# Endothelium-dependent frequency modulation of $Ca^{2+}$ signalling in individual vascular smooth muscle cells of the rat

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1. We visualized intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) changes, using fluo-3 as an indicator, of individual vascular smooth muscle cells and endothelial cells within intact rat tail arteries by confocal microscopy.

2. Using a piezo-driven objective, we focused on endothelial and smooth muscle cell layers alternately to obtain $Ca^{2+}$ images of their cells. In the presence of 1 $\mu$M acetylcholine (ACh), individual endothelial cells responded with intermittent increases in the $[Ca^{2+}]_i$ ($Ca^{2+}$ oscillations). At the same time, the frequency of $Ca^{2+}$ oscillations in smooth muscle cells induced by electrical stimulation of the perivascular sympathetic nerve was greatly decreased.

3. A $[Ca^{2+}]_i$ rise during the oscillations in the endothelial cells propagated in the form of a wave along the long axis of the cells.

4. In the presence of a NO synthase inhibitor, no significant inhibitory effect of ACh on the $Ca^{2+}$ signalling in the vascular smooth muscle cells was detected, although the $Ca^{2+}$ oscillations in the endothelial cells persisted.

5. The inhibitory effect of ACh on the frequency of $Ca^{2+}$ oscillations in the vascular smooth muscle cells was mimicked by 1 $\mu$M sodium nitroprusside, a NO donor.

6. These results indicate that $Ca^{2+}$ waves and oscillations in vascular endothelial cells regulate NO production, which modulates vascular tone by decreasing the frequency of $Ca^{2+}$ oscillations in smooth muscle cells activated by sympathetic agonists.

Vascular endothelial cells release various vasoactive substances including endothelium-derived relaxing factor (EDRF), which stimulates a series of reactions in vascular smooth muscle cells to decrease vascular tone. The principal EDRF has been identified as nitric oxide (NO), and an enzyme that catalyses NO synthesis from L-arginine is expressed in endothelial cells (Moncada, Palmer & Higgs, 1991). The activity of the endothelial NO synthase is regulated by calmodulin and is therefore dependent on the intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) (Griffith & Stuehr, 1995). The primary target of NO in smooth muscle cells is guanylyl cyclase, which catalyses the formation of cyclic GMP (cGMP), which in turn activates cGMP-dependent protein kinase (G-kinase). The vasorelaxant effect of NO is mimicked by chemicals from which NO is generated (NO donors) and membrane-permeable analogues of cGMP. These agents decrease the $[Ca^{2+}]_i$ of vascular smooth muscle cells and reduce the sensitivity of myofilaments to $Ca^{2+}$. Therefore, NO-induced vasorelaxation involves interaction between $Ca^{2+}$ signalling in endothelial cells and that in neighbouring vascular smooth muscle cells.

The mechanisms of NO-induced reduction of the $[Ca^{2+}]_i$ of smooth muscle cells are not fully understood, but have been proposed to include activation of $Ca^{2+}$-ATPase to increase sequestration of $Ca^{2+}$ from the cytoplasm, inhibition of inositol 1,4,5-trisphosphate ($IP_3$) formation, inhibition of $Ca^{2+}$ release from the intracellular $Ca^{2+}$ stores, and activation of $Ca^{2+}$-activated $K^+$ channels leading to hyperpolarization and blockade of voltage-dependent $Ca^{2+}$ channels (Lincoln & Cornwell, 1993). We have recently shown that $Ca^{2+}$ waves and oscillations are generated in individual vascular smooth muscle cells within intact rat tail arteries when the cells are stimulated via perivascular

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sympathetic nerves or with noradrenaline (Iino, Kasai & Yamazawa, 1994). The oscillatory patterns of $Ca^{2+}$ signalling in intact vascular smooth muscle cells had escaped detection in previous studies, because the $Ca^{2+}$ oscillations are not in phase among individual cells and oscillatory features were lost when the $Ca^{2+}$ response was averaged in multicellular preparations. In the light of this new finding, it is now important to study how NO modulates the $Ca^{2+}$ signalling in individual smooth muscle cells.

In the present study we made the most of a confocal digital imaging technique using a piezoelectric driver of the objective lens to collect the $Ca^{2+}$ signals of individual vascular smooth muscle cells and endothelial cells alternately without destroying the physiological structure of the vascular tissue. We show that agonist-stimulated $Ca^{2+}$ signalling occurs as $Ca^{2+}$ waves and oscillations in both endothelial cells and smooth muscle cells. Furthermore, we demonstrate that $Ca^{2+}$ oscillations in endothelial cells suppress the frequency of the $Ca^{2+}$ oscillations in smooth muscle cells via NO used as an intercellular messenger.

