

# Intercellular calcium signaling and nitric oxide feedback during constriction of rabbit renal afferent arterioles

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<sup>1</sup>Physiology and Pharmacology, University of Southern Denmark, Odense, Denmark; and <sup>2</sup>Department of Pharmacology, University of Hong Kong, Faculty of Medicine Building, Hong Kong SAR, China

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**Uhrenholt TR, Schjerning J, Vanhoutte PM, Jensen BL, Skøtt O.** Intercellular calcium signaling and nitric oxide feedback during constriction of rabbit renal afferent arterioles. *Am J Physiol Renal Physiol* 292: F1124–F1131, 2007. First published December 5, 2006; doi:10.1152/ajprenal.00420.2006.—Vasoconstriction and increase in the intracellular calcium concentration ( $[Ca^{2+}]_i$ ) of vascular smooth muscle cells may cause an increase of endothelial cell  $[Ca^{2+}]_i$ , which, in turn, augments nitric oxide (NO) production and inhibits smooth muscle cell contraction. This hypothesis was tested in microperfused rabbit renal afferent arterioles, using fluorescence imaging microscopy with the calcium-sensitive dye fura-2 and the NO-sensitive dye 4-amino-5-methylamino-2',7'-difluorescein. Both dyes were loaded into smooth muscle and endothelium. Depolarization with 100 mmol/l KCl led to a transient vasoconstriction which was converted into a sustained response by *N*-nitro-L-arginine methyl ester (L-NAME). Depolarization increased smooth muscle cell  $[Ca^{2+}]_i$  from  $162 \pm 15$  nmol/l to a peak of  $555 \pm 70$  nmol/l ( $n = 7$ ), and this response was inhibited by 80% by the L-type calcium channel blocker calciseptine. After a delay of 10 s,  $[Ca^{2+}]_i$  increased in endothelial cells immediately adjacent to reactive smooth muscle cells, and this calcium wave spread in a nonregenerative fashion laterally into the endothelial cell layer with a velocity of  $1.2 \mu\text{m/s}$ . Depolarization with 100 mmol/l KCl led to a significant increase in NO production ( $[NO]_i$ ) which was inhibited by L-NAME ( $n = 5$ ). Acetylcholine caused a rapid increase in endothelial  $[Ca^{2+}]_i$ , which did not transfer to the smooth muscle cells. L-NAME treatment did not affect changes in smooth muscle  $[Ca^{2+}]_i$  after depolarization, but it did increase the calcium sensitivity of the contractile apparatus. We conclude that depolarization increases smooth muscle  $[Ca^{2+}]_i$  which is transferred to the endothelial cells and stimulates NO production which curtails vasoconstriction by reducing the calcium sensitivity of the contractile apparatus.

blood flow; smooth muscle cell contraction

THE INTERACTION BETWEEN the endothelium and vascular smooth muscle in resistance arteries is of major importance for the local regulation of blood flow. The endothelium affects the smooth muscles by release of vasodilator and vasoconstrictor substances. However, the vascular smooth muscles may also influence the endothelium. Dora et al. (11) suggested the existence of an intravascular feedback mechanism between smooth muscle and endothelium in hamster cheek pouch arterioles. They observed that phenylephrine and KCl caused a vascular constriction that was accompanied by an elevation in endothelial cell intracellular  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ). They further observed an enhancement of the vasoconstriction induced by both agents after blockade of nitric oxide (NO) synthesis. Based on these findings, they suggested that a

vasoconstrictor-mediated increase in smooth muscle  $[Ca^{2+}]_i$  is transferred to the endothelial cells where it activates endothelial NO synthase and enhances formation of NO, which diffuses back to the smooth muscle and reduces/buffers contraction. On the other hand, deformation of the endothelium during vasoconstriction and consequent release of NO have also been described (22, 23).

However intriguing this hypothesis appears, it has proved difficult to provide unequivocal evidence in its favor. Thus the sequence of events that leads to transfer of calcium signals from smooth muscle to endothelium has not been directly demonstrated in the same blood vessel, and production of NO has not been measured by direct techniques in this setting.

