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Visualization of neural control of intracellular Ca^{2+} concentration in single vascular smooth muscle cells *in situ*

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The intermittent rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$ oscillation) has been observed in many types of isolated cells, yet it has not been demonstrated whether it plays an essential role during nerve stimulation *in situ*. We used confocal microscopy to study Ca^{2+} transients in individual smooth muscle cells *in situ* within the wall of small arteries stimulated with perivascular sympathetic nerves or noradrenaline. We show here that the sympathetic adrenergic regulation of arterial smooth muscle cells involves the oscillation of $[Ca^{2+}]_i$ that propagates within the cell in the form of a wave. Ca^{2+} release from intracellular stores plays a key role in the oscillation because it is blocked after the store depletion by ryanodine treatment. Ca^{2+} influx through the plasma membrane sustains the oscillation by replenishing the Ca^{2+} stores. These results demonstrate the involvement of $[Ca^{2+}]_i$ oscillations in the neural regulation of effector cells within the integrated system.

Key words: Ca^{2+} oscillations/confocal microscope/noradrenaline/sympathetic nerve/vascular tone

Introduction

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) often undergoes an intermittent rise (oscillation) in many types of isolated or cultured cells stimulated with receptor agonists. Several lines of evidence indicate that inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release from the intracellular Ca^{2+} storage organelle plays the key role in the oscillation (Berridge and Irvine, 1989; Meyer and Stryer, 1991; Berridge, 1993). The molecular mechanism of Ca^{2+} oscillation has attracted much attention and a number of hypotheses have been proposed. Although it has been reported that IP_3 concentration may fluctuate during the oscillations (Harootunian *et al.*, 1991), Ca^{2+} oscillations have been observed at a constant concentration of IP_3 or its analogue (Wakui *et al.*, 1989; Lechleiter and Clapham, 1992). Ca^{2+}-mediated positive feedback control of IP_3-induced Ca^{2+} release has been regarded as important for the initiation of the $[Ca^{2+}]_i$ rise (Iino and Endo, 1992; Berridge, 1993). The rate of Ca^{2+} uptake by the stores and Ca^{2+} influx through the plasmalemma may modulate the frequency of Ca^{2+} oscillations (Camacho and Lechleiter, 1993; Girard and Clapham, 1993; Petersen *et al.*, 1993; Yao and Parker, 1994). Oscillatory responses in the $[Ca^{2+}]_i$ have been considered more advantageous than a steady tonic increase because digitally coded responses are easier to control, and the intermittent rise may be effective only for highly cooperative responses without interfering with other cell functions that are influenced by a tonic rise in $[Ca^{2+}]_i$ (Woods *et al.*, 1986; Berridge and Galione, 1988; Jacob *et al.*, 1988; Meyer and Stryer, 1991). However, the conditions of cells may change upon isolation or in culture, and it remains to be demonstrated whether Ca^{2+} oscillation plays an essential role during nerve stimulation *in situ* within the integrated system.

Vascular smooth muscle cells are under the close control of the sympathetic nervous system which regulates blood pressure by changing the calibre of small arteries (Bevan *et al.*, 1980; Bülbring and Tomita, 1987; Hirst and Edwards, 1989; Mulvany and Aalkjaer, 1990). The contraction of vascular smooth muscle cells has been thought to be controlled by both Ca^{2+} release from the stores and influx through the sarcolemma. Although a transient contraction of smooth muscle cells is observed upon stimulation with the sympathetic neurotransmitter in the absence of extracellular Ca^{2+}, it is not maintained without the presence of Ca^{2+} outside the cells (Devine *et al.*, 1972). Therefore, the Ca^{2+} influx has been thought to be the key mechanism that controls the steady vascular tone, and the Ca^{2+} stores have been assumed to function only during the initial transient contraction (Nelson *et al.*, 1990). In cultured vascular smooth muscle cells, on the other hand, Ca^{2+} oscillations have been observed during constant stimulation by vasoconstrictive hormones (Johnson *et al.*, 1991; Blatter and Wier, 1992). However, cultured smooth muscle cells lose their most important function to contract, and there could be other alterations in their properties. Thus, the role of Ca^{2+} oscillations in the regulation of vascular smooth muscle contraction has not been seriously considered. We have studied the $[Ca^{2+}]_i$ change within the individual smooth muscle cells in the wall of small arteries that were stimulated with the perivascular sympathetic nerve activities. Our results now demonstrate oscillations of $[Ca^{2+}]_i$ in individual cells within the integrated tissue and indicate the important role of Ca^{2+} oscillations in the sympathetic control of vascular tone.

