Ca\(^{2+}\) Sparks and Their Function in Human Cerebral Arteries

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Background and Purpose—Local Ca\(^{2+}\) release events (Ca\(^{2+}\) sparks) caused by the opening of ryanodine-sensitive Ca\(^{2+}\) channels in the sarcoplasmic reticulum have been suggested to oppose constriction in cerebral arteries through the activation of large-conductance Ca\(^{2+}\)-activated K\(^+\) (BK) channels. We report the first identification and characterization of Ca\(^{2+}\) sparks and associated BK channel currents in smooth muscle cells isolated from human cerebral arteries.

Methods—Membrane currents and intracellular Ca\(^{2+}\) were measured with the use of the patch-clamp technique and laser scanning confocal microscopy.

Results—Ca\(^{2+}\) sparks with a peak fractional fluorescence change (F/F\(_{0}\)) of 2.02±0.04 and size of 8.2±0.5 μm\(^2\) (n=108) occurred at a frequency of approximately 1 Hz in freshly isolated, cerebral artery myocytes from humans. At a holding potential of −40 mV, the majority of, but not all, Ca\(^{2+}\) sparks (61 of 85 sparks) were associated with transient BK currents. Consistent with a role for Ca\(^{2+}\) sparks in the control of cerebral artery diameter, agents that block Ca\(^{2+}\) sparks (ryanodine) or BK channels (iberiotoxin) were found to contract human cerebral arteries.

Conclusions—This study provides evidence for local Ca\(^{2+}\) signaling in human arterial myocytes and suggests that these events may play an important role in control of cerebral artery diameter in humans. (Stroke. 2002;33:802-808.)

Key Words: calcium ■ cerebral arteries ■ muscle, smooth, vascular ■ potassium channels ■ ryanodine receptor calcium release channel

It is becoming increasingly evident that intracellular calcium ions are involved in the control of a vast array of cellular functions, playing a critical role in the regulation of events ranging from egg fertilization to programmed cell death.\(^1\) Within vascular smooth muscle, Ca\(^{2+}\) has long been known to be a fundamental part of the excitation-contraction process.\(^2\) However, it is now clear that the actions of Ca\(^{2+}\) as a second messenger in muscle are not restricted to the initiation of contraction, nor is the intracellular distribution of this ion homogeneous.

One example of localized variations in intracellular Ca\(^{2+}\) concentrations is the existence of events termed “Ca\(^{2+}\) sparks” that arise from the activation of ryanodine-sensitive Ca\(^{2+}\) release channels located in the sarcoplasmic reticulum (SR) membrane.\(^3,4\) In cerebral arterial muscle from rats and mice, these transient (100 to 200 ms) bursts of released intracellular Ca\(^{2+}\) typically occur in close proximity to the plasma membrane and affect <1% of the total surface area of the cell.\(^4,6\) Given their brief and localized nature, Ca\(^{2+}\) sparks have little impact (<1 nmol/L) on spatially averaged Ca\(^{2+}\) throughout the cell. Rather than directly altering global intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)\(_{\text{c}}\)],), Ca\(^{2+}\) sparks cause the activation of nearby large-conductance Ca\(^{2+}\)-activated K\(^+\) (BK) channels located on the plasma membrane.\(^4,5\) Enhanced BK channel activity causes membrane hyperpolarization and reduced Ca\(^{2+}\) influx by decreasing the open-state probability of dihydropyridine-sensitive L-type calcium channels (voltage-dependent Ca\(^{2+}\) channels).\(^7,8\) Thus, despite a dramatic local increase in Ca\(^{2+}\), Ca\(^{2+}\) sparks through enhanced BK channel activity paradoxically lead to decreased Ca\(^{2+}\) entry, decreased global [Ca\(^{2+}\)\(_{\text{i}}\)], and cerebral artery dilation.