## METHODS

### Tissue preparation

Male Wistar rats, weighing about 40–50 g, were stunned and exsanguinated, as approved by the local ethical committee. Tail arteries (width 300–400 $\mu$m) were excised and carefully cleaned of the surrounding connective tissue and cannulated at one end for luminal perfusion of physiological salt solution (PSS) containing 40 $\mu$M fluo-3 AM and 0·03% cremophor EL for ~1 h. During the dye loading the specimen was bathed in PSS at 30 °C with continuous bubbling of 100% $O_2$. A rectangular glass capillary (300 $\mu$m wide, 40 $\mu$m thick, 10 mm long) was then inserted into the lumen of a segment (about 8 mm long) cut from the specimen. Then it was mounted in a trough machined from a Perspex plate, containing PSS with a coverslip at the bottom. To suppress the movement of the smooth muscle cells we added to the PSS 10 $\mu$M cytochalasin D, a capping agent of actin filaments, which inhibits smooth muscle contraction without changing $[Ca^{2+}]_i$, through uncoupling of the force generation from the activated actomyosin $Mg^{2+}$-ATPase (Saito, Hori, Ozaki & Karaki, 1996).

### Confocal digital imaging

The experimental trough was mounted on the stage of an inverted fluorescence microscope (IX 70, Olympus, Tokyo) equipped with a confocal scanner (FLUOVIEW, Olympus). The temperature of the solution in the trough was kept at 28–30 °C, using an electrical warming plate (Microwarm Plate, Kitazato, Tokyo) which surrounded the trough. The arterial wall was viewed using a water-immersion objective (LUMPlanFL ×40; NA = 0·8; Olympus). The fluorescence intensity with excitation at 488 nm (krypton–argon laser) was digitized at 4096 levels. The 2-D images with 256 × 256 pixels (120 $\mu$m × 120 $\mu$m) were obtained at 1 frame $s^{-1}$, unless otherwise specified. The vertical resolution was < 2·5 $\mu$m, estimated as the full half-maximum width of the light intensity reflected at the edge of a coverslip in water. The image analysis was carried out on a personal computer using the program IP Lab (Signal Analytics Corporation, Vienna, VA, USA).

### Electrical stimulation and solution exchange

The proximal end of the specimen was electrically stimulated (20 V, 0·5 ms, 4 Hz) using a pair of platinum electrodes, one on each side of the preparation, connected to an electric stimulator (SEN-7203, Nihon Koden, Tokyo). The polarity of the electrodes was alternated to minimize polarization. For solution exchange, the solution in the experimental trough was aspirated and a new solution preheated to 30 °C was added to the trough.

### Materials

PSS contained (mM): 150 NaCl; 4 KCl; 2 $CaCl_2$; 1 $MgCl_2$; 5 Hepes; and 5·6 glucose; pH 7·4, adjusted with NaOH. Fluo-3 AM and 4-(4-diethylaminostyryl)-*N*-methylpyridium iodide (4-Di-2-ASP) were purchased from Molecular Probes. Cremophor EL, cytochalasin D, $N^G$-nitro-L-arginine (L-NA), $N^G$-nitro-L-arginine methyl ester (L-NAME) and sodium nitroprusside (SNP) were purchased from Sigma. All other chemicals were of the highest reagent grade available.

## RESULTS

### Imaging of nerve, smooth muscle and endothelial cell layers within intact arterial wall

Figure 1 shows sequential 2-D images of an intact rat tail artery wall stained with fluorescent dyes. The objective lens of the confocal microscope was moved by the piezoelectric driver perpendicular to the arterial wall at 3 $\mu$m intervals and the images were taken parallel to the arterial wall. The artery was loaded with fluo-3 AM, and a single layer of endothelial cells is seen on the luminal surface running parallel to the long axis. Perpendicular to the endothelial cells run smooth muscle cells, which usually form a monolayer but sometimes a two-cell layer. The perivascular sympathetic nerve network was difficult to observe with only fluo-3 loading, but it became clearly visible after brief (15 s) superfusion of the specimen with PSS containing 10 $\mu$M 4-Di-2-ASP (Iino *et al.* 1994).

### Simultaneous $Ca^{2+}$ imaging of endothelial cells and smooth muscle cells

As shown in Fig. 1, endothelial cells could be readily distinguished from smooth muscle cells. The objective lens was moved up and down alternately within 200 ms to focus on either the smooth muscle cell layer or the endothelial cell layer in order to obtain $Ca^{2+}$ images for each layer. Figure 2 shows typical results of such an experiment; the fluorescence images of fluo-3-loaded endothelial and smooth muscle cell layers are shown in Fig. 2*A* and *B*, respectively. The excursion distance of the piezo-electric driver between the layers of endothelial and smooth muscle cells was 6 $\mu$m in this specimen.