Results

Rat tail arteries were carefully dissected and loaded with a fluorescent Ca^{2+} indicator dye by the luminal perfusion of Fluo-3 AM (Minta *et al.*, 1989) for observation with a confocal microscope (Figure 1A). As shown in Figure 1B, we were able to clearly recognize a Fluo-3-loaded single layer of circular smooth muscle cells in the artery. By

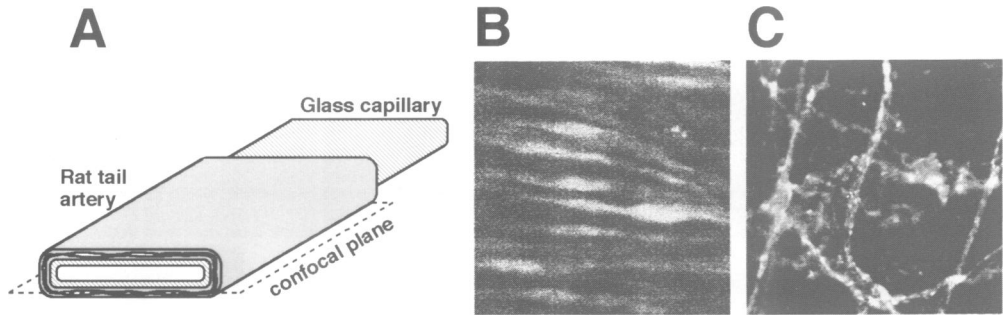

Fig. 1. Experimental specimen and confocal views of smooth muscle layer and perivascular nerve. (**A**) A schematic view of the arterial preparation over a glass capillary with a rectangular cross-section. (**B**) Confocal image of the circular smooth muscle layer stained with Fluo-3. This view was taken during nerve stimulation so that the fluorescence intensity of the indicator was higher than that at rest. The frame has a dimension of 115×115 μm. (**C**) Confocal view of the same area as in (B) but focused on the outer layer showing the sympathetic nerve networks stained with 4-Di-2-ASP.