BK channels are known to play a critical role in the regulation of cerebral artery diameter. BK channels have been identified in most, if not all, types of vascular and nonvascular smooth muscle, including human cerebral and coronary arteries.\(^9–13\) Activation of these ion channels has been implicated as an important negative feedback pathway to oppose pressure-induced (myogenic) membrane depolarizations and constrictions.\(^8,14\) BK channels are also targets of a variety of endogenous vasodilators activating cGMP-dependent protein kinase (eg, nitric oxide) or cAMP-dependent protein kinase (eg, adenosine or the neurotransmitter calcitonin-gene–related peptide).\(^6,15–17\) Furthermore, pharmacological activators of BK channels also exist, and these channels may represent a useful therapeutic target in the treatment of a number of pathological conditions, including cerebral artery vasospasm.
associated with subarachnoid hemorrhage. The importance of BK channels in the regulation of cerebral artery diameter is not surprising when one considers the high density and large conductance of this channel, combined with its activation by membrane depolarization and elevations in cytosolic Ca\(^{2+}\). Recent evidence suggests that the majority of Ca\(^{2+}\)-dependent BK activity in cerebral vascular smooth muscle may occur in response to local increases in Ca\(^{2+}\) (i.e., Ca\(^{2+}\) sparks) rather than in response to cellwide or global Ca\(^{2+}\) changes.

The goal of this present study was to explore whether Ca\(^{2+}\) sparks occur in human cerebral arterial muscle and, if so, to determine whether these intracellular Ca\(^{2+}\) release events contribute to the activation of plasmalemmal BK channels. This study provides evidence for local Ca\(^{2+}\) signaling in human arterial myocytes and suggests that these events, through the activation of BK channels, may play an important role in the control of cerebral artery diameter in humans.

**Subjects and Methods**

**Tissue Preparation**

Human cerebral arteries were obtained from consenting surgical patients. This study used arteries removed as a necessary part of a required surgical procedure, with the majority of specimens removed in conjunction with a planned surgical resection in individuals with either primary (and often recurrent) brain tumors or epilepsy. The University of Vermont has an approved assurance of compliance on file with the Department of Health and Human Services covering this activity (assurance identification number: M1375; institutional review board identification number: 01XB). The age range was 29 to 83 years, with a mean age of 49 years; tissue was obtained from a total of 21 individuals (6 women and 15 men). The health status of individuals from which tissue was obtained varied widely; however, in general, operative patients must meet a minimal level of systemic health. Cerebral arteries were dissected in cold, oxygenated physiological salt solution (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, glucose 11 (pH 7.4).

**Enzymatic Dissociation of Vascular Myocytes From Cerebral Artery Segments**

Smooth muscle cells from cerebral arteries were enzymatically isolated in a manner similar to that described previously. Briefly, cerebral artery segments 2 to 3 mm in length and 0.5 to 2.0 mm in diameter were opened longitudinally and placed in an enzyme solution containing 0.3 mg/mL papain for 22 minutes at 37°C. Arteries were then transferred to enzyme solution containing 100 μmol/L CaCl\(_2\), collagenase F (0.7 mg/mL), and collagenase H (0.3 mg/mL) for a period of 11 minutes at 37°C. The enzyme solution contained the following (in mmol/L): NaCl 55, sodium glutamate 80, KCl 5.6, HEPES 10, MgCl\(_2\) 1, 10 HEPES, EGTA 0.05 (pH 7.2). Amphotericin B was dissolved in dimethyl sulfoxide and diluted into the pipette solution to give a final concentration of 200 μg/mL. Experiments were performed at room temperature with the use of an extracellular solution containing HEPES-PSS (see above). Membrane currents were measured with an Axopatch 200A amplifier (Axon Instruments Inc) and recorded at a membrane potential of −40 mV. Currents were filtered at 500 Hz and digitized at 5 kHz. The threshold for detecting transient BK currents was 2.5 times the single BK channel current (i.e., 5 pA at −40 mV). Isometric Force Measurements

