Adrenergic stimulation of rat resistance arteries affects Ca\textsuperscript{2+} sparks, Ca\textsuperscript{2+} waves, and Ca\textsuperscript{2+} oscillations

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Received 17 October 2000; accepted in final form 30 January 2001

Mauban, Joseph R. H., Christine Lamont, C. William Balke, and W. Gil Wier. Adrenergic stimulation of rat resistance arteries affects Ca\textsuperscript{2+} sparks, Ca\textsuperscript{2+} waves, and Ca\textsuperscript{2+} oscillations. Am J Physiol Heart Circ Physiol 280: H2399–H2405, 2001.—Confocal laser scanning microscopy and fluo 4 were used to visualize local and whole cell Ca\textsuperscript{2+} transients within individual smooth muscle cells (SMC) of intact, pressurized rat mesenteric small arteries during activation of \(\alpha_1\)-adrenoceptors. A method was developed to record the Ca\textsuperscript{2+} transients within individual SMC during the changes in arterial diameter. Three distinct types of “Ca\textsuperscript{2+} signals” were influenced by adrenergic activation (agonist: phenylephrine). First, asynchronous Ca\textsuperscript{2+} transients were elicited by low levels of adrenergic stimulation. These propagated from a point of origin and then filled the cell. Second, synchronous, spatially uniform Ca\textsuperscript{2+} transients, not reported previously, occurred at higher levels of adrenergic stimulation and continued for long periods during oscillatory vasomotion. Finally, Ca\textsuperscript{2+} sparks slowly decreased in frequency of occurrence during exposure to adrenergic agonists. Thus adrenergic activation causes a decrease in the frequency of Ca\textsuperscript{2+} sparks and an increase in the frequency of asynchronous wavelike Ca\textsuperscript{2+} transients, both of which should tend to decrease arterial diameter. Oscillatory vasomotion is associated with spatially uniform synchronous oscillations of cellular [Ca\textsuperscript{2+}] and may have a different mechanism than the asynchronous, propagating Ca\textsuperscript{2+} transients.

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studies of the complete time course of Ca\(^{2+}\) signals during maintained exposure to adrenergic agonists, although this is expected to be important information, given the current concept that the importance of Ca\(^{2+}\) dependent mechanisms of force declines with time (18). In the present study, therefore, we sought to record, for the first time, the Ca\(^{2+}\) signals (Ca\(^{2+}\) sparks, Ca\(^{2+}\) waves, and synchronous whole cell Ca\(^{2+}\) oscillations) within the individual SMC of isobaric arteries during decreases in diameter and during vasomotion activated by the \(\alpha_1\)-adrenoceptor agonist phenylephrine (PE).

METHODS

Preparation of arteries. 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 200–330 g were anesthetized by intramuscular injection of pentobarbital sodium (Nembutal, 50–100 mg/kg) and euthanized by cervical dislocation. The mesenteric arcade was dissected and placed in a cold dissection chamber (7°C) containing a solution (the “dissection” solution) with the following composition (mM): 2 MOPS, 145 NaCl, 4.7 KCl, 2.0 CaCl\(_2\), 1.2 MgSO\(_4\), 1.2 NaH\(_2\)PO\(_4\), 0.02 EDTA, 2 pyruvate, and 5 glucose and 1.0% albumin (pH 7.4). Dissected arteries (12) were loaded with fluo 4 at room temperature for 3–4 h in the dissection solution (but without albumin) to which had been added 10 \(\mu\)M fluo 4-AM, 1.5% DMSO (vol/vol), and 0.03% cremophor EL (vol/vol). Arteries to be studied under isometric conditions were mounted over glass cannulas \(\sim 250 \mu m\) diameter. All arteries were equilibrated over \(-1 h\) to initial experimental conditions (22°C, 70 mmHg) studied in a modified Krebs solution of the following composition (in mM): 112 NaCl, 25.7 NaHCO\(_3\), 4.9 KCl, 2.5 CaCl\(_2\), 1.2 MgSO\(_4\), 1.2 KHPO\(_4\), 11.5 glucose, and 10 HEPES (pH 7.4 at 22°C); equilibrated with gas of 5% O\(_2\)-5% CO\(_2\)-90% N\(_2\). Most arteries were studied at 22°C rather than at 37°C because fluo 4 was much better retained at the lower temperature. In total, results from 35 arteries were included in this report. Initial studies in arteries free of dye at the two temperatures (see Results) established some of the differences in behavior at the two temperatures.