Electrical stimulation (4 Hz, 320 pulses) of the perivascular sympathetic nerve induced $Ca^{2+}$ oscillations in individual smooth muscle cells (Fig. 2*D*, areas 4–6, black traces) as shown previously (Iino *et al.* 1994). In the endothelial cells, however, no significant $[Ca^{2+}]_i$ changes were detected during perivascular sympathetic nerve stimulation (Fig. 2*C*, black traces). During the application of 1 $\mu$M ACh, $Ca^{2+}$ oscillations were detected in individual endothelial cells

(Fig. 2*C*, areas 1–3, red dotted traces), whereas the frequency of the sympathetically induced $Ca^{2+}$ oscillations in smooth muscle cells was significantly reduced (Fig. 2*D*, areas 4–6, red dotted traces; Fig. 2*E*). Although we selected the cells whose $Ca^{2+}$ oscillations remained in the presence of ACh in Fig. 2*D*, in the majority of the cells the $Ca^{2+}$ oscillations disappeared or occurred only once as shown in Fig. 2*E*. Therefore, there seemed to be hardly any response when $[Ca^{2+}]_i$ was averaged over the total area (Fig. 2*D*, average, red dotted trace). The effect of ACh was reversed when the drug was washed out: $Ca^{2+}$ oscillations of smooth muscle cells recovered (Fig. 2*E*) and $Ca^{2+}$ oscillations in endothelial cells disappeared (data not shown). We considered a $[Ca^{2+}]_i$ increase event in smooth muscle cells to be a $Ca^{2+}$ oscillation when the peak $F/F_0$ minus 1·0 exceeded 0·6, which was greater than at least 4 times the standard deviation of the baseline $F/F_0$ observed before the initiation of the electrical stimulation. Since the measurement was carried out every 2·4 s in Fig. 2, the peak size of $Ca^{2+}$ oscillations could have been underestimated.

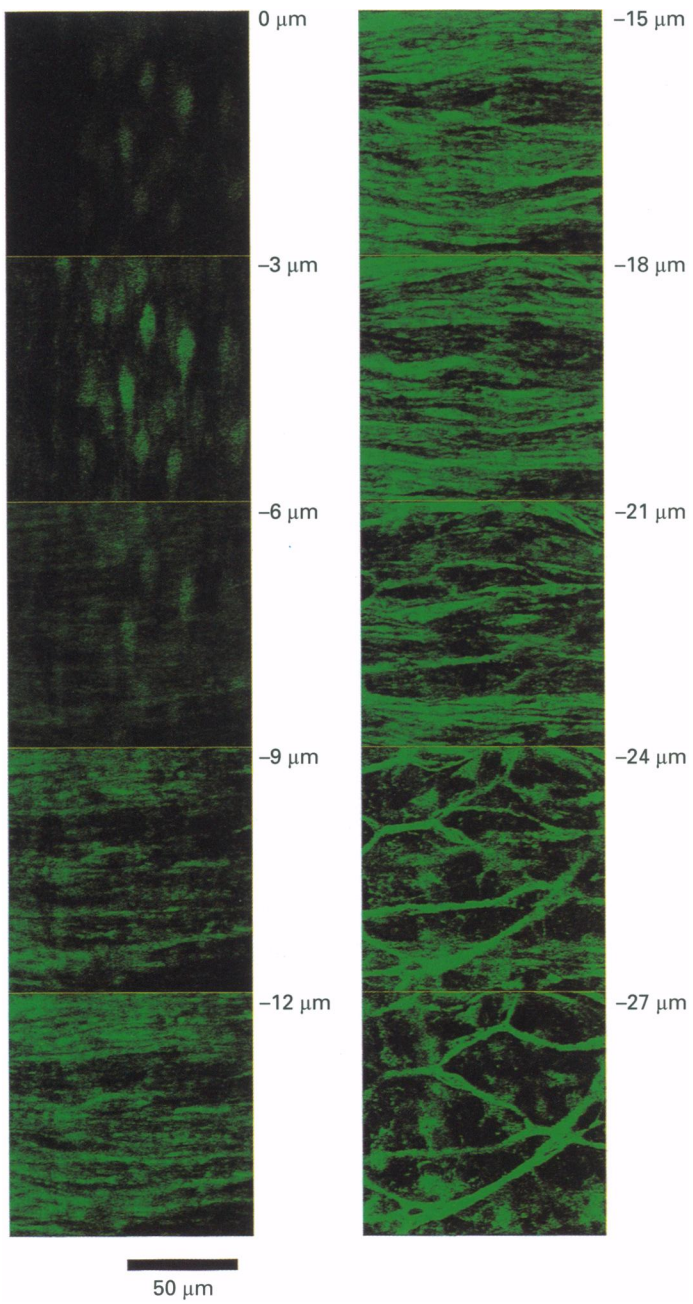


**Figure 1. Sequential fluorescence images of intact rat tail artery wall**

The images were taken at 3 μm intervals from the luminal side to the adventitial side as indicated by each panel. Endothelial and smooth muscle cells were loaded with fluo-3 AM and found to be oriented parallel and perpendicular, respectively, to the long axis of the artery. The perivascular sympathetic network was stained with 4-Di-2-ASP.