For several reasons, renal afferent arterioles are ideal candidates to test this hypothesis. First, they exhibit a transient response to depolarization, which is consistent with the above hypothesis; second, they only have a single layer of smooth muscle cells, which optimizes the ability of NO to influence vascular function. This feature also makes them ideally suited for digital imaging fluorescence techniques to visualize changes in  $[Ca^{2+}]_i$  in different vascular layers and at the same time to monitor changes in vascular diameter.

The NO feedback hypothesis was therefore tested in microperfused renal afferent arterioles that had been dissected from rabbits. Vascular diameters were observed directly, and a technique was developed that allowed endothelial and smooth muscle cell  $[Ca^{2+}]_i$  to be measured separately in the same individual blood vessels using fura-2 dye. NO was measured by the fluorescent dye DAF-FM.

## MATERIALS AND METHODS

*Isolation and microperfusion of renal arterioles.* Animal care followed the guidelines of National Institutes of Health, and permissions were obtained from the Danish Animal Experiments Inspectorate under the Danish Ministry of Justice. New Zealand rabbits (male, 2,000–2,700 g) obtained from Harlan AD were killed by a blow to the head and exsanguinated. This procedure conformed to the American Veterinary Medical Association guidelines for euthanizing laboratory animals (1). Male New Zealand rabbits ( $n = 47$ ) were given a standard chow and had free access to tap water. Renal afferent arterioles were microdissected and perfused with concentric glass pipettes in a movable track system (see online supplement; all supplementary material is available in the online version of this article) (16, 27). During a 30-min equilibration period, the temperature was raised to  $37^\circ\text{C}$ . In each experiment, a test stimulus of high potassium (100 mmol/l KCl) was given to demonstrate viability of the arteriole. All drugs were applied ablymally.

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**Fluorescence imaging.** Optical techniques were used to measure  $[Ca^{2+}]_i$  in smooth muscle and endothelial cells using fluorescent probe Fura-2/AM (Molecular Probes).  $[Ca^{2+}]_i$  was measured by using a  $Ca^{2+}$  imaging system (TILL Photonics GmbH, Gräfelfing, Germany; the online version of this article contains supplemental data). Fura-2/AM ( $8 \times 10^{-7}$  mol/l) was applied to the abluminal site of the afferent arterioles for 45 min ( $37^\circ C$ ) followed by 20 min of washout to ensure proper deesterification. Afferent arterioles were visualized by excitation at 380 nm using TILL Photonics Polychromator IV and Zeiss  $\times 40$  (0.75 numerical aperture) objective. Full-frame images were collected at both excitation wave lengths (340 and 380 nm) using a 510-nm long-pass filter. The images were collected at a frame rate of 2 Hz during stimulation and 0.5 Hz during washout. The 380-nm image collection was used to measure luminal diameter changes simultaneously with the calcium measurements. Ratio (R) images were generated by TILLVision Software (TILL Photonics GmbH) and analyzed by tracking smooth muscle or endothelial cells during contraction and relaxation. The ratio images were calculated on a pixel-to-pixel basis from the following equation:  $R = F_{340}(x,y,t)/F_{380}(x,y,t)$ . The relatively slow contraction did not cause any pixel shifts between the acquisition of the 340- and 380-nm images.  $[Ca^{2+}]_i$  was calculated according to Grynkiewicz et al. (13):  $[Ca^{2+}]_i = K_{eff} \cdot (R - R_{min}) / (R_{max} - R)$ , where  $R_{min} = 0.1$ ,  $R_{max} = 1.6$ , and  $K_{eff} = 2,673$  nmol/l, as determined by standard calibrations (see supplemental data).

A cell-permeable fluorescent NO indicator, DAF-FM DA, was used to detect NO production in afferent arterioles. The cells were abluminally loaded with 50  $\mu$ mol/l DAF-FM DA in 5% DMSO for 45 min ( $33^\circ C$ ) and then washed for 20 min. DAF-FM was excited at 488

nm, and emitted fluorescence was recorded using a 510-nm dichromatic mirror and a 510-nm long-pass filter. Regions of interest corresponding to one smooth muscle cell were selected, and mean intensity within the regions of interest was recorded. At the end of every experiment, maximal fluorescence was evaluated by adding  $10^{-4}$  mol/l sodium nitroprusside (SNP). Relative changes in DAF-FM fluorescence intensity (F) were calculated with the delta changes in F divided by  $F_{max}$ . Relative changes =  $(F_1 - F_0)/F_{max}$ .

