Evoked and Spontaneous Purinergic Junctional Ca^{2+} Transients (jCaTs) in Rat Small Arteries

Christine Lamont, W. Gil Wier

Confocal microscopy of fluo-4 fluorescence in pressurized rat mesenteric small arteries subjected to low-frequency electrical field stimulation revealed Ca^{2+} transients in perivascular nerves and novel, spatially localized Ca^{2+} transients in adjacent smooth muscle cells. These muscle Ca^{2+} transients occur with a very brief latency to the stimulus pulse (most <3 ms). They are wider (≈5 μm) and last longer ($t_{1/2}$, 145 ms) than Ca^{2+} sparks. They are abolished by the purinergic receptor (P2X) antagonist suramin, but they are totally unaffected by the α_1-adrenoceptor antagonist prazosin or by capsaicin (which inhibits the function of perivascular sensory nerves). We conclude that these novel Ca^{2+} transients represent Ca^{2+} entering smooth muscle cells through P2X receptors activated by ATP released from sympathetic nerves, and we therefore call them "junctional Ca^{2+} transients" or jCaTs. As expected from spontaneous neurotransmitter release, jCaTs also occur spontaneously, with characteristics identical to evoked jCaTs. Visualization of sympathetic neurotransmission shows that purinergic components dominate at low frequencies of sympathetic nerve fiber activation.

Sympathetic perivascular nerves of small arteries release the triad of sympathetic cotransmitters, ATP, norepinephrine (NE), and neuropeptide Y (NPY).[1] On the postjunctional membrane, ATP activates (ionotropic) purinergic receptors (P2X) to allow influx of Na^+ and Ca^{2+},[2] and NE activates (metabotropic) α_1-adrenoceptors to initiate phosphoinositide and other G protein–coupled signaling cascades[3] that result in Ca^{2+} waves.[4,5] Surprisingly, recent studies indicate a predominant role for purinergic mechanisms (rather than adrenergic) in activating neurogenic contractions of small, third- to sixth-order arteries[6]; the contractions of isolated rat mesenteric small arteries subjected to electrical field stimulation (EFS; 10 Hz, 1 second) are reduced to 65% of control values by the P2X receptor antagonist suramin. For the first time, we report novel Ca^{2+} signals, generated by neurogenic activation of purinergic receptors in the smooth muscle cells of intact, pressurized small arteries.

Materials and Methods

All experiments were carried out according to the guidelines of the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine. Male Sprague-Dawley rats, weighing 150 to 250 grams (Harlan, Indianapolis, Ind), were anesthetized with intramuscular ketamine (50 to 100 mg/kg) and killed by cervical dislocation. Mesenteric small arteries were dissected by methods described in detail previously.[5] Segments of third- or fourth-order arteries, 1 to 2 mm in length, were transferred to a recording chamber where their ends were mounted on glass pipettes (tip diameter 60 to 100 μm) and secured by 10-0 sutures. One pipette was attached to a servo-controlled pressure-regulating device (Living Systems), while the other was attached to a closed stopcock, and the intraluminal pressure was set to 40 mm Hg. The vessel was then loaded for 3 hours with calcium indicator in dissection solution[5] containing fluo-4/AM at 5 μmol/L, DMSO (vol/vol) 1.5%, and Cremaphor EL (vol/vol) 0.03%. Arteries were then equilibrated for 1 hour to experimental conditions (room temperature, 70 mm Hg). Physiological saline solution was composed of gassed Krebs solution containing (contents in mmol/L) NaCl 112, $NaHCO_3$ 25.7, KCl 4.9, $CaCl_2$ 2, $MgSO_4$ 1.2, $KHPO_4$ 1.2, glucose 11.5, and HEPES 10 (pH 7.4). EFS was performed via two platinum wires placed in the bath parallel to the long axis of the artery and connected to a stimulator (Astro-Med, Inc, model S48). Typical parameters of EFS were 0.67 Hz, 0.1 to 0.5 ms, and 50 V. The pulse duration was decreased until no vasoconstriction occurred (ie, subthreshold). Ryanodine, prazosin, and suramin were prepared in stock solutions and diluted in the superfusate reservoir. Cremaphor EL, capsaicin, and suramin were obtained from Sigma Chemical Co; fluo-4/AM was purchased from Molecular Probes; and ryanodine was obtained from Calbiochem Corp. For high spatial resolution in one dimension and high temporal resolution (3 ms), we used a custom-built confocal laser-scanning microscope[7,8] in line-scan mode. For two-dimensional confocal imaging, we used a real-time confocal imaging system (Solamere Technology Group) consisting of a Yokogawa confocal scanner (model CSU10) and an intensified CCD camera (model XR/Mega-10). Both confocal imaging systems had Nikon inverted microscopes equipped with water objective lenses (×60; numerical aperture 1.2).