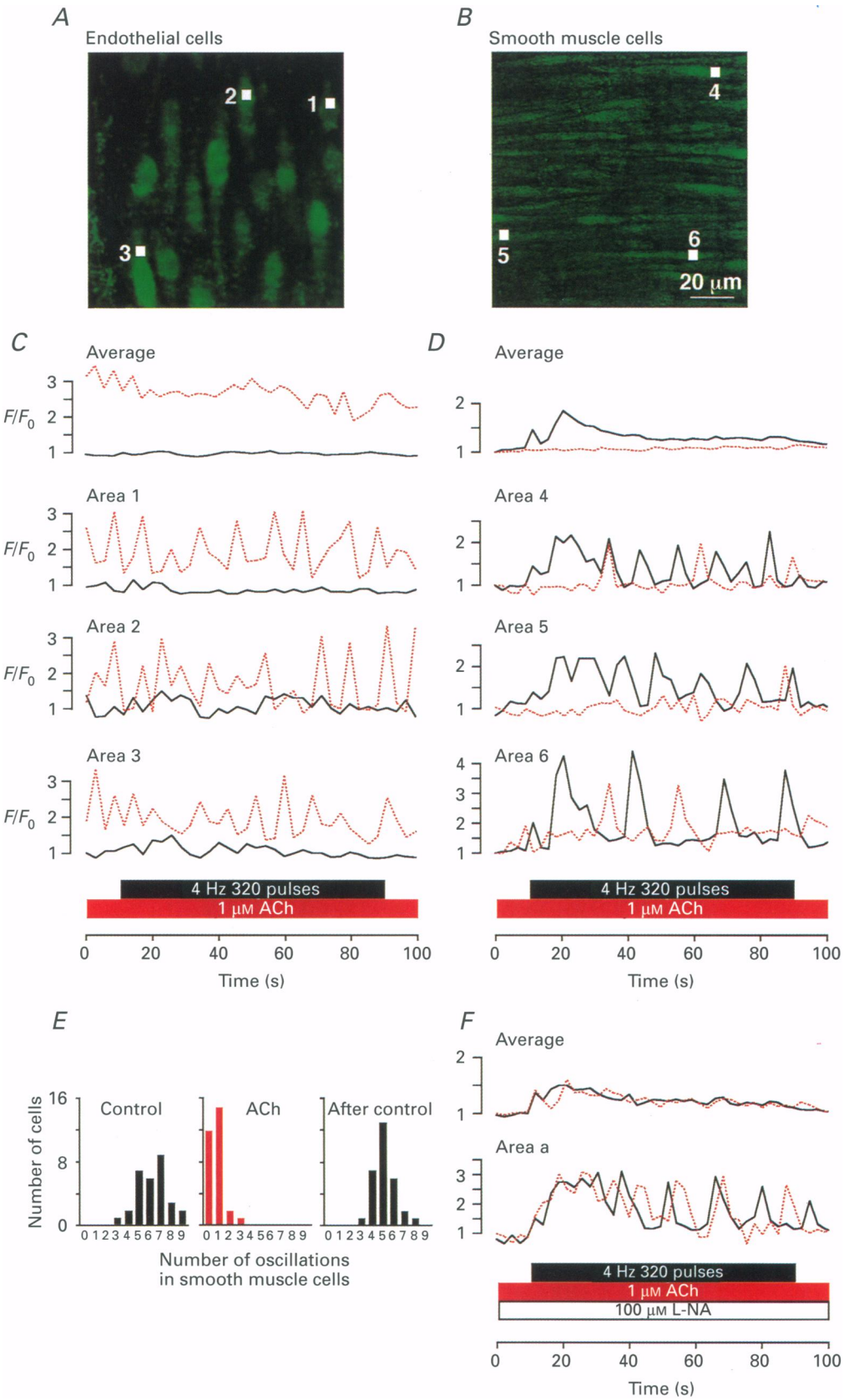


Figure 2. For legend see facing page.

### Effects of NO synthase inhibitor on the $Ca^{2+}$ signalling in smooth muscle cells

To determine whether the inhibitory effect of ACh on $Ca^{2+}$ oscillations in smooth muscle cells involves NO production, we observed the effects of ACh on $Ca^{2+}$ signalling in both endothelial cells and smooth muscle cells in the presence of a NO synthase inhibitor. Either 100–300 $\mu$M $N^G$-nitro-L-arginine (L-NA) or 100 $\mu$M $N^G$-nitro-L-arginine methyl ester (L-NAME) was added to the experimental solution 30 min before we studied the effect of ACh. Although we detected $Ca^{2+}$ oscillations in endothelial cells during ACh application in the presence of the NO synthase inhibitor, we observed no significant reduction of the averaged $Ca^{2+}$ signals and $Ca^{2+}$ oscillations in vascular smooth muscle cells (Fig. 2*F*). These results strongly suggest that the effect of ACh on $Ca^{2+}$ oscillations in smooth muscle cells is mediated by NO.

### $Ca^{2+}$ waves in endothelial cells

To investigate the spatial aspects of the ACh-induced $Ca^{2+}$ signalling in endothelial cells in more detail, we obtained fluorescence images every 0·6 s using a smaller imaging frame (318 × 46 pixels). Part of a representative frame is shown in Fig. 3*A*, which includes images of two endothelial cells. $Ca^{2+}$ images of one of the cells (surrounded by the rectangle) are shown in pseudocolour in Fig. 3*B* and *C*. The fluorescence intensity change of the cell was plotted against time (Fig. 3*D*, red dotted trace), and was compared with the control (black trace), which showed the baseline noise level. It can be seen that the increase in $[Ca^{2+}]_i$ propagated within the cell in the form of a wave along the long axis of the cell at a rate of ~0·3 Hz. Although the $Ca^{2+}$ waves usually started at the distal end (upper end) of the cell shown in Fig. 3*C*, this was not always the case. However, in general, the $Ca^{2+}$ waves tended to commence from either the proximal or the distal ends of the cells. On some occasions it seemed as if the $Ca^{2+}$ waves propagated intercellulary.

