.* 2000) and by increasing the driving force for Ca^{2+} from the SR. A significant proportion of Ca^{2+} sparks do not evoke a transient K_{Ca} current in human cerebral artery (28 %, Wellman *et al.* 2002), feline oesophageal (27 %, Kirber *et al.* 2001) or *Bufo marinus* stomach (21 %, ZhuGe *et al.* 2000) smooth muscle cells. If Ca^{2+} spark:transient K_{Ca} current coupling is not 1:1, an increase in Ca^{2+} spark amplitude may augment this coupling ratio, which would also increase transient K_{Ca} current frequency. Therefore, we measured the effect of an elevation in $[Ca^{2+}]_{SR}$ on Ca^{2+} spark and evoked transient K_{Ca} current properties, and the coupling ratio and amplitude relationship between these events. Simultaneous measurements of Ca^{2+} sparks and transient K_{Ca} currents were made in the same cells before tetracaine, and 90 s after tetracaine washout (50 μM, 7 min application), in the continued presence of diltiazem (50 μM).

Prior to tetracaine application, 100 % of detected Ca^{2+} sparks evoked a transient K_{Ca} current ($n = 4$ cells, $n = 33$ sparks) and 71.7 % of transient K_{Ca} currents were associated with a Ca^{2+} spark, suggesting that some sparks occurred outside the cytosolic volume detected by the

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confocal microscope (Fig. 5). The amplitude of a Ca^{2+} spark and that of the evoked transient K_{Ca} current were significantly correlated ($P < 0.0001$) (Fig. 6). In the same cells, tetracaine significantly reduced transient K_{Ca} current frequency, consistent with effects in non-fluo-4 loaded cells (Fig. 4). After tetracaine washout, when $[Ca^{2+}]_{SR}$ would have increased, mean Ca^{2+} spark frequency was ~2.7-fold higher than prior to tetracaine application (Fig. 5, $n = 88$ sparks). In contrast, mean Ca^{2+} spark amplitude (Fig. 5), half-time for decay ($t_{½}$; control, 52 ± 9 *vs.* tetracaine washout, 57 ± 6 ms) and spatial spread (FWHM; control, 2.23 ± 0.15 *vs.* tetracaine washout, 1.95 ± 0.17 μm) were not different ($P > 0.05$ for each). Consistent with effects in non-fluo-4 loaded cells, mean transient K_{Ca} current amplitude (control, 23.8 ± 2.8 *vs.* tetracaine washout, 23.1 ± 4.6 pA) also did not change ($P > 0.05$). After tetracaine washout, 100 % of Ca^{2+} sparks evoked a transient K_{Ca} current and 68.2 % of transient K_{Ca} currents were associated with a Ca^{2+} spark, suggesting that an elevation in $[Ca^{2+}]_{SR}$ did not change the coupling ratio between sparks and transient K_{Ca} currents. Furthermore, tetracaine washout did not significantly alter the amplitude correlation between Ca^{2+} sparks and evoked transient K_{Ca} currents (Fig. 6). Global F/F_0 was not altered by washout of tetracaine (99 ± 6 % of pre-tetracaine), suggesting that Ca^{2+} sparks were activated via an elevation in $[Ca^{2+}]_{SR}$ and not via an increase in cytosolic $[Ca^{2+}]_i$, and that the increase in Ca^{2+} spark frequency did not elevate global cytosolic $[Ca^{2+}]_i$. These data suggest that an elevation in $[Ca^{2+}]_{SR}$ increases Ca^{2+} spark frequency, but does not alter Ca^{2+} spark amplitude, decay, spatial spread, or the coupling ratio or amplitude correlation between sparks and transient K_{Ca} currents.

Thapsigargin progressively decreases SR Ca^{2+} load in cerebral arteries

If an elevation in $[Ca^{2+}]_{SR}$ increases Ca^{2+} spark frequency in arterial smooth muscle cells, then a decrease in $[Ca^{2+}]_{SR}$ should decrease Ca^{2+} spark frequency. Furthermore, because the $[Ca^{2+}]_{SR}$ establishes the driving force for Ca^{2+} from the SR, a decrease in $[Ca^{2+}]_{SR}$ may also reduce Ca^{2+}