**Drugs.** *N*-nitro-L-arginine methyl ester (L-NAME), SNP, and acetylcholine were from Sigma (Vallensbaek, Denmark) and calciseptine was from Alomone Labs (Jerusalem, Israel).

**Statistics.** Data were analyzed with IGOR PRO v5 (WaveMetrics, Lake Oswego, OR) and SigmaPlot v8 (Systat Software, Point Richmond, CA) software. Unless otherwise stated, the data were presented as means  $\pm$  SE. Statistical significance was tested with Student's *t*-test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## RESULTS

**L-NAME and  $K^+$ -induced vasoconstriction.** In microperfused afferent arterioles, elevated extracellular  $[K^+]$  caused a concentration-dependent reduction in arteriolar diameter. In the control arterioles, 65 mmol/l KCl was necessary to elicit half-maximal contraction, while at 40 mmol/l KCl, virtually no response was observed (Fig. 1A). After treatment with L-NAME ( $10^{-4}$  mol/l, 20 min), the  $K^+$  concentration-response curve was shifted significantly to the left. A small contraction

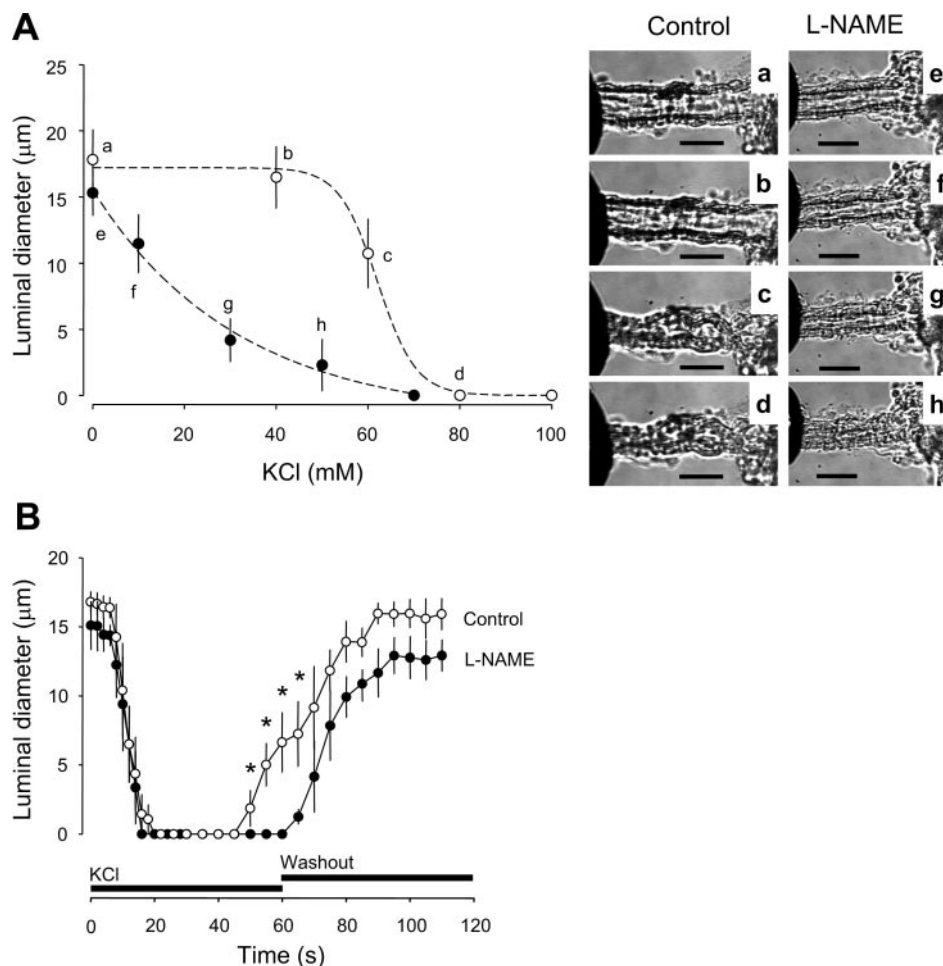


Fig. 1. *A*: concentration-response curve for KCl and time course of changes in vessel diameter. KCl (10–70 mmol/l) caused vasoconstriction in perfused arterioles pretreated with *N*-nitro-L-arginine methyl ester (L-NAME;  $10^{-4}$  mol/l, ●,  $n = 5$ ) while higher concentrations of KCl (60–100 mmol/l) was needed to induce a vasoconstriction in untreated perfused arterioles (○,  $n = 5$ ). Transmission images (a–h) correspond to different points on the concentration-response curve as indicated. *B*: time course of luminal diameter changes in perfused arterioles stimulated with 100 mmol/l KCl in control series ( $n = 7$ ) and in series treated with L-NAME ( $10^{-4}$  mol/l;  $n = 5$ ). In control arterioles, spontaneous relaxation was observed after 40 s. Data shown as means  $\pm$  SE. \* $P < 0.05$  compared with maximal contraction. Black bars represent 20  $\mu$ m.

was already seen at 10 mmol/l  $K^+$  and at 50 mmol/l  $K^+$ , the arterioles were maximally contracted. The half-maximal contraction was observed at 20 mmol/l KCl. In the absence of L-NAME, the response to 100 mmol/l KCl was transient vasoconstriction: arterioles were exposed to elevated KCl for 60 s, but 40–45 s after applying  $K^+$ , the arteriole consistently dilated (Fig. 1B). This dilatation during  $K^+$  depolarization was prevented by L-NAME ( $10^{-4}$  mol/l; Fig. 1).