### The effect of SNP on $Ca^{2+}$ oscillations in smooth muscle cells

If the effect of ACh on $Ca^{2+}$ oscillations in smooth muscle cells is mediated by NO, sodium nitroprusside (SNP), a NO donor, should have the same effect on the $Ca^{2+}$ signalling in smooth muscle cells. As shown in Fig. 4*A*, the average increase in the $[Ca^{2+}]_i$ of smooth muscle cells induced by electrical stimulation was decreased by 1 $\mu$M SNP application, and the $[Ca^{2+}]_i$ remained at almost the resting level even during the electrical stimulation of the perivascular sympathetic nerve (Fig. 4*A*, average, black *vs.* red trace). Within the representative individual smooth muscle cells, as shown in Fig. 4*A* (areas 1–3), the frequency of $Ca^{2+}$ oscillations in response to the sympathetic stimulation was decreased in the presence of SNP (Fig. 4*C*). However, the peak values of the $Ca^{2+}$ oscillations were almost the same as those of the control. There was no significant difference ($P > 0·5$) between the peak $F/F_0$: 2·03 ± 0·39 (mean ± S.D., $n = 36$, control) and 2·00 ± 0·38 ($n = 36$, SNP). These features are identical to those of ACh-induced inhibition of $Ca^{2+}$ signalling in smooth muscle cells (Fig. 2).

The very first $Ca^{2+}$ transient in the smooth muscle cells during the electrical stimulation of the perivascular sympathetic nerve is likely to have been mediated by ATP, a cotransmitter of noradrenaline (NA) at sympathetic nerve endings, as it was not blocked by $\alpha$-adrenergic antagonists but was inhibited in the presence of suramine, a $P_{2X}$ purinergic receptor antagonist (Iino *et al.* 1994). This initial $Ca^{2+}$ response to the electrical stimulation of the sympathetic nerve was also decreased in the presence of ACh (Fig. 2*D*, average, red trace) or SNP (Fig. 4*A*, average, red trace). This could have been due to an inhibitory effect of NO or cGMP on the ATP-induced response (Andriantsitohaina, Lagaud, Andre, Muller & Stoclet, 1995).

### Effect of SNP on NA-induced $Ca^{2+}$ oscillations in smooth muscle cells

A similar inhibitory effect of SNP on the $Ca^{2+}$ oscillations was observed when the smooth muscle cells were stimulated with NA instead of via the sympathetic nerve as shown in Fig. 4*B*. The application of 1 $\mu$M SNP reduced the increase in the $[Ca^{2+}]_i$ by reducing the frequency of $Ca^{2+}$ oscillations in individual smooth muscle cells (Fig. 4*B*, areas 4–6; Fig. 4*D*). At the same time there was some delay until the first $Ca^{2+}$ response from the initiation of NA application in the presence of SNP. The responsiveness of the smooth muscle cells to NA was recovered after the removal of SNP (data not shown). Despite the significant reduction in the frequency of

**Figure 2. Simultaneous $Ca^{2+}$ imaging of endothelial cells and smooth muscle cells**

Confocal 2-D images of the endothelial cell layer (*A*) and the smooth muscle cell layer (*B*) of the same artery. $Ca^{2+}$ images of the endothelial and smooth muscle cell layers were alternately collected at intervals of 1·2 s. *C* and *D*, fluorescence intensity normalized by the resting value ($F/F_0$) plotted against time for six selected areas (white rectangles in *A* and *B*) of the endothelial cells (areas 1–3) and smooth muscle cells (areas 4–6). The mean $F/F_0$ for the total area of each layer was also plotted against time (Average). Electrical stimulation (4 Hz, 320 pulses) was applied in the absence (black traces) or presence of 1 $\mu$M ACh (red dotted traces). *E*, histogram of the number of $Ca^{2+}$ oscillations in smooth muscle cells during electrical stimulation counted within 30 cells in the imaging field with or without ACh. Representative result of 14 experiments. *F*, $F/F_0$ in smooth muscle cells, plotted in the same manner as in *D*, in the presence (red dotted traces) and absence (black traces) of ACh after pretreatment with 100 $\mu$M L-NA for 30 min. Representative results of a different set of experiments ($n = 3$ for L-NA and $n = 3$ for L-NAME).

$Ca^{2+}$ oscillations, the peak $[Ca^{2+}]_i$ was almost the same with or without SNP. The peak $F/F_0$ were $2{\cdot}35 \pm 0{\cdot}47$ and $2{\cdot}30 \pm 0{\cdot}37$ for control and SNP, respectively ($n = 14$, $P > 0{\cdot}5$).