Measurements of fluorescence and arterial diameter. We used a custom confocal laser scanning microscope described in detail previously (27, 32). The “water” objective lens \((\times60);\) numerical aperture, 1.2) provided excellent spatial resolution but a small field-of-view. To obtain a larger field-of-view during large contractions, we used a “dry” low-power objective lens \((\times20; 0.4\) numerical aperture\). Resolution with this objective lens was still adequate to resolve individual SMC. As shown in Fig. 1, the optical sections we obtained in the present study were of two types. “Radial” sections (Fig. 1A) are relatively uninfluenced by arterial wall motion and show individual SMC in cross section. A digital video (DV) clip (Fig. 1) illustrates the fact that individual SMC can be successfully “tracked” during vasomotion (DV clips for Figs. 1, 3A, and 4A are available at the AJP: Heart and Circulatory Physiology web site). “Tangential” sections (Fig. 1B) (usually of arteries on glass cannulas) are strongly affected by motion but more easily reveal Ca\(^{2+}\) sparks and are required to investigate propagating Ca\(^{2+}\) waves. To improve temporal resolution when recording Ca\(^{2+}\) sparks, SMC were imaged in the tangential line-scan mode in which the same line, 50 \(\mu m\) in length, was scanned once every 3 ms for 1.5 s, thus creating a single line-scan image. Between successive images, however, the position of the line was moved randomly to a new position within a 50 \(\times\) 50-\(\mu m\) planar area of the artery, a technique we refer to as “randomized confocal line scanning.” This allows objective measurements of Ca\(^{2+}\) spark frequency, because the measurements cannot be biased by observer selection. With this technique, Ca\(^{2+}\) spark frequency is presented in terms of the number of Ca\(^{2+}\) sparks recorded per unit of distance (\(\mu m\)) scanned per unit of time (s). Measurements of arterial wall position were made either by using the edges of the fluorescence image (e.g., Fig. 1A, a and b) or in arteries not containing fluo 4, from transmitted light images recorded at 2 s\(^{-1}\). Fluorescence images were corrected for photobleaching of fluo 4. Although we may refer to the images as “Ca\(^{2+}\) images,” all the images are simply of Ca\(^{2+}\)-dependent fluo 4 fluorescence. Autofluorescence was negligible in these arteries in these experiments. All image analysis was done with custom computer procedures written in IDL (Research Systems; Boulder, CO). DV clips were constructed from the original data using digital video editing software (Adobe Systems; San Jose, CA). The DV clips may
RESULTS

Effects of temperature. Figure 2 illustrates the basic contractile responses of isobaric arteries to PE as well as some characteristics of the responses at the two different temperatures. At 37°C, exposure of isobaric arteries (70 mmHg) to relatively low concentrations of PE (≤300 nM) caused a decrease in diameter to a constant level, which we call “steady” vasoconstriction (Fig. 2A, top trace). Application of higher concentrations (e.g., 3.0 μM, Fig. 2A, bottom trace) caused a larger initial decrease, which was then followed by vasomotion. Cumulative concentration-effect curves are shown in Fig. 2B for arteries at 22°C (3 arteries) and 37°C (5 arteries). Oscillations were usually absent at PE concentration of <300 nM. When vasomotion was present, the average diameter was used. The two concentration-effect curves, obtained at the two different temperatures, are not significantly different (unpaired t-test, P > 0.05), both having an EC₅₀ of ~300 nM. The maximum frequency of physical oscillations (Fig. 2D) at 37°C was 0.20 ± 0.01 Hz, significantly different from that at 22°C, 0.059 ± 0.004 Hz (unpaired t-test, P < 0.05). The amplitude of the oscillations in diameter (Fig. 2C) (relative to the maximum diameter) at 37°C was also significantly larger than that at 22°C, 0.089 ± 0.003 as opposed to 0.054 ± 0.001 (unpaired t-test, P < 0.05). These results indicated that the sensitivity of the arteries to PE was not significantly different at the experimental temperature, but that the frequency and relative amplitudes of vasomotion were less at the lower temperature.