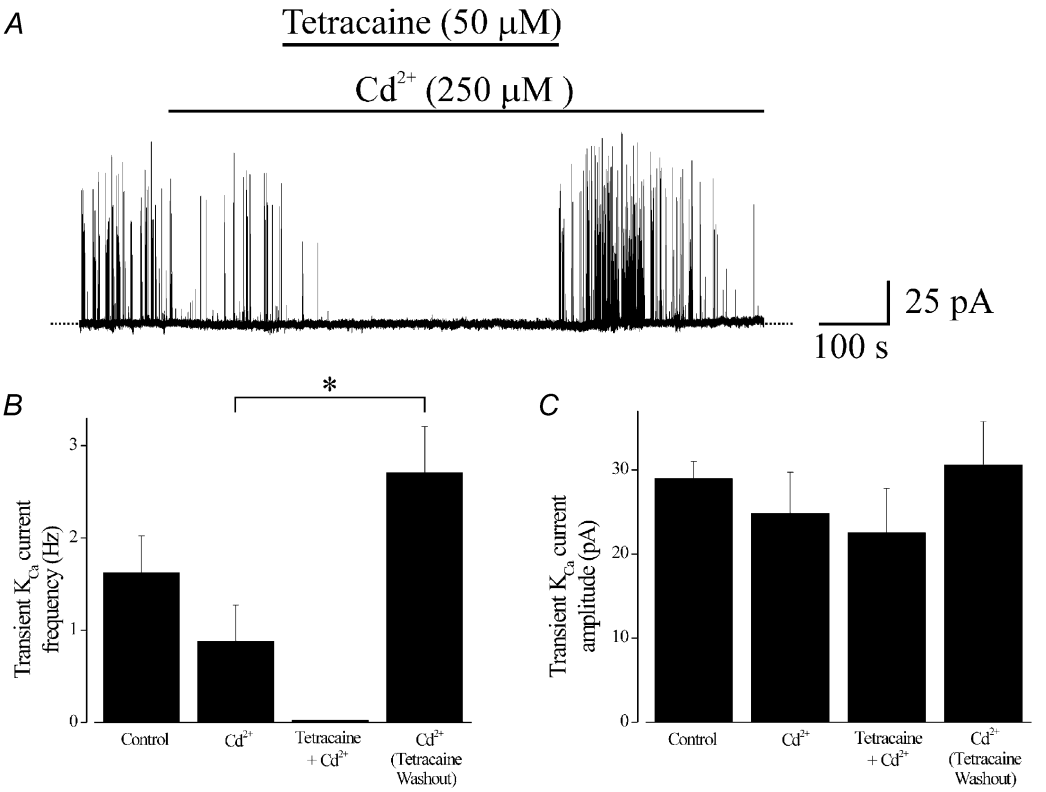

Figure 4. Regulation of transient K_{Ca} currents by tetracaine

A, original record illustrating transient K_{Ca} currents in a cerebral artery smooth muscle cell voltage-clamped at −40 mV using the perforated-patch configuration. Cd^{2+} (250 μM) reduced transient K_{Ca} current frequency. Tetracaine (50 μM, 7 min application), applied in the continued presence of Cd^{2+}, further reduced transient K_{Ca} currents. Immediately after tetracaine washout, transient K_{Ca} current frequency was significantly higher than before tetracaine application, although transient K_{Ca} current amplitude was not significantly different. Transient K_{Ca} current frequency returned to pre-tetracaine levels approximately 5 min after tetracaine washout. Average effects of Cd^{2+} (250 μM), tetracaine (50 μM) + Cd^{2+}, and 1–3 min after tetracaine washout + Cd^{2+} on transient K_{Ca} current frequency (*B*) and amplitude (*C*). * $P < 0.05$; Student-Newman-Keuls test.