## DISCUSSION

The present results suggest that $Ca^{2+}$ waves and oscillations in endothelial cells induce NO production, which then modulates the $Ca^{2+}$ signalling of neighbouring smooth muscle cells, via a decrease in the frequency of $Ca^{2+}$ oscillations in individual cells. It has been shown that at different concentrations (0·1–1 $\mu$M) of NA, the frequency of the $Ca^{2+}$ oscillation in the smooth muscle cells is altered with little effect on the amplitude of individual $Ca^{2+}$ oscillations and the baseline $[Ca^{2+}]_i$ (Iino *et al.* 1994). This, together with the present findings, suggests that the frequency modulation of $Ca^{2+}$ oscillation in smooth muscle cells seems to be important in the regulation of vascular tone.

### $[Ca^{2+}]_i$ increase of endothelial cells

Endothelium-dependent relaxation of vascular smooth muscle cells occurs in response to a wide variety of stimuli, including ACh, bradykinin, substance P, histamine, thrombin, adenine nucleotides and mechanical stress. These agonists induce $Ca^{2+}$ oscillations in cultured endothelial cells (Jacob, Merritt, Hallam & Rink, 1988; Missiaen, Lemaire, Parys, De Smedt, Sienaert & Casteels, 1996). $Ca^{2+}$ imaging of intact lung

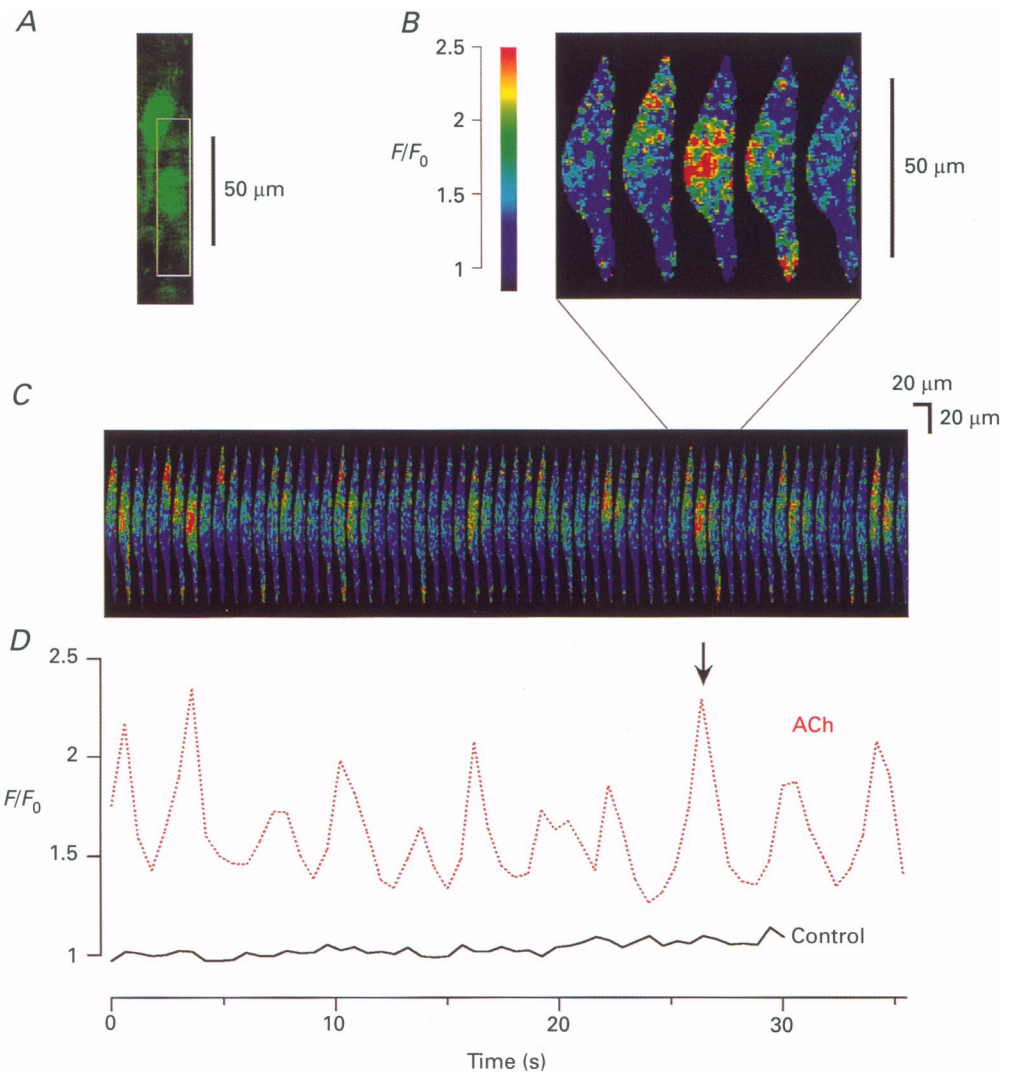


**Figure 3. $Ca^{2+}$ waves and oscillations in endothelial cells within arterial wall**

*A*, fluo-3 fluorescence intensity of two endothelial cells. A subframe (220 × 46 pixels) of the imaging frame is shown. The cell marked with the white rectangle was used for the analysis shown in *B–D*. *B*, selected sequential pseudocolour images (one frame per 0·6 s) showing the fluorescence intensity change in the single endothelial cell stimulated with 1 $\mu$M ACh. *C*, repetitive $Ca^{2+}$ waves in the endothelial cell. The images were compressed in the horizontal direction for clarity. *D*, average fluorescence intensity changes of the cell in *C* (red dotted trace). The control data without ACh were also plotted (black trace). The results are representative of 24 experiments.