Ca²⁺ transients in individual SMC at low levels of adrenergically stimulated vasoconstriction. In isobaric arteries, application of PE at concentrations near the EC₅₀ caused an initial rapid, nearly synchronous Ca²⁺ transient in all the individual SMC (see Fig. 3, A and B,b, and the DV clip). At a PE concentration over the range of 300 nM-3 μM, this initial calcium transient, produced by most of the cells in an artery, varied in duration from 19 to 87 s (36.34 ± 5.05 s, n = 5 arteries). At the lower end of this range of PE concentration, the decrease in diameter then developed relatively slowly, and asynchronous Ca²⁺ transients in individual SMC then occurred, as can be seen particularly clearly in the video clip. The frequency of these oscillations and

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Fig. 2. Adrenergic activation of isobaric rat mesenteric small arteries. Concentration-effect curves of phenylephrine (PE) and effects of temperature. A: time courses of vasoconstriction induced by α₁-adrenoceptor agonist PE in pressurized (70 mmHg) arteries at concentrations of 300 nM (top trace) and 3.0 μM (bottom trace). The lower concentration induced a steady vasoconstriction, which relaxed rapidly upon removal of PE. At the higher concentration, the recording ended before the PE was removed. Arterial diameter refers to the outside diameter, as measured using digitized brightfield images of the arteries. B: concentration-effect curves summarizing data from the type of experiment illustrated in A, at 37°C (n = 5, open circles) and 22°C (n = 3, solid circles). Max Diameter, diameter in the absence of external Ca²⁺. On average, arteries developed more tone at 37°C (~16%) than at 22°C (~8%). The approximate EC₅₀ for the two temperatures was the same, however, 300 nM. When oscillatory vasomotion occurred, the average diameter was used. C: maximum amplitude of the oscillations was much less at 22°C (~5.4% of maximum diameter) than at 37°C (~9.3% of maximum diameter). D: similarly, the maximum frequency was much less at 22°C (0.06 Hz) than at 37°C (0.20 Hz).
the number of cells producing such oscillations was dependent on the PE concentration. In 87 cells, taken from 4 arteries exposed to PE over the range of concentrations of 300 nM–3 μM and imaged during both the initial development of vasoconstriction and during steady vasoconstriction, the frequency of these asynchronous oscillations was 0.051 ± 0.002 Hz. It has been shown recently, in venous smooth muscle (28) and in arterial smooth muscle (33), that the number of cells producing these asynchronous Ca2+ transients depends on the concentration of PE. Although not quantified, it appeared that, if synchronous Ca2+ oscillations did not develop (see Ca2+ transients in individual SMC at high levels of adrenergically stimulated vasoconstriction), the frequency of the asynchronous Ca2+ oscillations declined throughout the period of exposure to PE.