Two stainless steel wires (35 gauge) were placed through the lumen of intact artery segments 3 mm in length. One wire was attached to a fixed support, while the other was attached to a Grass (model FT 03) isometric force transducer. Arteries were allowed to equilibrate for 30 minutes in 50 mL water-jacketed tissue baths containing HEPES-PSS.
gassed with 95% O₂/5% CO₂ and heated to 37°C. A resting tension of 100 mg was then applied to each artery segment, placing them at an optimal position on their length-tension curve (determined in preliminary studies). Isometric force measurements were acquired onto a PC with the use of a waveform recording system (DI-700 with WinDaq waveform recording software, Dataq Instruments Inc).

**Statistical Analysis**

Data are presented as mean±SEM. Correlation coefficients (r) for Ca²⁺ spark data were determined by linear regression fit. Isometric force contractions are expressed (mean±SEM) as a percentage of the maximum force generation. Maximal force generation in the presence of a combination of 120 mmol/L extracellular K⁺, histamine (10⁻⁵ mol/L), and serotonin (10⁻⁵ mol/L) was determined in each artery. Isometric force measurements were analyzed by 1-way ANOVA followed by Student-Newman-Keuls multiple comparison test. Statistical significance was considered at the level of P<0.05 or P<0.01.

**Results**

**Identification of Ca²⁺ Sparks in Human Cerebral Artery Myocytes**

To explore whether discrete SR Ca²⁺ release events (ie, Ca²⁺ sparks) exist in human cerebral artery myocytes, changes in fluorescence intensity were monitored by laser scanning confocal microscopy in freshly isolated cells loaded with the Ca²⁺ indicator dye fluo-3. Ca²⁺ sparks were observed in isolated human cerebral artery myocytes at a frequency of 0.8±0.1 Hz (n=10 cells), with an average of 2.3±0.3 distinct spark sites in these cells. These events were asynchronous and represented localized and transient increases in intracellular Ca²⁺, which typically lasted for a period of 100 to 200 ms. A typical Ca²⁺ spark observed in a human cerebral artery myocyte is illustrated in Figure 1. The majority of Ca²⁺ sparks were found to occur in regions of the cell near the plasma membrane. The amplitude of these events, as measured by changes in local fractional fluorescence (F/F₀; see Subjects and Methods for details), was 2.02±0.04 (n=108 sparks from 10 cells). Spark size (defined as the area measured at 50% peak spark amplitude) was 8.2±0.5 μm² (n=108 sparks from 10 cells), representing <1% of the estimated total surface area of these cells (approximately 1500 μm²) as estimated from cell membrane capacitance (15.6±1.0 pF; n=10). Consistent with Ca²⁺ sparks occurring through the activation of intracellular ryanodine-sensitive Ca²⁺ release channels (ryanodine receptors), these events were abolished by a 30-minute exposure of cells to ryanodine (10 μmol/L; n=3) (Figure 2).

**Simultaneous Measurements of Ca²⁺ Sparks and Large-Conductance Ca²⁺-Activated K⁺ Channel Currents**

In other species, nearly every Ca²⁺ spark occurring in cerebral artery myocytes causes the transient activation of nearby BK channels located on the plasma membrane. To explore whether Ca²⁺ sparks also activate BK channels in human cerebral artery smooth muscle, patch-clamp electrophysiology was combined with laser scanning confocal microscopy to simultaneously measure Ca²⁺ sparks and transient outward K⁺ currents attributable to BK channel activation. In myocytes voltage clamped to a physiological membrane potential.
of −40 mV, Ca2+ sparks were observed at a frequency of 0.9±0.2 Hz (n=6 cells from 3 patients). Under these conditions (ie, −40 mV), Ca2+ sparks had a rise time of 28.8±2.1 ms (n=60 sparks), half-width (defined as the time from when a Ca2+ spark reaches 50% of peak until it reaches the midpoint of decay) of 64.3±4.5 ms (n=60), and a half-time of decay of 53.1±3.9 ms (n=60). Although the majority of Ca2+ sparks were associated with BK channel currents, a substantial number of these intracellular Ca2+ release events did not cause transient outward currents. In fact, as illustrated in Figure 3, only approximately 72% (61 of 85 sparks) were associated with BK currents recorded at a holding potential of −40 mV. Blue and red tracings represent F/F0 measurements obtained from 2.2×2.2-μm areas centered on distinct Ca2+ spark sites (sampling frequency of 120 Hz).