capillaries devoid of smooth muscle cells revealed the occurrence of pacemaker-generated inter-cellular $Ca^{2+}$ waves in capillary endothelial cells (Ying, Minamiya, Fu & Bhattacharya, 1996). In the present study we performed $Ca^{2+}$ imaging of arterial endothelial cells within intact arterial walls. We detected $Ca^{2+}$ waves and oscillations during the application of 1 $\mu$M ACh (Fig. 3). $Ca^{2+}$ waves were also evoked upon application of bradykinin or substance P (Y. Kasai, unpublished observation). We identified no typical pacemaker cells in our experiments. Further study is necessary to determine whether $Ca^{2+}$ waves propagate intercellulary in the rat tail artery.

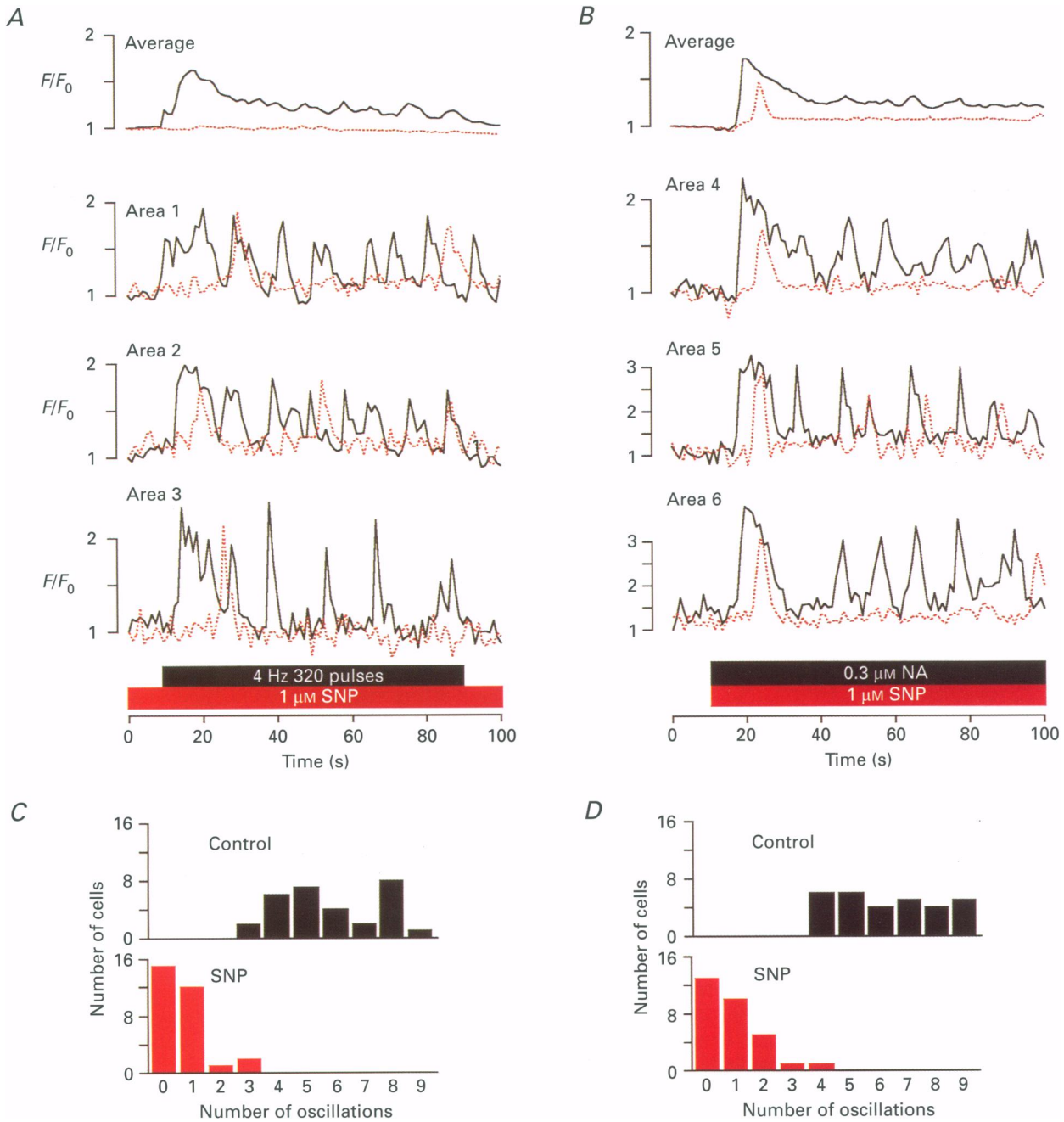


**Figure 4. The effect of SNP on $Ca^{2+}$ oscillations in smooth muscle cells**

Time course of $[Ca^{2+}]_i$ change induced by electrical stimulation (*A*) or 0·3 $\mu$M NA (*B*) in the absence (black traces) or presence of 1 $\mu$M SNP (red dotted traces). The $Ca^{2+}$ signals were collected from a small area within a cell (areas 1–6) or averaged over all the cells in the imaging field (average). The histograms show the numbers of $Ca^{2+}$ oscillations during electrical stimulation (*C*) or NA application (*D*) counted in 30 areas with or without SNP. *A* and *B* are from different preparations. The results are representative of 15 and 8 experiments for *A* and *B*, respectively.

### EDHF and inducible NO synthase

It has been shown that endothelial cells in small resistance arteries release, in addition to NO, a still uncharacterized factor called endothelium-derived hyperpolarizing factor (EDHF). EDHF increases the $K^+$ conductance in smooth muscle cells and shifts the membrane potential closer to the equilibrium potential for $K^+$ (Garland, Plane, Kemp & Cocks, 1995). The resulting hyperpolarization inhibits the activity of voltage-dependent $Ca^{2+}$ channels and $Ca^{2+}$ influx, leading to smooth muscle relaxation. In our experiments, the fact that the effects of ACh on $Ca^{2+}$ oscillations in smooth muscle cells were inhibited by NO synthase inhibitors suggests that they were due mainly to NO. However we cannot completely rule out the possibility that EDHF was additionally involved in the vasorelaxant activity of ACh in our preparations.

NO may also be produced by the inducible NO synthase which may be expressed in smooth muscle cells. Although the function of the inducible NO synthase was not systematically studied, we did not find a significant direct effect of the NO synthase inhibitor on the $Ca^{2+}$ signals in the smooth muscle cells induced by the sympathetic stimulation (Fig. 2*F*, black line).