**$[Ca^{2+}]_i$  in vascular smooth muscle in response to elevated  $K^+$ .** One-hundred mmol/l  $K^+$  caused an increase in  $[Ca^{2+}]_i$  in the vascular smooth muscle cells from a resting level of  $162 \pm 15$  nmol/l ( $n = 7$ ) to a peak of  $555 \pm 70$  nmol/l ( $n = 7$ ; Fig. 2). Treatment with the L-type calcium channel inhibitor calciseptine (100 nmol/l,  $n = 5$ ) did not affect baseline calcium concentration (Fig. 2B), whereas the peak calcium concentration after depolarization was inhibited by 78% (Fig. 2C). The ability of calciseptine to inhibit depolarization-induced increase in  $[Ca^{2+}]_i$  was fully reversible (Fig. 2A).

**Sequential changes in  $[Ca^{2+}]_i$  in vascular smooth muscle cells and endothelial cells.** When loading of the Fura2-AM was achieved simultaneously in the smooth muscle and endothelial cells, it was possible to follow sequential changes in  $[Ca^{2+}]_i$  in both cell types in the same afferent arteriole. Elevated extracellular  $[KCl]$  led to a rapid increase in vascular smooth muscle cell  $[Ca^{2+}]_i$  and contraction (Fig. 3A). Ten to 15 s later, the

$[Ca^{2+}]_i$  increased in the underlying endothelial cells (Fig. 3, A and B). The endothelial  $[Ca^{2+}]_i$  reached a maximal plateau  $\sim 40$  s after addition of  $K^+$ , which coincided with the onset of relaxation (compare Figs. 3B and 1B). The maximal rate of the increase in  $[Ca^{2+}]_i$  during depolarization was equal to the  $[Ca^{2+}]_i$  decrease during washout (Fig. 3C). The response to KCl was heterogeneous. Not all vascular smooth muscle cells responded to KCl. Only cells that responded with an increase in  $[Ca^{2+}]_i$  also displayed contraction. This behavior made it possible to observe a calcium wave that propagated into the endothelial cell layer. It originated in endothelial cells close to the contracting smooth muscle cell and propagated in both upstream and downstream directions. The velocity of the calcium wave was 1.13–1.44  $\mu\text{m/s}$  ( $n = 6$ ) and its amplitude diminished with increasing distance from the initiation site. The calcium wave did not show regenerative characteristics (Fig. 3D).