Figure 3. Simultaneous measurements of Ca2+ sparks and BK currents in human cerebral artery myocytes. Time courses of whole cell current and F/F0 changes recorded from 2 different human cerebral artery myocytes (A and B) are shown. Black tracings represent membrane K+ currents recorded at a holding potential of −40 mV. Blue and red tracings represent F/F0 measurements obtained from 2.2×2.2-μm areas centered on distinct Ca2+ spark sites (sampling frequency of 120 Hz).

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Blockers of BK Channels and Ryanodine Receptors Contract Human Cerebral Artery Segments

Isometric force measurements were next used to explore the impact of Ca2+ sparks and BK channel activation on the contractility of intact human cerebral arteries. Since cerebral arteries are maintained in a partially constricted state in vivo, initial experiments were performed on cerebral artery segments bathed in a PSS containing 25 mmol/L K+. Increasing the extracellular K+ concentration from 6 to 25 mmol/L contracted human cerebral artery segments to 52.4±9.5% of their maximum level of force development, and the subsequent addition of iberiotoxin (100 nmol/L; a selective inhibitor of BK channels8,21) caused a further contraction to 80.3±6.4% of tissue maximum (P<0.05 versus 25 mmol/L K+; n=6) (Figure 5A and 5B). In the presence of 25 mmol/L K+ and iberiotoxin, ryanodine (10 μmol/L; a selective inhibitor of ryanodine receptors) did not significantly affect isometric force generation in this preparation (P=0.77; n=6) (Figure 5A and 5B). Consistent with the involvement of membrane depolarization and enhanced Ca2+ influx via voltage-dependent Ca2+ channels, diltiazem (50 μmol/L) reversed contractions in the combined presence of 25 mmol/L extracellular K+, iberiotoxin, and ryanodine (P<0.01; Figure 5A and 5B).

To examine the impact of ryanodine receptor activity (ie, Ca2+ sparks) on human cerebral arteries with functional BK channels, arterial segments were exposed to ryanodine in the absence of iberiotoxin. In 25 mmol/L extracellular K+, ryanodine (10 μmol/L) contracted human cerebral artery segments from 48.7±5.6% to 75.6±7.0% of tissue maximum (P<0.01; n=6) (Figure 5C). In the presence of 25 mmol/L K+ and ryanodine, addition of iberiotoxin was without effect on these partially contracted human cerebral arteries (P=0.78; n=6) (Figure 5C). Diltiazem again reversed contractions in the combined presence of 25 mmol/L extracellular K+, ryanodine, and iberiotoxin. To better understand the impact of voltage-dependent Ca2+ channel blockade on ryanodine-induced contractions, cerebral arteries were also examined in an extracellular solution (PSS) containing 6 mmol/L K+ rather than 25 mmol/L K+. Unlike arteries bathed in 25 mmol/L K+ PSS, arteries in 6 mmol/L K+ PSS were not partially contracted before the addition of ryanodine.