### Mechanism of NO-induced modulation $Ca^{2+}$ signalling in vascular smooth muscle cells

How does NO or NO donors reduce the frequency of $Ca^{2+}$ oscillations in vascular smooth muscle cells? Since a decrease in the $Ca^{2+}$ oscillation frequency in vascular smooth muscle cells was also detected with a decrease in the NA concentration applied to rat tail arteries (Iino *et al.* 1994), NO may inhibit the $\alpha_1$-adrenergic receptor–G protein-phospholipase C pathway, resulting in reduced production of $IP_3$ (Hirata, Kohse, Chang, Ikebe & Murad, 1990). NO activates G-kinase, which in turn catalyses the phosphorylation of a multitude of proteins in smooth muscle cells. Phosphorylation of phospholamban by G-kinase results in activation of $Ca^{2+}$ pumps in the endoplasmic reticulum (Eggermont, Raeymaekers & Casteels, 1989). This has often been assumed to result in a decrease in the $[Ca^{2+}]_i$ due to enhanced sequestration of $Ca^{2+}$ in the intracellular $Ca^{2+}$ stores. However, an increased frequency of $Ca^{2+}$ oscillation was detected in *Xenopus* oocytes that overexpress $Ca^{2+}$-pump ATPase (Camacho & Lechleiter, 1993). Therefore, further study is necessary to determine whether in fact the phosphorylation of phospholamban leads to a decrease in the $Ca^{2+}$ oscillation frequency in smooth muscle cells. G-kinase also phosphorylates the $IP_3$ receptor type 1 at serine 1755 (Koga, Yoshida, Cai, Islam & Imai, 1994; Komalavilas & Lincoln, 1994). However, the functional significance of the $IP_3$ receptor phosphorylation is not well understood. If it results in decreased affinity of the $IP_3$ receptor to $IP_3$ or inhibition of gating, it may account for the decreased frequency of $Ca^{2+}$ oscillations, although results inconsistent with this hypothesis have been reported in hepatocytes (Rooney, Joseph, Queen & Thomas, 1996). The frequency of $Ca^{2+}$ oscillation may also depend on the influx of $Ca^{2+}$ through the plasma membrane in certain cell types (Girard & Clapham, 1993). G-kinase enhances the activity of $Ca^{2+}$-activated $K^+$ channels through activation of phosphatase 2A (Zhou, Ruth, Schlossmann, Hofmann & Korth, 1996), leading to hyperpolarization of the plasma membrane and a decrease in the rate of $Ca^{2+}$ influx through voltage-dependent $Ca^{2+}$ channels. Although direct inhibition by NO of the activity of voltage-dependent $Ca^{2+}$ channels has been detected in vascular smooth muscle cells (Clapp & Gurney, 1991), conflicting results have been obtained for guinea-pig bladder cells (Bonev & Isenberg, 1992). Further work is necessary to clarify the molecular mechanism of NO-induced modulation of $Ca^{2+}$ signalling in smooth muscle cells.


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