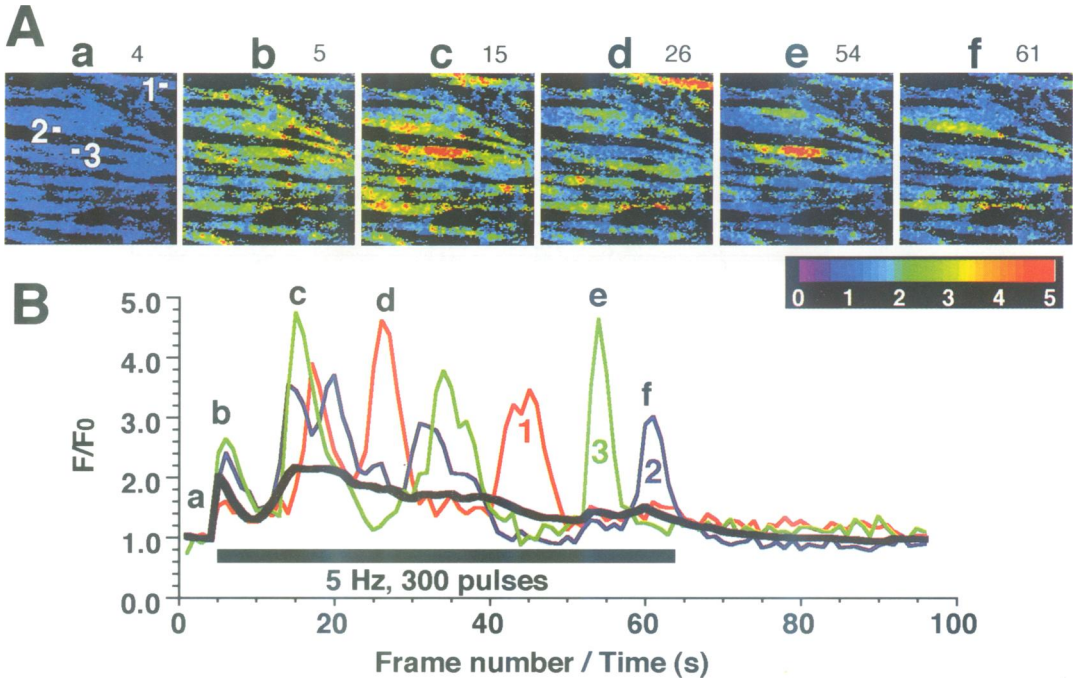

Fig. 2. Confocal images of the smooth muscle layer of rat tail artery with electrical stimulation of the perivascular nerve. (**A**, parts a−f) Six frames selected from 96 consecutive 2-D confocal images (one frame per second) of the fluorescent intensity change of Fluo-3 in the smooth muscle cells. The frame numbers are shown above each panel. Increase in the fluorescence intensity was displayed in a pseudo-colour code. Electrical stimulation (5 Hz, 300 pulses) was applied between frames 5 and 64. (**B**) Fluorescence intensity change (red, blue and green lines) was plotted against time in the three selected areas of the image (shown by the white boxes in A, part a) and the average value of all the cells in the field (thick black line). The lower case letters (a−f) in (B) indicate the times at which the frames in (A) were taken. These data are representative of seven experiments.

changing the focal plane, we could also observe dye-loaded endothelial cells and perivascular connective tissues, but the fluorescence intensity of these cells did not change upon electrical stimulation. The presence of perivascular nerves that formed a network surrounding the smooth muscle layer (Bevan *et al.*, 1980) was clearly observed after incubation of the preparation for 60 s in 10 μM 4-(4-diethylaminostyryl)-*N*-methylpyridium iodide (4-Di-2-ASP), a fluorescent probe for nerve fibres (Magrassi *et al.*, 1987; Figure 1C).

We obtained 2-D confocal images of smooth muscle cells every second while electrical stimulation was applied. In Figure 2A we show frames selected from 96 consecutive images taken from the same cells as in Figure 1B. To show the time course of $[Ca^{2+}]_i$ change in individual cells, the fluorescence intensity changes at three representative areas of the field (Figure 2A, part a) are displayed in Figure 2B. Upon the initiation of electrical stimulation (5 Hz, 300 pulses) all the cells responded with a sudden rise in $[Ca^{2+}]_i$ (Figure 2A, part b), which was followed by intermittent rises in $[Ca^{2+}]_i$ in individual cells (Figure 2A, parts c−f, and 2B). Similar responses were obtained at lower rates of stimulation (2.0 or 3.3 Hz), although the frequency of the Ca^{2+} oscillations was lower. These responses were elicited by transmitters released from the perivascular sympathetic nerves because they were completely abolished by the addition of 1 μM tetrodotoxin that blocks action potentials in nerve fibres (n = 6). Except for the initial transient, all other $[Ca^{2+}]_i$ rises were abolished by the addition of 1 μM phentolamine (n = 2) or 0.1 μM prazosin (n = 2), α- and α_1-adrenergic antagonists, respectively. It has been suggested that the