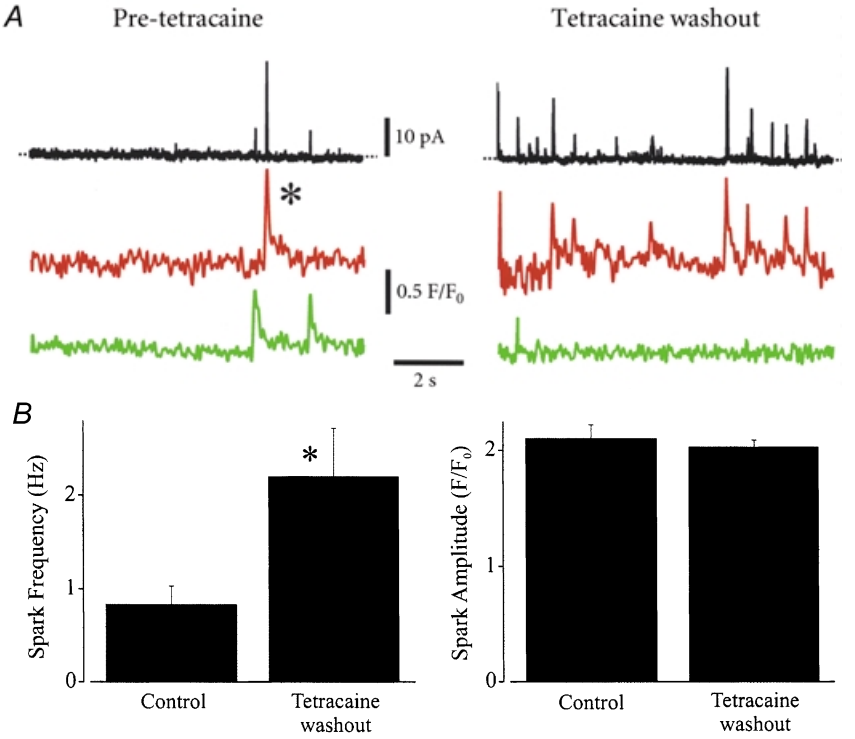

Figure 5. Tetracaine washout elevates Ca^{2+} spark frequency

A, original simultaneous recordings of Ca^{2+} sparks and transient K_{Ca} currents in a voltage-clamped (−40 mV) cerebral artery smooth muscle cell. The black line illustrates whole cell K^+ current. Red and green lines illustrate fluorescence changes (F/F_0) measured in two different 1.54 μm × 1.54 μm (i.e. 2.37 μm^2) areas of the cell in which Ca^{2+} sparks occurred. Traces indicate activity before tetracaine (50 μM) application (pre-tetracaine) and 90 s after tetracaine washout. Tetracaine was applied for 7 min. The experiment was performed in the continued presence of diltiazem (50 μM). *B*, summary of Ca^{2+} spark frequency and amplitude pre-tetracaine (n = 33 sparks, n = 4 cells) and after tetracaine washout (n = 88 sparks). * $P < 0.05$; Student's paired t test.

spark amplitude. To investigate the effect of decreasing $[Ca^{2+}]_{SR}$ on Ca^{2+} sparks and transient K_{Ca} currents, thapsigargin, a selective blocker of the SR Ca^{2+}-ATPase, was used. In smooth muscle cells, thapsigargin has most commonly been applied at a high concentration (~100 nM), to rapidly deplete the SR of Ca^{2+} (e.g. see Jaggar, 2001). To determine the effect of a low concentration of thapsigargin (20 nM) on $[Ca^{2+}]_{SR}$, caffeine-induced $[Ca^{2+}]_i$ transients were measured in control, and 5, 10 and 15 min after application of thapsigargin (20 nM) in the same endothelium-denuded cerebral artery segments.

Thapsigargin (20 nM) induced a time-dependent decrease in caffeine-induced $[Ca^{2+}]_i$ transients (Fig. 7, n = 9 arteries). Five, ten and fifteen minutes after addition of thapsigargin, caffeine-induced $[Ca^{2+}]_i$ transients decreased to ~76, ~57 and ~41 % of control, respectively. In contrast, 5, 10 and 15 min after thapsigargin application, steady-state arterial wall $[Ca^{2+}]_i$ was 99 ± 2, 101 ± 2 and 102 ± 2 nM, respectively, which were not significantly different from control ($P > 0.05$ for each, Fig. 7). To ensure that $[Ca^{2+}]_{SR}$ was reduced by thapsigargin, and not by repetitive addition of caffeine or insufficient recovery of $[Ca^{2+}]_{SR}$ after each caffeine application, caffeine-induced $[Ca^{2+}]_i$ transients were measured in six separate arteries in the absence of thapsigargin using the same time protocol. In the absence of thapsigargin, caffeine-induced $[Ca^{2+}]_i$ transients were not significantly different at 0, 5, 10 or 15 min ($P > 0.05$ for each, Fig. 7). In these control arteries, arterial wall $[Ca^{2+}]_i$ also did not change over the same time course (0 min, 99 ± 3 nM; 15 min, 96 ± 5 nM, $P > 0.05$). These data demonstrate that a low concentration of thapsigargin (20 nM) progressively decreases $[Ca^{2+}]_{SR}$ in cerebral arteries, and that significant depletion of $[Ca^{2+}]_{SR}$ does not alter arterial wall $[Ca^{2+}]_i$.

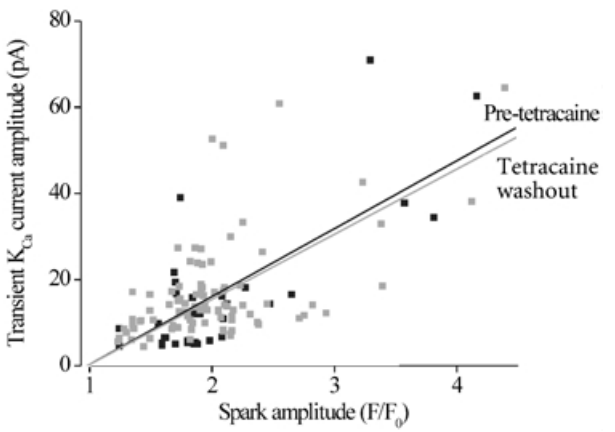

Figure 6. Tetracaine washout does not alter the amplitude correlation between Ca^{2+} sparks and transient K_{Ca} currents

Scatter plot of Ca^{2+} spark and evoked transient K_{Ca} current amplitude at −40 mV before tetracaine application (pre-tetracaine, black), and after tetracaine washout (grey). The amplitudes of Ca^{2+} sparks and evoked transient K_{Ca} currents were significantly correlated, both for pre-tetracaine ($P < 0.0001$) and tetracaine washout data ($P < 0.0001$). Correlation co-efficients (r) were: control, 0.55; tetracaine washout, 0.58. The amplitude correlations before, and after, tetracaine were not significantly different from each other ($P = 0.79$).

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Thapsigargin progressively decreases Ca^{2+} spark frequency, amplitude and spatial spread

We sought to examine the effect of progressively decreasing $[Ca^{2+}]_{SR}$ on Ca^{2+} sparks and transient K_{Ca} currents. Simultaneous measurements of Ca^{2+} sparks and transient K_{Ca} currents were made in the same voltage-clamped (−40 mV) cerebral artery smooth muscle cells in control, and 5, 10 and 15 min after addition of thapsigargin (20 nM).