**Acetylcholine and  $[Ca^{2+}]_i$ .** Abluminal application of acetylcholine ( $10^{-6}$  mol/l,  $n = 5$ , data not shown) induced a rapid sustained increase in  $[Ca^{2+}]_i$  in the endothelial cells (Fig. 4A). In contrast to the calcium response in smooth muscle cells that propagated into the endothelial cell layer, the  $[Ca^{2+}]_i$  increase which originated in endothelial cells did not propagate into the vascular smooth muscle (Fig. 4A). The velocity of the increase in endothelial  $[Ca^{2+}]_i$  induced by acetylcholine was 10 times faster (Fig. 4B) compared with depolarization-induced increase in  $[Ca^{2+}]_i$ . The fall in endothelial  $[Ca^{2+}]_i$  during washout of acetylcholine was similar to that observed after washout of KCl (Fig. 4B, from 60 s).

**Vasoconstriction and NO.** During the full constriction that was achieved after exposure to elevated  $[K^+]$ , the vascular geometry was unchanged. This made possible to measure NO production in a time period of 40 s (Fig. 5A). The DAF-FM fluorescence signal increased significantly at 40 s. This coincided with the time at which endothelial  $[Ca^{2+}]_i$  achieved the maximal level (Fig. 3) and was followed by vasodilatation (Fig. 1). In the presence of L-NAME ( $10^{-4}$  mol/l,  $n = 5$ ), NO production did not increase (Fig. 5B).

**NO and calcium in the smooth muscle cell layer.** After exposure to KCl, smooth muscle  $[Ca^{2+}]_i$  increased to reach a peak at  $\sim 30$  s after the initiation of depolarization (Fig. 6A). Thereafter, SMC  $[Ca^{2+}]_i$  decreased. Incubation with L-NAME ( $10^{-4}$  mol/l,  $n = 5$ ) had no significant effect on  $[Ca^{2+}]_i$  at any point during depolarization or during washout (Fig. 6A). As shown in Fig. 6B, the rates of changes in smooth muscle  $[Ca^{2+}]_i$  were unaffected by L-NAME. At the time where  $[Ca^{2+}]_i$  in the endothelium was maximal (30–60 s), the rate of  $[Ca^{2+}]_i$  change was similar in the presence or absence of L-NAME.

The simultaneous measurements of smooth muscle  $[Ca^{2+}]_i$  and luminal diameter in individual experiments made it possible to characterize the relationship between these parameters within a complete contraction cycle after exposure to KCl (Fig. 7). In the untreated arterioles (Fig. 7B), the resting diameter was  $\sim 16$   $\mu\text{m}$ , and  $[Ca^{2+}]_i$  was  $\sim 165$  nmol/l. Exposure to 100 mmol/l KCl led to a vasoconstriction, which was complete at  $\sim 20$  s, and was associated with an increase in  $[Ca^{2+}]_i$  to  $\sim 420$  nmol/l. The calcium concentration continued to increase for the next 10 s while the vascular lumen was still occluded. Then, at 40, 50, and 60 s, when  $[Ca^{2+}]_i$  was still over 400 nmol/l, a