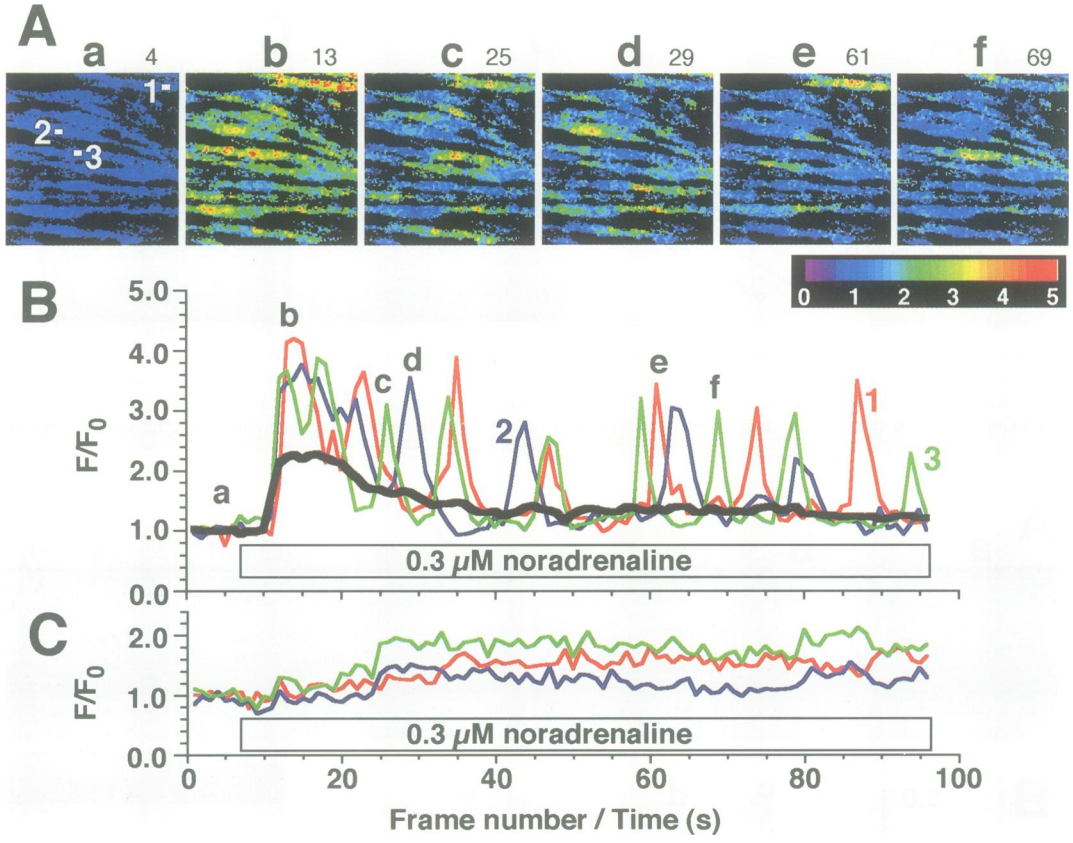

Fig. 3. Confocal images of the smooth muscle layer of rat tail artery stimulated with 0.3 μM noradrenaline. (**A**, a–f) 2-D confocal image of the fluorescent intensity change of Fluo-3 in the smooth muscle cells. Frame numbers are indicated above each panel. The bathing solution was changed to PSS containing 0.3 μM noradrenaline at frame 7. (**B**) Fluorescence intensity change (red, blue and green lines) plotted against time in the three selected areas of the image (shown by the white boxes in A, part a) and the average value of all the cells (thick black line). The lower case letters (a–f) in (B) indicate the time at which the frames in (A) were taken. (**C**) Similar to (B) but was obtained in PSS after co-application of 30 μM ryanodine and 25 mM caffeine for 2 min. Ryanodine and caffeine had been washed out from the experimental trough when noradrenaline was applied. These data are representative of six experiments.

non-α-adrenergic early response is due to ATP, a cotransmitter of noradrenaline at the sympathetic nerve endings, and is sensitive to either α,β-methylene ATP or suramine (Sneddon and Burnstock, 1985; Bao and Stjärne, 1993). In accordance with this notion, the initial $[Ca^{2+}]_i$ transient was inhibited in the presence of 500 μM suramine, and it subsided rapidly during continuous electrical stimulation as reported previously (Msghina and Stjärne, 1993).