Figure 4. Relationship between Ca2+ sparks and transient BK currents in human cerebral artery myocytes. A, Scatterplot of Ca2+ spark amplitude vs peak transient BK current amplitude at −40 mV. B, Scatterplot of Ca2+ spark size vs transient BK current amplitude at −40 mV.
Discussion

In this study we report the first visualization of subcellular Ca2+ release events (Ca2+ sparks) in human cerebral artery myocytes. Simultaneous measurements of Ca2+ sparks and membrane currents suggest that the majority of these SR Ca2+ release events cause the activation of BK channels located on the plasma membrane of these myocytes. While blockers of BK channels (iberiotoxin) and Ca2+ sparks (ryanodine) both contract human cerebral arteries, the effects of these agents are not additive. We propose that in the human cerebral vasculature, Ca2+ sparks via the activation of plasmalemmal BK channels promote membrane hyperpolarization and cerebral artery relaxation (Figure 6).

BK channels are thought to play an important role in the control of cerebral artery diameter in a number of species.5,7 Activation of BK channels by Ca2+ sparks causes membrane hyperpolarization and thus a decrease in the open-state probability of voltage-dependent L-type Ca2+ channels to promote vasodilation. In this manner, BK channel activation is thought to act as an important negative feedback system in response to physiological vasoconstrictor stimuli, such as pressure-induced contractions (often referred to as myogenic tone).14,22,23 Although numerous in vivo and in vitro studies have documented the role of BK channels in the regulation of cerebral artery diameter in a number of species,7,8 relatively little is known with respect to the human vasculature. The use of pharmacological inhibitors of BK channels has suggested a role for this ion channel in the regulation of membrane potential and force development in human cerebral artery segments.10,24 Results of the present study suggest that Ca2+ sparks are the underlying subcellular events leading to BK channel activation in this tissue.

It has been estimated that during a Ca2+ spark, intracellular Ca2+ transiently rises from a basal level of 100 nmol/L to concentrations as high as 10 to 100 μmol/L in localized regions (approximately 10 μm2 in area) near the surface of the plasma membrane.5,20 Given the relatively low affinity of BK channels for Ca2+ at physiological membrane potentials (eg, −40 mV),20,25 Ca2+ sparks provide sufficient Ca2+ to activate BK channels to influence membrane potential and thereby voltage-dependent Ca2+ channels. As in other types of vascular and nonvascular smooth muscle, Ca2+ sparks in human cerebral artery myocytes appear to be the result of the activation of ryanodine receptors located on the SR. Ryanodine (10 μmol/L), a selective inhibitor of ryanodine receptors, completely abolished Ca2+ sparks in human cerebral artery myocytes (Figure 2). The spatiotemporal characteristics (ie, size, amplitude, and decay) of Ca2+ sparks in cells freshly isolated from human cerebral arteries are similar to measurements obtained from smooth muscle of other species.3,15,25,26 However, we found a lower percentage of Ca2+ sparks leading to transient BK channel activity in human cerebral artery myocytes compared with previous studies in cerebral arteries from rodents.5,25 At a physiological membrane potential of −40 mV, nearly 100% of Ca2+ sparks observed in rat and mouse cerebral artery myocytes caused transient BK channel currents, while we report that only 72% of Ca2+ sparks in human cerebral artery myocytes were similarly associated with BK channel activity (Figure 3). The percentage of Ca2+ sparks not associated with BK currents that we have observed in human cerebral artery myocytes is similar to that noted in nonvascular smooth muscle isolated from Bufo marinus stomach muscularis27 and feline esophagus.28 In the present study we detected several Ca2+ spark sites where not every Ca2+ spark caused BK channel activation (for example, see Figure 3B).

We also found a poor correlation between Ca2+ spark amplitude and BK current amplitude in human cerebral artery myocytes compared with previous studies in cerebral arteries from rat5 and mouse25 and guinea pig urinary bladder.26

Ryanodine contracted these arteries by an average of 27.7±3.1% (n=5) of tissue maximum, and the subsequent addition of iberiotoxin did not significantly alter this level of contraction (36.9±4.7% of tissue maximum in the combined presence of ryanodine and iberiotoxin; n=5). As with arteries partially contracted with 25 mmol/L K+, diltiazem (50 μmol/L) completely reversed contractions to ryanodine in arteries bathed in 6 mmol/L K+ PSS.