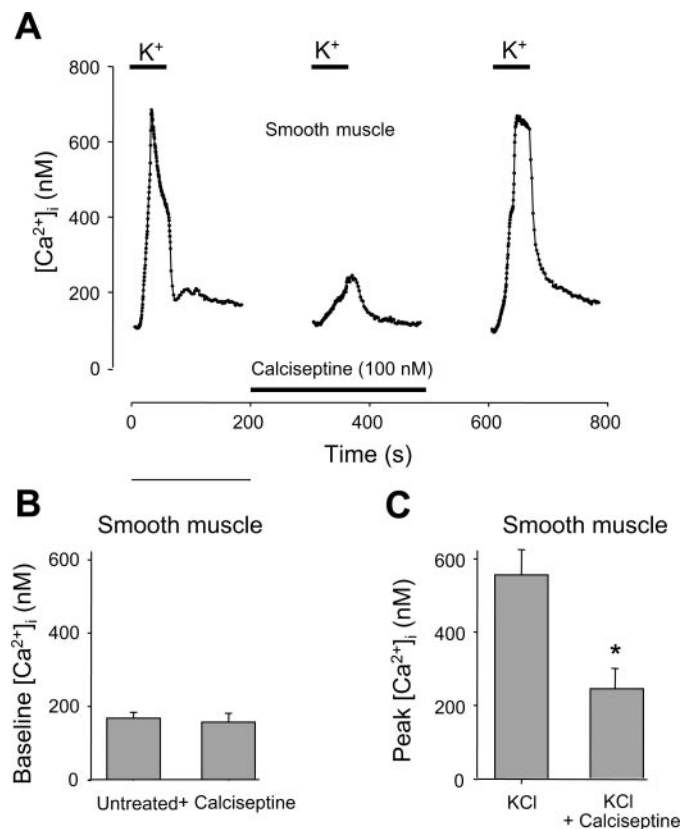


Fig. 2. A: depolarization-induced changes in  $[Ca^{2+}]_i$  in a smooth muscle cell in the vascular wall before, during, and after treatment with calciseptine (100 nmol/l). B: baseline  $[Ca^{2+}]_i$  in smooth muscle cells in untreated perfused arterioles ( $n = 7$ ), and arterioles treated with calciseptine (100 nmol/l,  $n = 5$ ). C: KCl induced peak in  $[Ca^{2+}]_i$  in smooth muscle cells in perfused arterioles ( $n = 7$ ) and perfused arterioles treated with calciseptine (100 nmol/l,  $n = 5$ ). Data are mean values  $\pm$  SE. \* $P < 0.05$ .