To distinguish whether the oscillatory responses were due to intermittent activities of the sympathetic nerve terminals or to oscillatory properties of the response of smooth muscle cells to a constant level of stimulation, we observed responses to 0.3 μM noradrenaline in the same artery (Figure 3A). After a few seconds of delay all cells showed a $[Ca^{2+}]_i$ rise, followed by oscillations of $[Ca^{2+}]_i$ as during nerve stimulation (Figure 3B). Therefore, the oscillatory mechanism functions at a constant noradrenaline concentration and seems to be intrinsic to smooth muscle cells.

The frequency of the oscillation was dependent on the noradrenaline concentration. Figure 4 shows $[Ca^{2+}]_i$ changes in four different cells at 0.1, 0.3 and 1.0 μM noradrenaline concentrations. Figure 4A and B shows $[Ca^{2+}]_i$ changes in the same areas as 1 and 3, respectively, of Figures 2 and 3. Figure 4C and D were obtained from two different cells in another tail artery. The frequency of the oscillation increased with the increase in the noradrenaline concentration. At 1 μM noradrenaline the oscillation became irregular and apparent fusion of the oscillatory responses was observed so that the distinct rise and fall of $[Ca^{2+}]_i$ became less discernible (especially in Figure 4C).

A $[Ca^{2+}]_i$ rise during the oscillations occurred within each cell in the form of a wave, as shown by the sequential images of a representative cell (Figure 5). The first noradrenaline-induced $[Ca^{2+}]_i$ transient had a very rapid rise time throughout the cell, and the subcellular patterns of $[Ca^{2+}]_i$ change were barely resolved (Figure 5A). However, during the following oscillations it took ⩾4 s for the Ca^{2+} waves to travel along the cell (Figure 5B). Thus, the wave velocity was ~20 $\mu m.s^{-1}$. There seemed to be no anatomically specialized region for the initiation of waves. On some occasions Ca^{2+} waves propagated from both ends of the cell, collided and were annihilated (Figure 5C). Similar $[Ca^{2+}]_i$ waves were observed during sympathetic stimulation (Figure 5D).

To study the role of Ca^{2+} stores in the Ca^{2+} oscillations, we examined the effect of ryanodine and caffeine treatment, which is known to deplete the Ca^{2+} stores (Hwang and van Breemen, 1987; Iino *et al.*, 1988; Kanmura *et al.*, 1988). Ryanodine (30 μM) was applied with 50 mM caffeine because ryanodine binding is facilitated by the opening of ryanodine receptors. Since the effect of ryanodine is virtually irreversible, both drugs were removed