In control, 98.2 % of detected Ca^{2+} sparks evoked a transient K_{Ca} current (Fig. 8, Table 1, $n = 55$ sparks, $n = 6$ cells). The amplitudes of coupled Ca^{2+} sparks and evoked transient K_{Ca} currents were correlated (Fig. 8). In control, one Ca^{2+} spark out of a total of 56 did not evoke a transient K_{Ca} current. The amplitude (F/F_0) of this uncoupled Ca^{2+} spark was 1.26, which was significantly smaller than the mean amplitude of coupled sparks (Table 1), and is only just larger than our designated threshold for a Ca^{2+} spark, which is an F/F_0 greater than 1.2. In addition, the spatial spread of the uncoupled spark was smaller (0.96 μm) and the decay was faster (18.0 ms) than the mean value for coupled sparks (Table 1). In the same cells, thapsigargin (20 nM) induced a time-dependent decrease in mean Ca^{2+} spark frequency, amplitude and spatial spread, which reduced transient K_{Ca} current frequency and amplitude (Table 1). Five, ten or fifteen minutes after addition of thapsigargin, 100 % of Ca^{2+} sparks evoked a transient K_{Ca} current, suggesting that uncoupling of Ca^{2+} sparks from K_{Ca} channels did not occur, even when mean spark amplitude and spread were reduced to 47 and 56 % of control, respectively (Table 1). The amplitudes of Ca^{2+} sparks and transient K_{Ca} currents were correlated at 5, 10 or 15 min after thapsigargin (Fig. 8). Furthermore, the amplitude correlation for each time point after thapsigargin addition was not significantly different when compared with the amplitude correlation in control (Fig. 8). These data suggest that although a decrease in $[Ca^{2+}]_{SR}$ reduces Ca^{2+} spark amplitude and spatial spread, the amplitude correlation between a spark and a transient K_{Ca} current is maintained (Fig. 8). Thapsigargin did not change global F/F_0 (103 ± 5 % of control at 15 min, $P > 0.05$), suggesting that all modifications in Ca^{2+} sparks were due to a decrease in $[Ca^{2+}]_{SR}$ and not due to alterations in cytosolic $[Ca^{2+}]_i$.

To investigate if the repetitive confocal acquisition protocol was responsible for the observed changes in Ca^{2+} sparks and transient K_{Ca} currents, the same imaging procedure was performed in voltage-clamped (−40 mV) cerebral artery smooth muscle cells in the absence of thapsigargin. In six cerebral artery smooth muscle cells, Ca^{2+} spark frequency was not different at 0 min (first laser exposure, 0.76 ± 0.1 Hz) and 15 min (fourth laser exposure, 0.81 ± 0.1 Hz, $P > 0.05$ for each). Similarly, Ca^{2+} spark amplitude (F/F_0; 0 min, 2.21 ± 0.16 $n = 48$; 15 min, 2.15 ± 0.11, $n = 51$) and transient K_{Ca} current amplitude (0 min, 18.8 ± 2.2 pA; 15 min, 17.6 ± 2.2 pA) were unchanged ($P > 0.05$ for each). These data indicate that the repetitive confocal Ca^{2+} imaging protocol did not induce changes in Ca^{2+} sparks and transient K_{Ca} currents. In summary, progressively decreasing $[Ca^{2+}]_{SR}$ reduced Ca^{2+} spark frequency, amplitude and spatial spread, which reduced transient K_{Ca} current frequency and amplitude. However, even when Ca^{2+} spark amplitude and spread were reduced by 47 and 56 % of control, respectively, the coupling ratio or the amplitude correlation between Ca^{2+} sparks and transient K_{Ca} currents was not altered.