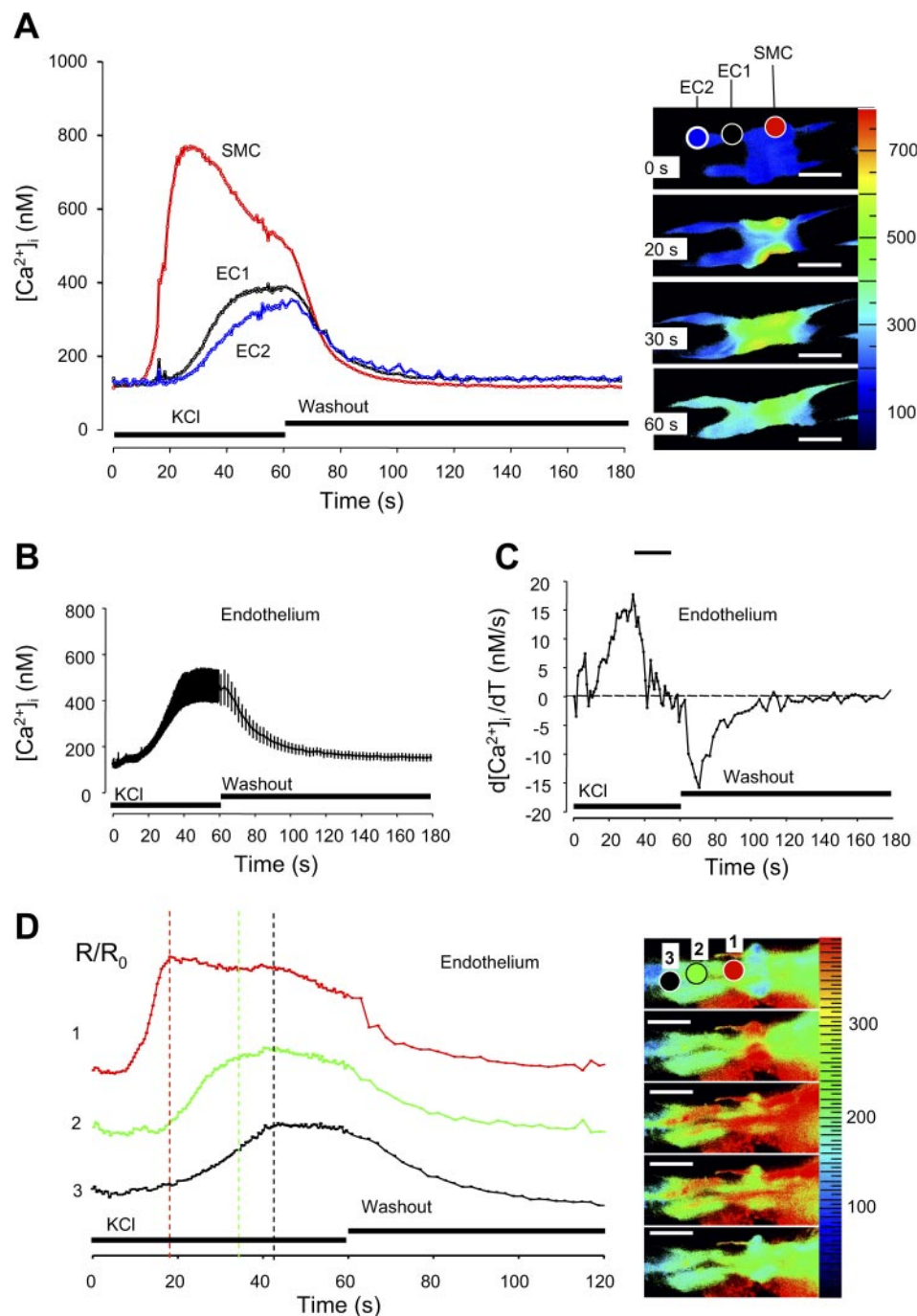


Fig. 3.  $[Ca^{2+}]_i$  in smooth muscle and endothelial cells during depolarization-induced vasoconstriction. *A, left*: experiment from one arteriole showing changes in  $[Ca^{2+}]_i$  in smooth muscle (SMC) and in two endothelial cells (EC1, EC2) after exposure to 100 mmol/l  $K^+$  and during washout. *Right*: ratiometric ( $F_{340}/F_{380}$ ) pseudocolor images of the arteriole at 0, 20, 30, and 60 s. *Right* scale bar represents pseudocolor translation into  $[Ca^{2+}]_i$  in nmol/l. *B*: average  $[Ca^{2+}]_i$  in the endothelial cell closest to the corresponding active smooth muscle cell measured during and after exposure to 100 mmol/l  $K^+$ . The response maximum was  $459 \pm 69$  nmol/l ( $n = 5$ ). Data shown as means  $\pm$  SE. *C*: differentiation of curve in *B* shows the rate of increase and decrease of  $[Ca^{2+}]_i$  in endothelial cells. The mean peak rates for increase and removal of  $[Ca^{2+}]_i$  were equal ( $12 \text{ nmol} \cdot \text{l}^{-1} \cdot \text{s}^{-1}$ ). *D*: time course of changes in endothelial  $[Ca^{2+}]_i$  closest to the active smooth muscle cell (1), and in two cells further downstream (2, 3). Vertical dashed lines represent time markers used for velocity measurements. Wave velocity and amplitude were highest in the beginning (velocity =  $1.13\text{--}1.44 \mu\text{m/s}$ ;  $n = 4$ ). Values in traces are  $R/R_0$ . *Right*: ratiometric images at 0, 10, 30, 40, and 120 s. *Right* scale bar represents translation of pseudocolors into calcium concentrations (in nmol/l). *Left*: areas used to generate the 3 traces are shown as circles in the top panel. White bars represent  $20 \mu\text{m}$ .

significant continuous vasodilatation took place. This vasodilatation occurred simultaneously with the increase in endothelial  $[Ca^{2+}]_i$  (Fig. 3). After 60 s, KCl was washed out, the calcium concentration decreased, and the arteriole dilated back to the resting level. In the presence of L-NAME, the relationship between smooth muscle  $[Ca^{2+}]_i$  and vascular diameter was similar to that of untreated arterioles up to 40 s after exposure to high KCl (Fig. 7C). However, from 40 to 60 s, where the  $[Ca^{2+}]_i$  in the smooth muscles decreased from  $\sim 500$  to  $\sim 420$  nmol/l, the lumen of the arterioles was still totally occluded. After 60 s, the high KCl was removed,  $[Ca^{2+}]_i$  decreased, and the arteriole dilated back to the resting diameter.