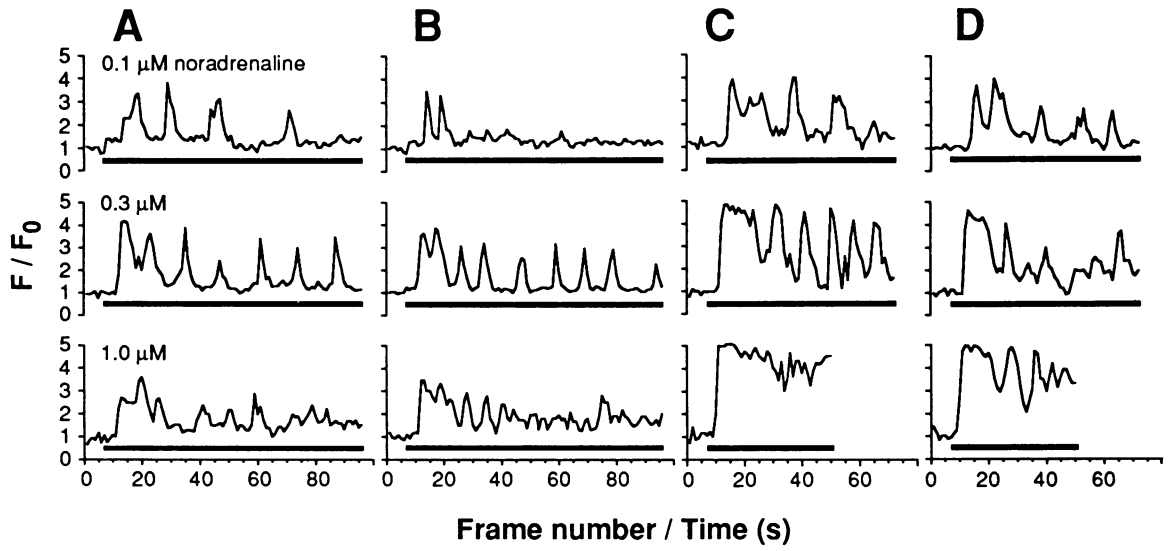

Fig. 4. Dependence of $[Ca^{2+}]_i$ oscillations on noradrenaline concentration. Representative results from four cells are shown. The noradrenaline concentration was 0.1 (top row), 0.3 (middle row) and 1.0 μM (bottom row). Black bars below the trace indicate the duration of noradrenaline application. (**A**) and (**B**) were obtained from the same spots as shown in Figures 2 and 3, and (**C**) and (**D**) were obtained from two different cells in another artery.

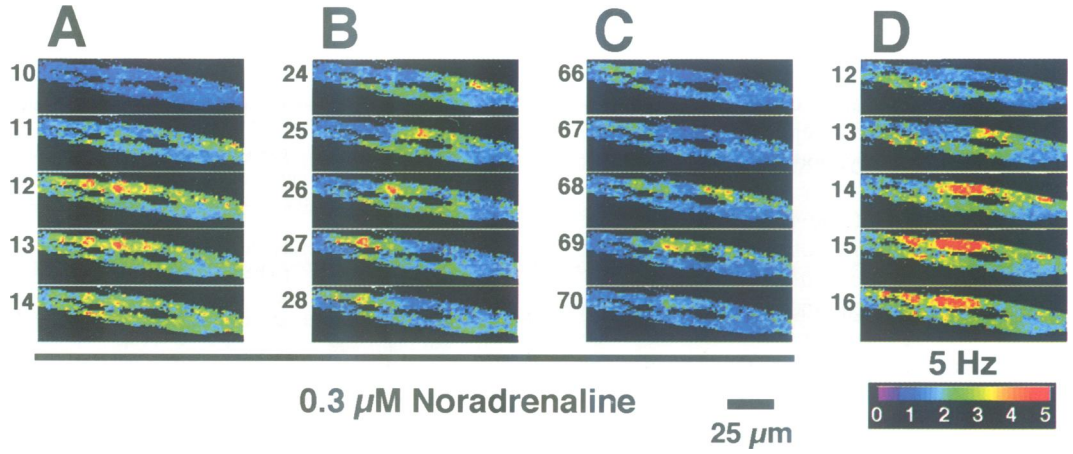

Fig. 5. Waves of $[Ca^{2+}]_i$ during Ca^{2+} oscillations. Consecutive images of two cells in the centre of the field shown in Figure 1B stimulated with 0.3 μM noradrenaline (**A**−**C**) or with sympathetic activity (**D**). Frames are selected to show the Ca^{2+} wave in the upper cell and their numbers correspond to those in Figures 2 and 3. (A) Initial $[Ca^{2+}]_i$ transient spread rapidly throughout the cell after noradrenaline application. (B) The following Ca^{2+} waves had a slower propagation speed. (C) On some occasions two Ca^{2+} waves originating from both ends of the cell collided and were annihilated. (D) Ca^{2+} waves were also seen during sympathetic nerve stimulation.

from physiological salt solution (PSS) before subsequent experiments. The noradrenaline-induced $[Ca^{2+}]$ oscillations were abolished after the ryanodine treatment (Figure 3C). This indicates the involvement of Ca^{2+} stores in the formation of Ca^{2+} oscillations.