Ca2+ transients in individual SMC at high levels of adrenergically stimulated vasoconstriction. Relatively high PE concentration (≥1.0 μM) generally resulted in vasoconstriction immediately followed by vasomotion in isobaric arteries. The spatiotemporal pattern of intracellular [Ca2+] was fundamentally different from that at lower PE concentrations, as illustrated in Fig. 4 and in the DV clip. Arterial diameter decreased more rapidly and to a greater extent than at lower PE concentration. The Ca2+ in the arterial wall was remarkably uniform during the decrease in diameter. During the oscillatory vasomotion, the peaks of the intracellular Ca2+ transients in different cells all coincided with each other and with the point of maximum diameter (relaxation). In seven isobaric arteries, the delay between peak Ca2+ and minimum diameter (maximum vasoconstriction) at concentrations of PE
ranging from 300 nM to 3.0 μM was 3.0 to 19.9 s (8.17 ± 0.38 s). It has been reported that such oscillatory vasomotion requires an intact endothelium and a functional sarcoplasmic reticulum (SR) (14). To investigate the possible role of nitric oxide (NO) in these Ca2+ transients, we preincubated (1 h) the lumen of two arteries with inhibitors of NO synthesis (N^G-nitro-L-arginine methyl ester, 30.0 μM). No oscillatory vasomotion could be elicited by PE in these arteries.

Ca2+ sparks and Ca2+ waves during adrenergic activation. The radial sections shown in Figs. 3 and 4 do not reveal whether the asynchronous Ca2+ transients are actually propagating Ca2+ waves or what effect adrenergic activation might have on Ca2+ sparks. Therefore, we used arteries mounted on glass cannulas so that tangential sections could be obtained. Figure 5 illustrates the effects of exposure to high concentrations of PE on the frequency of Ca2+ sparks and illustrates the Ca2+ waves that occurred upon exposure to PE. The three images in Fig. 5A were obtained before, at 2 min during exposure to PE at a concentration of 5.0 μM, and 4 min after removal of PE. A Ca2+ transient was identified as a “wave” if it exhibited a constant propagation velocity at least 20 μm. The frequency of Ca2+ sparks was obtained from images such as those in Fig. 5A by counting the number of Ca2+ sparks and dividing by the total time of scanning of the image (usually 1.5 s) and then by the total distance scanned (usually 50 μm). To be counted as a “spark,” a local Ca2+ transient had to have a width at half-maximum of <5.0 μm and a peak amplitude (in terms of fluorescence ratio, ΔF/ΔF₀) of at least 1.3. Figure 5B shows the spark frequency (in each frame) as a function of time before, during, and after the exposure to PE in a typical experiment. Under the control conditions, the average frequency of Ca2+ sparks in six arteries during a 100-s period was 0.025 ± 0.0084 sparks s⁻¹μm⁻¹. During the third and fourth minutes of exposure to PE (120 s), the spark frequency declined to a significantly different level, 0.0130 ± 0.0033 sparks s⁻¹μm⁻¹ (paired t-test, P < 0.016). Histograms of the peak amplitudes (∆F/ΔF₀) of the sparks are shown in Fig. 5C. No differences were found in the distribution of peak amplitudes. As a control experiment, arteries were also exposed to 30 mM KCl. In this case, the frequency of Ca2+ sparks increased dramatically, as expected if Ca2+ sparks are activated by the entry of Ca2+ through L-type Ca2+ channels (17, 21). Thus, although PE results in depolarization, a separate mechanism must exist that reduces Ca2+ spark frequency. During the exposure to PE, the Ca2+ transients were revealed to be propagating Ca2+ waves of the type shown in Fig. 5A, middle. The average velocity of the front of such waves in four arteries exposed to PE at a concentration of 5.0 μM was 32.4 ± 1.7 μm/s (73 waves). The waves were asynchronous between cells and did not propagate between cells.