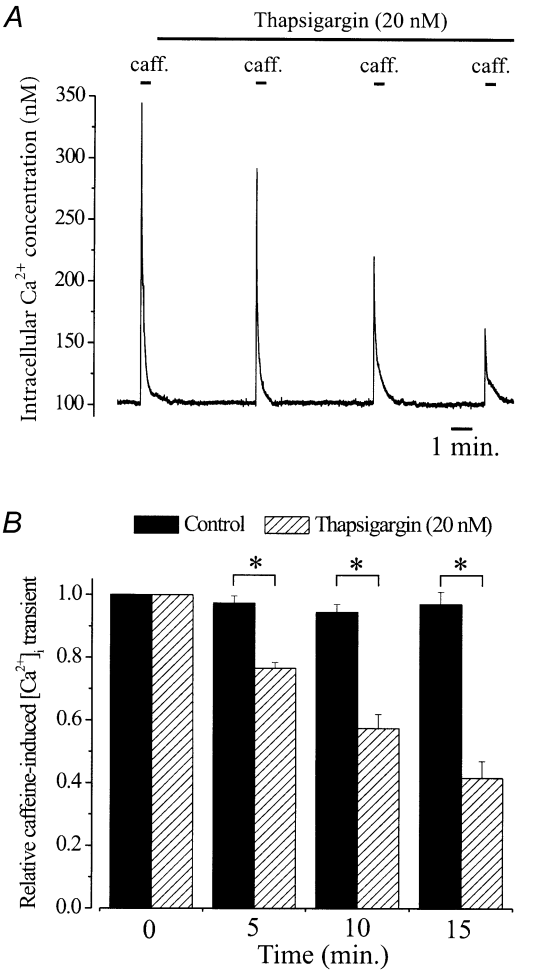

Figure 7. Thapsigargin reduces $[Ca^{2+}]_{SR}$ in cerebral arteries

A, intracellular Ca^{2+} concentration and caffeine (20 mM)-induced $[Ca^{2+}]_i$ transients in control and 5, 10 and 15 min after thapsigargin application (20 nM) in an endothelium-denuded cerebral artery segment. *B*, relative time-dependent change in caffeine-induced $[Ca^{2+}]_i$ transients when compared with control in the absence (black bars) or presence of thapsigargin (20 nM, hatched bars). Mean caffeine-induced $[Ca^{2+}]_i$ transients for each time point were (control *vs.* thapsigargin): 0 min, 240 ± 11 *vs.* 245 ± 14 nM; 5 min, 235 ± 8 *vs.* 184 ± 10 nM; 10 min, 229 ± 8 *vs.* 136 ± 7 nM; 15 min, 234 nM ± 9 *vs.* 98 ± 8 nM. * $P < 0.05$ when compared using unpaired Student's *t* test.

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Table 1. Summary of the effects of thapsigargin on Ca^{2+} spark and transient K_{Ca} currents

	Control	Post thapsigargin (20 nM) time (min) 5	10	15
Ca^{2+} sparks				
Frequency (Hz)	1.0 ± 0.23 (6 cells)	0.43 ± 0.14 (6 cells) *	0.32 ± 0.15 (6 cells) *	0.16 ± 0.07 (6 cells) *
Amplitude (F/F_0)	1.97 ± 0.07 (55)	1.65 ± 0.05 (19) *	1.55 ± 0.03 (16) *	1.46 ± 0.07 (5) *
Decay ($t_{½}$, ms) †	40.9 ± 4.4 (29)	35.6 ± 2.5 (14)	32.6 ± 3.1 (12)	30.6 ± 2.8 (5)
Spread (FWHM, μm)	2.11 ± 0.19 (18)	2.07 ± 0.08 (11)	1.21 ± 0.11 (11) *	1.18 ± 0.21 (11) *
K_{Ca} transients				
Amplitude (pA)	23.7 ± 2.4 (61)	17.0 ± 2.3 (36) *	15.0 ± 1.3 (29) *	12.2 ± 2.6 (11) *
Decay ($t_{½}$, ms) ‡	16.6 ± 0.9 (61)	15.8 ± 0.9 (36)	15.2 ± 0.8 (29)	14.8 ± 1.0 (11)
Rise time (10–90 %, ms)	11.8 ± 0.6 (61)	11.5 ± 0.7 (36)	11.7 ± 0.7 (29)	10.7 ± 1.0 (11)

All Ca^{2+} spark data refer to events associated with transient K_{Ca} currents. Only one Ca^{2+} spark was detected in this series of experiments that did not evoke a transient K_{Ca} current and this spark occurred in control conditions. Transient K_{Ca} current data also includes events for which an associated Ca^{2+} spark was not detected. Values of n shown in parentheses refer to number of sparks or transient K_{Ca} currents used for analysis, unless specified otherwise. † Ca^{2+} spark decays were fit with a second-order exponential. ‡ Transient K_{Ca} current decays were fit with a first-order exponential. $t_{½}$: time for decay to half-maximal amplitude. FWHM: full width at half maximal amplitude. * $P < 0.05$ when compared with control; Student-Newman-Keuls test.