The phase of $[Ca^{2+}]_i$ oscillations varied from cell to cell (except for the first transient), both in nerve-stimulated or noradrenaline-induced responses (Figures 2 and 3). This shows that each arterial smooth muscle cell functions independently under the influence of the neurotransmitter. As a consequence of the phase variation, the average response of all the cells in the field appeared as a tonic submaximal response after an initial phasic response (Figures 2B and 3B, thick black lines).

Discussion

Ca^{2+} oscillations and waves have been observed in dispersed or cultured cells to which a digital imaging technique is easy to apply. However, it has not been entirely clear whether the same $[Ca^{2+}]_i$ responses take place when the cells remain in the integrated system and are stimulated with physiological signals. We have succeeded in the $[Ca^{2+}]_i$ imaging of individual smooth muscle cells within the wall of rat tail artery that can be stimulated with the perivascular sympathetic nerve fibres. The present results now demonstrate Ca^{2+} waves and oscillations in cells *in situ* within an intact tissue stimulated with innervating nerves. Similar observations in various other intact tissues are awaited.

We have shown that arterial smooth muscle cells respond with Ca^{2+} oscillations to constant sympathetic activities. Quite contrary to what has been generally believed, a graded response to different levels of the sympathetic transmitter (Bao and Stjärne, 1993) seems to be accomplished not by a graded response within each smooth muscle cell, but by a graded number of active cells within the vascular wall. This is reminiscent of the force control in

skeletal muscle. However, in skeletal muscle there is the close 1:1 relationship between action potentials in the motor nerve endings and muscle fibres. Here in arterial smooth muscle there is no such tight coupling.

Our observation demonstrates the importance of Ca^{2+} stores in $[Ca^{2+}]_i$ regulation of arterial smooth muscle cells in the form of pharmaco-mechanical coupling (Somlyo and Somlyo, 1968). The velocity of the propagation of $[Ca^{2+}]_i$ change during oscillations is too slow to be attributed to the propagation of electrical activities along the cell, and is likely to be due to $[Ca^{2+}]_i$ waves resulting from Ca^{2+} release from intracellular stores. The wave velocity of ~20 $\mu m.s^{-1}$ is comparable with those found in freshly dispersed intestinal smooth muscle cells (33.6 ± 11.4 $\mu m.s^{-1}$; Iino *et al.*, 1993) and in various other cell types (Jaffe, 1991), although a slower wave velocity (~10 $\mu m.s^{-1}$) has been found in cultured smooth muscle cells (Neylon *et al.*, 1990). In accordance with the important roles of the Ca^{2+} stores, noradrenaline <1 μM concentration has been shown to have little depolarizing action in these smooth muscle cells (Holman and Surprenant, 1980; Itoh *et al.*, 1983). Furthermore, the noradrenaline-induced $[Ca^{2+}]_i$ oscillations were abolished after ryanodine and caffeine treatment, which is known to deplete the Ca^{2+} stores (Hwang and van Breemen, 1987; Iino *et al.*, 1988; Kanmura *et al.*, 1988). The effect of ryanodine and caffeine treatment does not necessarily indicate the role of the ryanodine receptor in the formation of the Ca^{2+} wave, because ryanodine-sensitive stores overlap with the IP_3-sensitive stores (Iino *et al.*, 1988). Indeed, it has been shown that the ryanodine receptors are not involved in the formation of the Ca^{2+} wave in single smooth muscle cells (Iino *et al.*, 1993).