DISCUSSION

The results support a model of adrenergic constriction of pressurized arteries in which asynchronous propagating Ca2+ waves underlie vasoconstriction. Low levels of adrenergic activation and spatially uniform, synchronous Ca2+ transients underlie oscillatory vasomotion at high levels of activation. We suggest below that these two types of Ca2+ signals are fundamentally different in their mechanisms of origin and in their role as Ca2+ signals for force production. An additional component of Ca2+ signaling is the decline...
in the frequency of Ca$^{2+}$ sparks during adrenergic activation. This may be expected to enhance vasoconstriction by further depolarizing the SMC, consequent to a reduction of Ca$^{2+}$-activated hyperpolarizing membrane currents (25). Other possible components of Ca$^{2+}$ signaling in arterial smooth muscle, such as the spontaneous Ca$^{2+}$ “ripples” and “flashes” reported in wide-field imaging studies (1) were not observed in the present study, as discussed further below.

**Developing and steady vasoconstriction: asynchronous, propagating Ca$^{2+}$ waves.** The initial release of Ca$^{2+}$ and the asynchronous propagating Ca$^{2+}$ waves in the isobaric arteries we studied are almost certainly dependent on ryanodine receptors and release of Ca$^{2+}$ from the SR (2). Agonist activation of single isolated mesenteric artery myocytes with norepinephrine has shown brief Ca$^{2+}$ transients (4, 5) dependent on SR function. Ca$^{2+}$ waves, dependent on SR function, were elicited by norepinephrine in isolated venous myocytes (6). Propagating Ca$^{2+}$ waves have also been recorded from isometric preparations of the rabbit inferior vena cava (28). The initial Ca$^{2+}$ release after PE stimulation tends to occur synchronously between cells in the optical section and can account for the initial Ca$^{2+}$ “spike” observed in previous studies that did not utilize confocal imaging techniques. The asynchronous waves that follow may then account for the lower levels of average Ca$^{2+}$ seen in nonimaging studies (18), because asynchronous Ca$^{2+}$ transients would not “sum” effectively. Average [Ca$^{2+}$] would therefore be low, despite the existence of asynchronous Ca$^{2+}$ transients. The decline in average Ca$^{2+}$ recorded previously has been taken to indicate the development of Ca$^{2+}$-independent mechanisms of force generation or the development of Ca$^{2+}$ sensitization of the contractile proteins. Our experiments show that propagating Ca$^{2+}$ waves continue to occur within individual SMC in the presence of PE concentration. This raises the possibility that Ca$^{2+}$ is still playing a role, although the efficacy of these Ca$^{2+}$ waves in activating force remains unknown. During the time when Ca$^{2+}$ waves are asynchronous, the diameter may be fairly constant. Individual SMC did not appear to move independently of the arterial wall.

**Vasomotion: synchronous Ca$^{2+}$ oscillations.** An important component of the adrenergic response is the vasomotion and the slow, spatially uniform, synchronous Ca$^{2+}$ transients that can be seen in isobaric arteries in the presence of higher concentrations of PE. As mentioned, early studies of Ca$^{2+}$ signals during adrenergic activation generally showed that average arterial wall [Ca$^{2+}$]$_i$ rose and then fell to a low maintained level, although oscillations in arterial wall [Ca$^{2+}$] were sometimes seen (29). The present study is the first to utilize high-resolution imaging of Ca$^{2+}$ in individual SMC during vasomotion. The synchronous Ca$^{2+}$ oscillations we observed in the SMC were “phase-locked” to the vasomotion. The peak of Ca$^{2+}$ elevation did not occur at the point of maximum vasoconstriction but instead during the relaxed phase of the diameter changes. The timing and synchronous nature of the Ca$^{2+}$ elevations during vasomotion strongly suggests that the mechanism of generation involves primarily changes in membrane potential (which should be “synchronous” in different SMC). Oscillatory force production is known to be accompanied by oscillations in membrane potential (15). Depolarization could allow for coordinated L-type Ca$^{2+}$ channel activation and Ca$^{2+}$ influx (26). The mechanisms of oscillatory contractions or vasomotion (13) are still obscure, although it is known that vasomotion in the rat mesenteric artery requires endothelial NO release and subsequent elevation of cGMP levels in the SMC (14). Adrenergic vasoconstriction of the rat arterial mesenteric bed in situ is also associated with release of both NO and cGMP (8). It has been suggested that the stimulus for the production of NO in this case is either a rise in endothelial cell [Ca$^{2+}$], produced by Ca$^{2+}$ diffusing from SMC through myoendothelial gap junctions (11), or an elevation of shear stress. Shear stress was not a factor in our experiments because flow was not occurring. Regardless of the stimulus for NO production, it seems likely that NO diffuses from endothelial cells to the SMC, where it stimulates the production of cGMP and inhibits Ca$^{2+}$ entry through L-type Ca$^{2+}$ channels (3). The mechanism of oscillatory changes in membrane potential is not known in detail. Nevertheless, we postulate that the origin of the slow, synchronous Ca$^{2+}$ transients involves changes in membrane potential, because only this agent would seem capable of synchronizing the activity of all the cells.