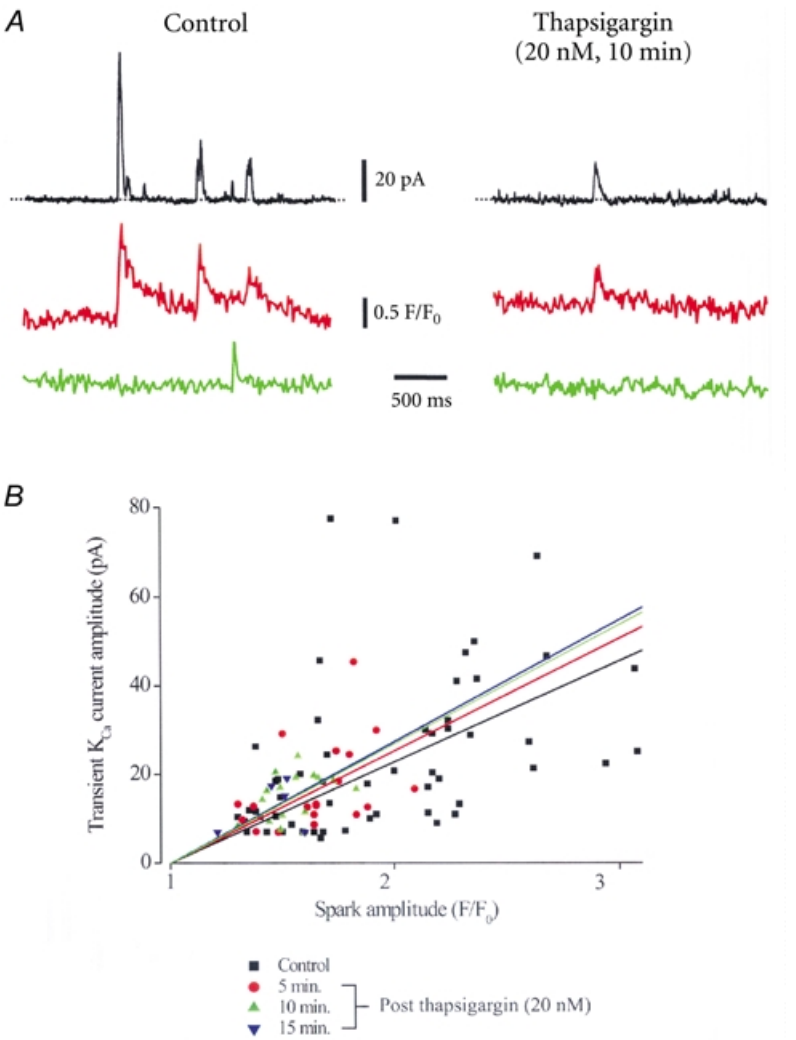

Figure 8. Regulation of Ca^{2+} sparks and transient K_{Ca} currents by thapsigargin

A, original simultaneous recordings of Ca^{2+} sparks (red and green lines) and whole-cell current (black lines) in the same voltage-clamped (−40 mV) cerebral artery smooth muscle cell in control and 10 min after thapsigargin (20 nM) application. *B*, the amplitudes of Ca^{2+} sparks and evoked transient K_{Ca} currents were correlated for control ($P = 0.002$), and data obtained 5 ($P = 0.04$), 10 ($P < 0.0001$), or 15 ($P < 0.0001$) min after thapsigargin. Linear correlation coefficients (r) were: control (black), 0.46; thapsigargin: 5 min (red), 0.41; 10 min (green), 0.38; 15 min (blue), 0.32. Amplitude correlations for each time point after thapsigargin application were not significantly different when compared with control (significance values: 5 min, $P = 0.76$; 10 min, $P = 0.94$; 15 min, $P = 0.83$).

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DISCUSSION

We demonstrate that an acute increase or decrease in $[Ca^{2+}]_{SR}$ regulates Ca^{2+} sparks and evoked transient K_{Ca} currents in cerebral artery smooth muscle cells. An elevation in $[Ca^{2+}]_{SR}$ increased Ca^{2+} spark and transient K_{Ca} current frequency, but did not change Ca^{2+} spark amplitude, spatial spread or decay, or the coupling ratio or amplitude correlation between sparks and transient K_{Ca} currents. Decreasing $[Ca^{2+}]_{SR}$ reduced Ca^{2+} spark frequency, amplitude and spatial spread, and these effects reduced the frequency and amplitude of evoked transient K_{Ca} currents, although the coupling ratio or amplitude correlation between Ca^{2+} sparks and transient K_{Ca} currents was not affected.

An elevation in $[Ca^{2+}]_{SR}$ increases Ca^{2+} spark frequency in arterial smooth muscle cells

$[Ca^{2+}]_{SR}$ was elevated in cerebral artery smooth muscle cells by: (1) selectively increasing the activity of the SR Ca^{2+}-ATPase, or (2) blocking RyR channels. To activate the SR Ca^{2+}-ATPase we used a monoclonal antibody raised against phospholamban that elevates $[Ca^{2+}]_{SR}$ in cardiac myocytes (Suzuki & Wang, 1986; Sham *et al.* 1991; Lukyanenko *et al.* 2001). The anti-phospholamban antibody increased mean transient K_{Ca} current frequency, but did not alter transient K_{Ca} current amplitude. $[Ca^{2+}]_{SR}$ was also elevated with tetracaine, a reversible RyR channel blocker. Local anaesthetics block RyR channels by inducing a long closed state rather than by reducing the single channel conductance or the mean open times of the channel (Zahradnikova & Palade, 1993). Tetracaine was applied in the continued presence of a voltage-dependent Ca^{2+} channel blocker, to prevent any non-specific effects on voltage-dependent Ca^{2+} channels that would also regulate Ca^{2+} sparks (Jaggar *et al.* 1998; Jaggar, 2001). After tetracaine washout when $[Ca^{2+}]_{SR}$ had increased, Ca^{2+} sparks and transient K_{Ca} currents were significantly more frequent than prior to tetracaine application, but Ca^{2+} spark amplitude, decay and spatial spread were not altered, nor were transient K_{Ca} current amplitude or the coupling ratio or amplitude correlation between Ca^{2+} sparks and transient K_{Ca} currents. $[Ca^{2+}]_{SR}$ and transient K_{Ca} current frequency returned to control levels ~5 min after tetracaine washout, presumably due to the elevation in Ca^{2+} release.