Results

The subthreshold electrical stimulation excited perivascular nerve fibers (Figure 1A), as evidenced by rapid increases in their fluorescence in confocal line-scan images (Figure 1B) but not vasoconstriction, as evidenced by the lack of any motion. Nerve fibers were often brighter than muscle cells (perhaps because their greater surface-to-volume ratio permits greater influx of fluo-4/AM). Therefore, in line-scan images, nerve fibers appear as bright streaks (see top of Figure 1B). Because the increases in neuronal fluorescence were spatially confined (did not spread laterally with time) and always occurred coincidentally with the EFS pulse (arrowheads), such nerve fiber Ca^{2+} transients characteristically appeared in line-scan images as transiently increased fluorescence within a narrow horizontal streak. At 4.83 seconds, a Ca^{2+} transient occurred in the lower region of one of the smooth muscle cells, close to where a small nerve fiber can be seen to cross the cell (white arrow). This Ca^{2+} transient, which occurs coincidentally with the stimulus pulse, is seen clearly in the line-scan image (Figure 1B) and in a video clip that is available as an online data supplement (see http://www.circresaha.org).

To study objectively the neurogenic muscle Ca^{2+} transients, to which we now refer, putatively, as "junctional Ca^{2+} transients" or jCaTs, we used randomized line scanning[9] with the custom microscope. This instrument provided higher temporal resolution (in line-scan mode), higher magnification, and higher spatial resolution (in the z-axis or depth axis)

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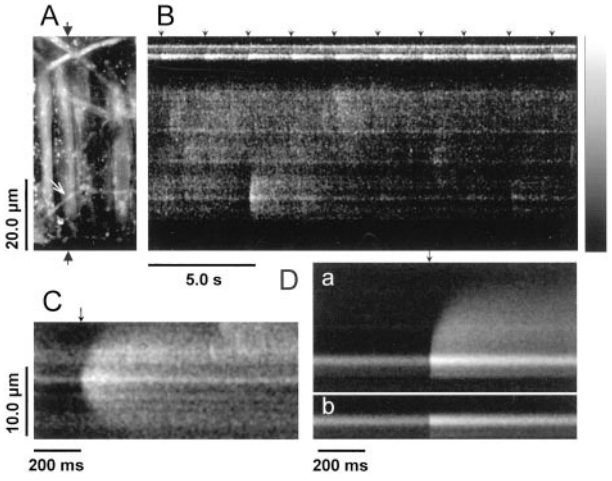

Figure 1. A, Images of perivascular nerves and smooth muscle cells (SMCs) in a pressurized rat mesenteric small artery. The image is an average of 30 frames (1 second total), from a set of 600 images obtained with the real-time confocal microscope during EFS. B, Virtual line-scan image was derived from these data, by taking a single column of pixels (extending through the image vertically, between the two black arrowheads) from each of the 600 frames and stacking them horizontally to produce the line-scan image shown. The chosen line crosses prominent nerve fibers at the top of the image, bisects a smooth muscle cell longitudinally, and crosses a region (white arrow) in which a small nerve fiber crosses the muscle cell. In the line-scan image, nerve fiber Ca^{2+} transients in the large fibers at the top are seen as periodic (0.5 s^{-1}) increases in fluorescence occurring at the time of the stimulus pulses (black arrowheads). A putative jCaT is seen in the SMC at 4.8 seconds in the line-scan image. C, jCaTs often arise at streaks in line-scan images. D, Region where a jCaT occurred was scanned repeatedly. In (a), a jCaT occurred. In (b), no jCaT occurred, but a fluorescence transient occurred in the streak. We identify such transients as nerve fiber Ca^{2+} transients. These images demonstrate that jCaTs can arise precisely in the region of an electrically excitable nerve fiber.