Figure 5. Inhibitors of Ca2+ sparks and BK channels contract human cerebral arteries. A, Isometric force recording from a human cerebral artery segment. Solid bars represent the addition of various agents to the PSS bathing the artery. B, Summary of isometric force recordings of cerebral artery segments. Conditions were similar to that described in B, with the exception that the order of ryanodine addition was reversed. *P<0.05, **P<0.01 (NS, P>0.05).
However, a weak correlation between BK current amplitude and Ca$^{2+}$ spark amplitude has also been observed in cells isolated from feline esophagus$^{28}$ and toad stomach. $^{27}$ Several possibilities could explain the lack of correlation that we have observed between the amplitudes of these events. For example, BK channels in the spark microdomain could be saturated with respect to Ca$^{2+}$ (ie, the open probability for BK channels is approaching one) with even the lowest-amplitude Ca$^{2+}$ sparks. Other differences, including variations in BK channel density, differences in the Ca$^{2+}$ and/or voltage dependence of BK channel activation, as well as modest differences in dye distribution, could also influence the correlation between these events. We did, however, observe a significant correlation between the size of a Ca$^{2+}$ spark and BK current amplitude (Figure 4B). This observation would be consistent with spark size being proportional to the total amount of Ca$^{2+}$ released during a Ca$^{2+}$ spark. A larger spark size would be expected to encompass a greater plasma membrane surface area with sufficient levels of Ca$^{2+}$ to cause the activation of additional BK channels. Regardless of these somewhat subtle apparent differences with respect to Ca$^{2+}$ spark properties in human cerebral artery myocytes, our present data clearly suggests that ryanodine-sensitive Ca$^{2+}$ release channel activity in the form of Ca$^{2+}$ sparks can cause significant BK channel activity. Furthermore, inhibition of Ca$^{2+}$ sparks or BK channel activity causes contraction of human cerebral artery segments, suggesting a functional vasodilator role for this pathway. The demonstration of Ca$^{2+}$ sparks and associated BK currents in isolated arterial myocytes clearly illustrates the genesis of these events in vascular smooth muscle. While the role of the vascular endothelium was not examined in the functional experiments included in this study, a modulatory role of the endothelium is certainly possible. Although Ca$^{2+}$ sparks have not been reported in endothelial cells, direct effects of iberiotoxin and ryanodine on the endothelium cannot be ruled out in the isometric force measurements performed in this study.

A number of cellular events can lead to an increase in Ca$^{2+}$ spark frequency, including increases in the level of cytoplasmic Ca$^{2+}$. The effect of increased cytosolic Ca$^{2+}$ may be direct, through an increase in ryanodine-sensitive Ca$^{2+}$ release channel activity, $^{3}$ or indirect, through increased Ca$^{2+}$ uptake into the SR, which also increases ryanodine-sensitive Ca$^{2+}$ release channel activity. $^{29}$ In this fashion, the Ca$^{2+}$ spark to BK channel pathway may act as a negative feedback system to limit vasoconstrictions in response to stimuli such as elevated intravascular pressure. Vasodilators acting through either cAMP-dependent protein kinase (protein kinase A) or cGMP-dependent protein kinase also increase Ca$^{2+}$ spark frequency and BK channel activity to promote membrane hyperpolarization. $^{6,15}$ Given the large number of both endothelial-dependent and -independent stimuli that can influence Ca$^{2+}$ spark and BK current frequency, this pathway is likely to play an important role in the regulation of cerebral blood flow in humans.

In conclusion, we provide the first demonstration of Ca$^{2+}$ sparks and their associated BK channel currents in human cerebral artery myocytes. Furthermore, we provide support that this pathway plays a role in the regulation of human cerebral artery contraction.

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