Ca^{2+} spark amplitude should depend on the number and activity of contributing RyR channels and the driving force for Ca^{2+} from the SR. However, although Ca^{2+} sparks in smooth muscle cells exhibit a wide range of amplitudes (see Fig. 6, Jaggar *et al.* 2000; ZhuGe *et al.* 2000), data suggest that an acute elevation in $[Ca^{2+}]_{SR}$ did not change mean Ca^{2+} spark amplitude. Conceivably, this could be because resting $[Ca^{2+}]_{SR}$ saturates Ca^{2+} flux through RyR channels. If this is the case, the SR Ca^{2+}-ATPase maintains this saturating $[Ca^{2+}]_{SR}$ at a cytosolic $[Ca^{2+}]_i$ of ~100 nM, and the wide range of Ca^{2+} spark amplitudes that occur at this saturating $[Ca^{2+}]_{SR}$ may be due to different numbers and/or activities of RyR channels that contribute to sparks. The decline in mean Ca^{2+} spark amplitude and spatial spread that occurs when $[Ca^{2+}]_{SR}$ is reduced could also be due to a decrease in the driving force for Ca^{2+} from the SR in addition to a reduction in the number or activity of contributing RyR channels. However, mechanisms that regulate Ca^{2+} spark amplitude may be more complex and may involve multiple processes, particularly since Ca^{2+} activation and inactivation sites may be located on the luminal (Ching *et al.* 2000) and cytosolic side (Herrmann-Frank *et al.* 1991; Xu *et al.* 1994; Herrmann-Frank & Lehmann-Horn, 1996; Tripathy & Meissner, 1996) of RyR channels. In addition, during a Ca^{2+} spark, the localized free Ca^{2+} concentration may partially deplete near the luminal side of RyR channels, and this may also influence Ca^{2+} spark amplitude.

Our finding that an acute increase in SR Ca^{2+} load elevates Ca^{2+} spark frequency but mean Ca^{2+} spark amplitude does not change is consistent with findings in arterial smooth muscle cells of a phospholamban-deficient mouse, wherein SR Ca^{2+} load is chronically elevated (Wellman *et al.* 2001). However, modifications in Ca^{2+} signalling modalities in smooth muscle cells regulate the transcription factor, CREB, the immediate-early gene, c-*fos* (Cartin *et al.* 2000) and the transcription factor, NFAT (Stevenson *et al.* 2001). Conceivably, long-term modifications in Ca^{2+} signalling modalities, particularly during development, could modify the expression levels of Ca^{2+} signalling proteins, such as RyR channels, and this could have led to the observation that a chronic elevation in SR Ca^{2+} load does not change Ca^{2+} spark amplitude. Forskolin, an activator of adenylyl cyclase, also elevates SR load and Ca^{2+} spark frequency in arterial smooth muscle cells, but does not change mean spark amplitude (Porter *et al.* 1998). However, cAMP-dependent protein kinase also activates RyR channels (Porter *et al.* 1998; Wellman *et al.* 2001), which could have masked a change in Ca^{2+} spark amplitude. Our data, which examined the regulation of Ca^{2+} sparks and transient K_{Ca} current by an acute elevation in SR Ca^{2+} load, also suggest that frequency is increased, but amplitude does not change. When comparing our data with that in previous studies, findings suggest that an acute, or chronic, elevation in $[Ca^{2+}]_{SR}$ elevates Ca^{2+} spark frequency, but the amplitude and decay of Ca^{2+} sparks are unchanged.

A decrease in $[Ca^{2+}]_{SR}$ reduces Ca^{2+} spark frequency, amplitude and spatial spread

Thapsigargin induced a progressive reduction in $[Ca^{2+}]_{SR}$ and Ca^{2+} spark frequency, amplitude and spatial spread, and these changes decreased transient K_{Ca} current

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frequency and amplitude. Presumably, transient K_{Ca} current amplitude was reduced because: (1) a lower amplitude Ca^{2+} spark would be a less effective activator of K_{Ca} channels, and (2) a smaller spark spread would envelop a reduced sarcolemmal surface area and impact a lower number of K_{Ca} channels. The thapsigargin-induced decrease in Ca^{2+} spark amplitude (47 % of control) and spatial spread (56 % of control) did not induce uncoupling of Ca^{2+} sparks from K_{Ca} channels. In this study, out of a total of 216 sparks, only one uncoupled Ca^{2+} spark was detected, suggesting a coupling ratio of sparks to transient K_{Ca} currents of 99.5 %, which is similar to previous reports in rat cerebral artery smooth muscle cells (Perez *et al.* 1999). The amplitude (F/F_0) and spatial spread of this uncoupled spark were significantly smaller than the mean value for coupled sparks (Table 1), which may partially explain why it did not evoke a transient K_{Ca} current. Mechanisms that lead to uncoupling are unclear, but may involve a reduced density (ZhuGe *et al.* 2000) or Ca^{2+} sensitivity (Bayguinov *et al.* 2001) of K_{Ca} channels, in addition to the properties of the Ca^{2+} release event. The variability in coupling ratio that occurs in different smooth muscle preparations (ZhuGe *et al.* 2000; Kirber *et al.* 2001; Wellman *et al.* 2002) may reflect species- and tissue-specific diversity in Ca^{2+} signalling between RyR channels and K_{Ca} channels.