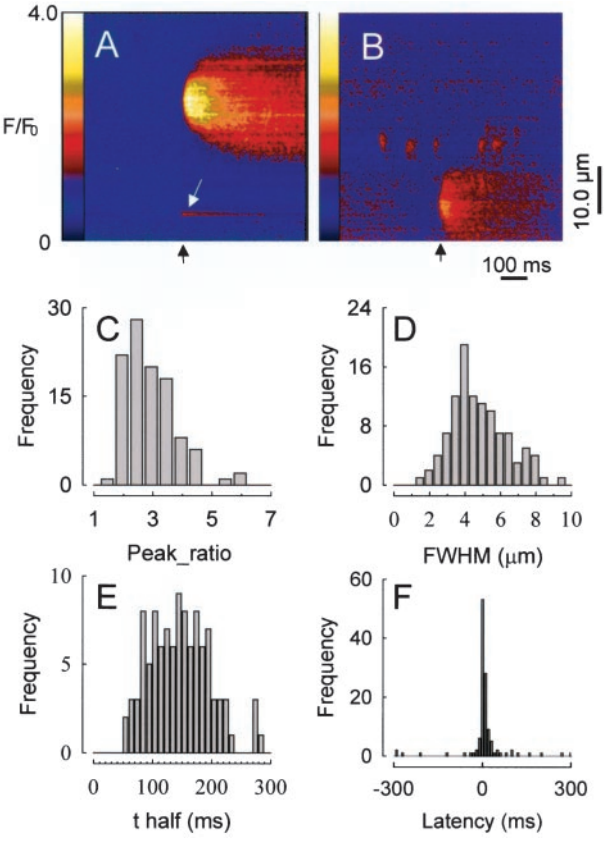

Figure 2. Junctional Ca^{2+} transients in line-scan images, expressed as fluorescence pseudo-ratios (F/F_0). A, Black arrow indicates time of stimulus pulse. White arrow indicates (unrelated) nerve fiber Ca^{2+} transient. B, jCaT with spontaneous Ca^{2+} sparks. C through E, Probability density histograms of peak F/F_0, FWHM, and $t_{1/2}$ for all jCaTs (F). Probability density histogram of latencies of all jCaTs, showing a few spontaneous jCaTs.

than the real-time instrument. An evoked jCaT was considered to be any spatially spreading local Ca^{2+} transient that began within 24 ms of the stimulus pulse and had a $t_{1/2}$ of decline >50 ms. A representative line-scan image is shown in Figure 1C. The jCaT seemed to originate at a streak. Direct evidence that such streaks can be excitable nerve fibers, perhaps sympathetic varicosities, is shown in the images in Figure 1D, a and b. In image (a), the stimulus pulse did elicit a jCaT, and it originated at a streak. Repeated scanning of the same line, however, elicited only confined increases in fluorescence in the streak with every pulse, typical of that resulting from excitation of nerve fibers (Figure 1B). Although protocols of the type used in Figure 1D could be used in the future to define the (low) probability of sympathetic neuromuscular transmission at defined varicosities, the present study was carried out with randomized line scans. In such images, the probability of observing a jCaT was extremely low, 0.031±0.0029 (mean±SEM; 7 arteries, 3 runs of 100 line-scan images per artery, one stimulus pulse per line-scan image, 50 μm of cell length scanned). To characterize jCaTs quantitatively, fluorescence pseudo-ratio images (F/F_0) were constructed (Figures 2A and 2B, see spontaneous Ca^{2+} sparks in panel B, for comparison to jCaT), and the spatial and temporal parameters of 144 jCaTs from 8 arteries (control conditions and in presence of capsaicin, see below) were measured. Probability density histograms of peak F/F_0, FWHM, and $t_{1/2}$ for such Ca^{2+} transients are shown in Figures 2C through 2E). The distribution of peak F/F_0 is, as might be expected, not parametric or normal, because the measurement of peak F/F_0 would be expected to be subject to the same confocal sampling issues as Ca^{2+} sparks.[10] The putative jCaTs are much wider (mean FWHM, 4.68 μm) and longer lasting (mean $t_{1/2}$, 145 ms) than Ca^{2+} sparks measured by others in smooth muscle (FWHM, 2.4 μm; $t_{1/2}$, 48 to 56 ms; values from Table 3 of Jaggar et al[11]) or by us, in the present experiments (FWHM, 2.3 μm; $t_{1/2}$, 24 ms; 92 sparks, 5 arteries). Because the putative jCaTs are readily distinguishable from spontaneous Ca^{2+} sparks, we also sought to discover spontaneous jCaTs, which would be expected from the spontaneous neurotransmitter release that generates spontaneous excitatory junction potentials. A probability density histogram of the latencies from the stimulus pulse of all local Ca^{2+} transients having a $t_{1/2}$ >50 ms (2 times the mean $t_{1/2}$ of sparks) showed that, indeed, a few spontaneous Ca^{2+} transients did occur before the stimulus pulse or long after it (Figure 2F). Their characteristics were indistinguishable from those of evoked jCaTs (Table).