Upon stimulation with noradrenaline, the arterial smooth muscle contracts in two phases: the initial phasic response and the sustained tonic response. In the absence of extracellular Ca^{2+}, the tonic response to noradrenaline was abolished (Xiao and Rand, 1989). These observations suggested previously that Ca^{2+} stores are involved only in the phasic response to receptor agonists, and the tonic component was due to Ca^{2+} influx that directly elevated $[Ca^{2+}]_i$ (Nelson *et al.*, 1990). However, the Ca^{2+} oscillation continues in the tonic phase, and $[Ca^{2+}]_i$ between Ca^{2+} oscillations returned to the pre-stimulation level in individual cells at the low noradrenaline concentrations (Figures 2B and 3B). When noradrenaline was applied in the absence of extracellular Ca^{2+} (5 mM EGTA), $[Ca^{2+}]_i$ oscillations were observed only a few times and then disappeared (unpublished observation). Taken together, our observations suggest that the influx of Ca^{2+} is used mainly to assist the replenishment of the Ca^{2+} stores that were released intermittently to produce Ca^{2+} oscillations. $[Ca^{2+}]_i$ showed a steady increase upon stimulation with noradrenaline after the function of Ca^{2+} stores was impaired after ryanodine and caffeine treatment (Figure 3C). This might suggest the presence of noradrenaline-activated Ca^{2+} influx pathways which would assist the loading of the Ca^{2+} stores during noradrenaline stimulation. Further studies are required to clarify this point.

It is now shown that the Ca^{2+} influx can be coupled with Ca^{2+} release and the two sources of Ca^{2+} may work in a collaborative manner throughout the transmitter-induced contraction of vascular smooth muscle cells. Therefore, the present results cast a new light on the cellular mechanism of sympathetic regulation of blood pressure and may provide new strategies for the study of cardiovascular functions and diseases, or for the development of vasoactive drugs.

Materials and methods

Preparation

Tail arteries (outer diameter ~300 μm, length ~30 mm) were carefully dissected from young rats (60–90 g), cleaned of the surrounding connective tissue and cannulated at one end for luminal perfusion of PSS containing 40 μM Fluo-3 AM and 0.005% pluronic F-127 for 4–6 h at 35°C. The artery was then cut to the length of ~8 mm and a rectangular glass capillary (400 μm wide, 40 μm thick, 12 mm long) was inserted into the lumen of the specimen (Figure 1A). Then the artery was mounted in a trough containing PSS with a coverslip at the bottom. PSS had the following composition (mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES and 10 glucose, and its pH was adjusted to 7.4 with NaOH. Cytochalasin D (5 μM) was added to PSS to suppress the movement of smooth muscle cells (Iino *et al.*, 1993). The proximal end of the artery was stimulated with electrical shocks (20 V, 0.2 ms duration) using a pair of platinum wires placed on both sides of the preparation.

Confocal digital imaging

The trough was mounted on the stage of an inverted microscope (IMT-2, Olympus) equipped with a confocal scanner (MRC-600, Bio-Rad). The temperature of the trough was kept near 30°C. The arterial wall was viewed using a water-immersion objective (WPLAN ×40 UV, NA = 0.7, Olympus). The fluorescence intensity with excitation at 488 nm (argon laser) was digitized at 256 levels. The 2-D images had 512×512 pixels (Figure 1) or 128×128 pixels (Figures 2 and 3) and a dimension of 115×115 μm. The contrast of Figure 1B and C was enhanced using the NIH image programme on a personal computer (Quadra 800, Apple Macintosh).

The fluorescence intensity change of intracellularly loaded Fluo-3 was used to estimate the $[Ca^{2+}]_i$ change upon stimulation. To exclude regions of the image outside the cell and near the cell periphery, 12 frames during peak response to stimulation (when the fluorescence level of Fluo-3 was high) were averaged and the pixels with lower intensity than a threshold value were not used for the following analysis. Values for the threshold were chosen so that the cell borders could be easily discerned. The excluded regions were shown by black. Three frames before the stimulation were averaged pixel by pixel to obtain resting fluorescence (F_0). All frames were then divided by F_0 in a pixel-by-pixel basis, and the normalized fluorescence intensity values were displayed in a pseudo-colour code. We did not convert the fluorescence intensity change to absolute values of $[Ca^{2+}]_i$ because it was difficult to unambiguously determine the background fluorescence intensity and the resting $[Ca^{2+}]_i$, both of which are required for $[Ca^{2+}]_i$ determination using non-ratiometric dyes.

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