Regardless of the mechanism of generation of the synchronous Ca$^{2+}$ oscillations, their function is more easily identified. Synchronous Ca$^{2+}$ oscillations are always present during vasomotion and were never observed in arteries that did not exhibit some degree of vasomotion. Although Ca$^{2+}$ sensitization of the contractile proteins may develop during prolonged adrenergic activation, synchronous Ca$^{2+}$ oscillations underlie the oscillatory force production that generates the oscillatory changes in diameter.

**Other types of Ca$^{2+}$ signals during adrenergic stimulation.** Recently, two other types of spontaneous Ca$^{2+}$ signals, “ripples” and “flashes,” have been observed with the use of wide-field fluorescence microscopy, in muscle cells of the rat tail artery (1). It was suggested that the Ca$^{2+}$ ripples are generated via inositol 1,4,5-trisphosphate-induced Ca$^{2+}$ release in response to locally produced angiotensin II. It is possible that the frequency of ripples or proportion of SMC generating Ca$^{2+}$ ripples and flashes could be affected by adrenergic stimulation. In the present study, however, we did not seek to determine any such possible effects. Interestingly, the amplitude of the Ca$^{2+}$ ripples was reported to be about 0.04 ($\Delta F/F_0$) in peak amplitude and the Ca$^{2+}$ oscillations induced by norepinephrine in that study were reported to have an average amplitude ($\Delta F/F_0$) of just <0.4. This is far less than the peak amplitude of the synchronous Ca$^{2+}$ oscillations illustrated in Fig. 3B,b ($-2.4 \Delta F/F_0$). The quantitative difference in the apparent amplitude of the adrenergically stimulated Ca$^{2+}$ transients in the two studies may reflect differences in the optical techniques used.
(viz. confocal vs. wide-field microscopy) or in the preparations (isobaric vs. isometric).

Effects of adrenergic stimulation on Ca\(^{2+}\) sparks. We observed that adrenergic activation decreased the frequency of Ca\(^{2+}\) sparks. This is in accord with the fact that activators of protein kinase C (which is activated during adrenergic stimulation) decrease Ca\(^{2+}\) spark frequency (7). It has been postulated that this is a direct effect on the ryanodine receptors, because the Ca\(^{2+}\) content of the SR (which would affect Ca\(^{2+}\) spark frequency) was not affected by activators of PKC (7). The effects of adrenergic activation on Ca\(^{2+}\) sparks within intact arteries that we have observed could be more complex, however, because Ca\(^{2+}\) content of the SR is believed to decline during adrenergic activation (4, 5) and because of possible effects other than activation of PKC, such as depolarization and increased availability of L-type Ca\(^{2+}\) channels.

We gratefully acknowledge the assistance of Dr. Victor A. Miriel in helping to develop methods for studying isobaric arteries and of M. R. Saunders for help with video editing.

This study was supported by National Institutes of Health Research Grant HL-60748 (to W. G. Wier) and by Training Grant AR-07592 to the University of Maryland Training Program in Muscle Biology.

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