In cardiac myocytes, Ca^{2+} sparks decay primarily due to diffusion, but some of the released Ca^{2+} is also sequestered into the SR by the SR Ca^{2+}-ATPase (Gomez *et al.* 1996; Santana *et al.* 1997). In our experiments, partially blocking the SR Ca^{2+}-ATPase reduced spark amplitude and spread, and slightly decreased half-time for decay ($t_{½}$). If the SR Ca^{2+}-ATPase sequestered a portion of the released Ca^{2+}, thapsigargin should have prolonged decay ($t_{½}$), which was not observed. In experiments with tetracaine where $[Ca^{2+}]_{SR}$ was increased, spark spread and decay were unaltered. This finding suggests that decreasing the driving force for SR Ca^{2+} uptake also has no effect on spark decay. Thus, the SR Ca^{2+}-ATPase does not appear to contribute directly to spark decay by sequestering the released Ca^{2+} into the SR. Data suggest that amplitude largely determines the decay of a spark, since spread and decay ($t_{½}$) decreased when amplitude was reduced (Table 1). Possible explanations for a correlation between amplitude and spread/decay may be that spark diffusion is space limited or that sparks saturate cytosolic Ca^{2+} buffers within the immediate vicinity of the release site. Thus, lower amplitude sparks would decay more rapidly and would exhibit lower spread due to a higher ratio of cytosolic buffering molecules to Ca^{2+}.

A decrease in $[Ca^{2+}]_{SR}$ elevates sarcolemmal Ca^{2+} entry in a variety of cell types via activation of store-operated Ca^{2+} channels, which are also referred to as capacitative Ca^{2+} entry channels or Ca^{2+}-release activated Ca^{2+} (CRAC) channels (Parekh & Penner, 1997). Although a store-operated Ca^{2+} entry pathway has been demonstrated in arterial smooth muscle (Xu & Beech, 2001), this observation is not universal (e.g. see Knot *et al.* 1998). In the present study, a decrease or an increase in $[Ca^{2+}]_{SR}$ did not alter cytosolic $[Ca^{2+}]_i$ (Figs 3 and 7), suggesting that store-operated Ca^{2+} entry is not significant in rat cerebral artery smooth muscle cells, at least in the conditions used in our experiments.

Physiological relevance of modifications in $[Ca^{2+}]_{SR}$ in arterial smooth muscle cells

An elevation in $[Ca^{2+}]_{SR}$ that increases Ca^{2+} spark and transient K_{Ca} current frequency should lead to membrane hyperpolarization, a decrease voltage-dependent Ca^{2+} channel activity, a reduction in global $[Ca^{2+}]_i$, and dilation (Jaggar *et al.* 2000). An elevation in Ca^{2+} spark frequency may also increase the driving force for Ca^{2+} for sarcolemma extrusion mechanisms that are located within the vicinity of the release site, such as the Na^+–Ca^{2+} exchanger or the Ca^{2+}-ATPase. A similar mechanism, referred to as the 'superficial buffer barrier hypothesis' (van Breemen *et al.* 1995), suggests that Ca^{2+} entering smooth muscle cells is buffered by the SR and is discharged vectorially towards the sarcolemma without any effect on global $[Ca^{2+}]_i$. Elevating Ca^{2+} release from the SR may also enhance Ca^{2+}-dependent inactivation of sarcolemma voltage-dependent Ca^{2+} channels, an additional mechanism that would further reduce Ca^{2+} entry. Thus, an increase in $[Ca^{2+}]_{SR}$ may be intimately involved in directly regulating a number of negative feedback mechanisms via activation of Ca^{2+} sparks. Conversely, a decrease in $[Ca^{2+}]_{SR}$ would reduce Ca^{2+} spark frequency, amplitude and spatial spread, which would have the opposite effect.

In summary, our data suggest that an elevation in $[Ca^{2+}]_{SR}$ increases Ca^{2+} spark and transient K_{Ca} current frequency in cerebral artery smooth muscle cells, but Ca^{2+} spark amplitude, spatial spread or decay, or the coupling ratio or amplitude correlation between sparks and transient K_{Ca} currents are not altered. A decrease in $[Ca^{2+}]_{SR}$ reduces Ca^{2+} spark frequency, amplitude and spatial spread, and the frequency and amplitude of evoked transient K_{Ca} currents, although the coupling ratio or amplitude correlation between sparks and transient K_{Ca} currents remains unchanged. These findings demonstrate that acute changes in $[Ca^{2+}]_{SR}$ regulate Ca^{2+} sparks and transient K_{Ca} currents in cerebral artery smooth muscle cells.

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