To determine the postjunctional receptors that might be involved in jCaTs, we used selective purinergic and adrener-

Characteristics of jCaTs

Condition	jCaTs, No.	Arteries, No.	Peak Ratio		Half-Time, ms		FWHM, μm	
			Mean	Median	Mean	Median	Mean	Median
Control	70	4	2.801	2.585	145.0	148.0	4.680	4.875
Capsaicin	74	4	2.712	2.594	128.5	124.5	4.330	4.000
Prazosin	31	3	3.181	3.230	166.9	169.0	5.020	4.650
Ryanodine	27	2	2.427*	2.220	129.6	127.5	4.681	4.600
Suramin	...	2	...	...	...	...	...	...
Spontaneous	33	7	2.758	2.710	127.4	130.5	4.330	4.700

FWHM indicates full width at half-maximum.
*$P<0.002$; Mann-Whitney rank sum test.

gic receptor blockers (Table). Evoked jCaTs were unaffected by the α_1-adrenoceptor antagonist prazosin at a concentration (10.0 μmol/L) sufficient to block completely the contraction elicited by the exogenous α_1-adrenoceptor agonist PE (3 arteries). They were also completely unaffected by capsaicin (1.0 μmol/L), indicating that they do not arise from sensory perivascular nerves.[12] Suramin, the potent P2X receptor antagonist[13] (300 μmol/L), blocked the jCaTs completely. In 2 arteries and 600 images, the probability of a jCaT occurring in a particular random line-scan image before suramin was 0.037, zero in suramin (none were observed), and recovered to 0.025 on removal of suramin. Although coreleased NPY may be involved in some way in the jCaTs, it does not cause smooth muscle Ca^{2+} to rise directly, as no increases were observed in the presence of suramin. JCaTs were largely unaffected by ryanodine treatment, although a small decrease in mean peak amplitude occurred. From the very small effect of ryanodine on the peak of the jCaT, we conclude that, if present at all, Ca^{2+} release from internal stores during jCaTs is small, both through ryanodine and through inositol (1,4,5) tris-phosphate receptors (as might occur consequent to activation of P2Y2 purinergic receptors).

Discussion

The data indicate that the local Ca^{2+} transients we observed are due to activation of P2X receptors by neurotransmitter (ATP) released from sympathetic perivascular nerves. It seems likely that jCaTs represent the Ca^{2+} that enters the cell via the P2X1 receptors that are known to carry the current that generates excitatory junction potentials. From the fact that spontaneous jCaTs are the same as evoked jCaTs, we postulate that jCaTs arise from one or a few quanta of sympathetic neurotransmitter. JCaTs are distinct from Ca^{2+} transients activated in isolated venous myocytes by exogenously applied ATP.[14] In the venous myocytes, ATP produced either a rather uniform increase in $[Ca^{2+}]_i$ at low ATP concentrations or propagating Ca^{2+} waves at higher ATP concentrations.[14] In our preparation, jCaTs were never observed to trigger Ca^{2+} waves. The lack of effect of prazosin may be attributed to the necessity for NE to accumulate at sympathetic varicosities before there is significant metabotropic activation. The rarity of the events probably reflects the extremely low probability of neurotransmitter release at individual sympathetic varicosities, as has been determined from electrophysiological studies.[15] Similarly, the variability of the jCaTs may be due to variability in the size of receptor patches and/or the size of neurotransmitter quanta.[15] Recently, similarly appearing Ca^{2+} transients have been recorded in a distinctly different (nonvascular) type of smooth muscle, vas deferens, and have been termed "neuroeffector Ca^{2+} transients" or NCTs.[16]

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