## DISCUSSION

This study presents direct evidence in intact microperfused renal afferent arterioles that a depolarization-induced increase in smooth muscle cell  $[Ca^{2+}]_i$  is followed by increased endothelial  $[Ca^{2+}]_i$  and production of NO, which curtails contraction most likely by a decreased calcium sensitivity of adjacent smooth muscle cells.

To test the hypothesis that an initial increase in  $[Ca^{2+}]_i$  in the smooth muscle cells was followed by an increase in  $[Ca^{2+}]_i$  in the endothelium, a technique was developed to allow measurements simultaneously and in real time of changes in diameter and  $[Ca^{2+}]_i$  in smooth muscle and endothelial cells

Fig. 4.  $[Ca^{2+}]_i$  in smooth muscle and endothelium during stimulation with acetylcholine. *A*: acetylcholine ( $10^{-6}$  mol/l) induced a rapid increase in endothelial  $[Ca^{2+}]_i$ , while no changes were seen in the smooth muscle cells. The vessel selected for ratio images in this time series is the same vessel as shown in Fig. 3A. For a movie of this experiment, see online supplement. *B*: differentiation of the data presented in *A* shows a fast rate of calcium increase during stimulation with acetylcholine while the rate of decrease of  $[Ca^{2+}]_i$  during the washout period (60–180 s) was much slower. The results are compared with the rate of changes in  $[Ca^{2+}]_i$  during and after exposure to high KCl. White bars represent 20  $\mu$ m.

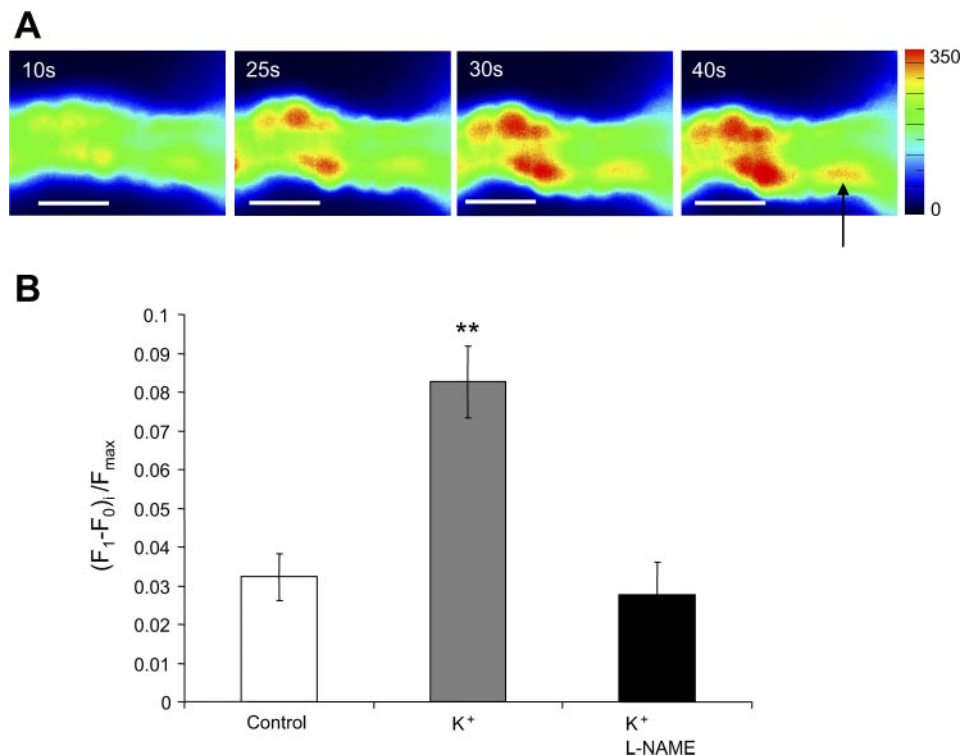
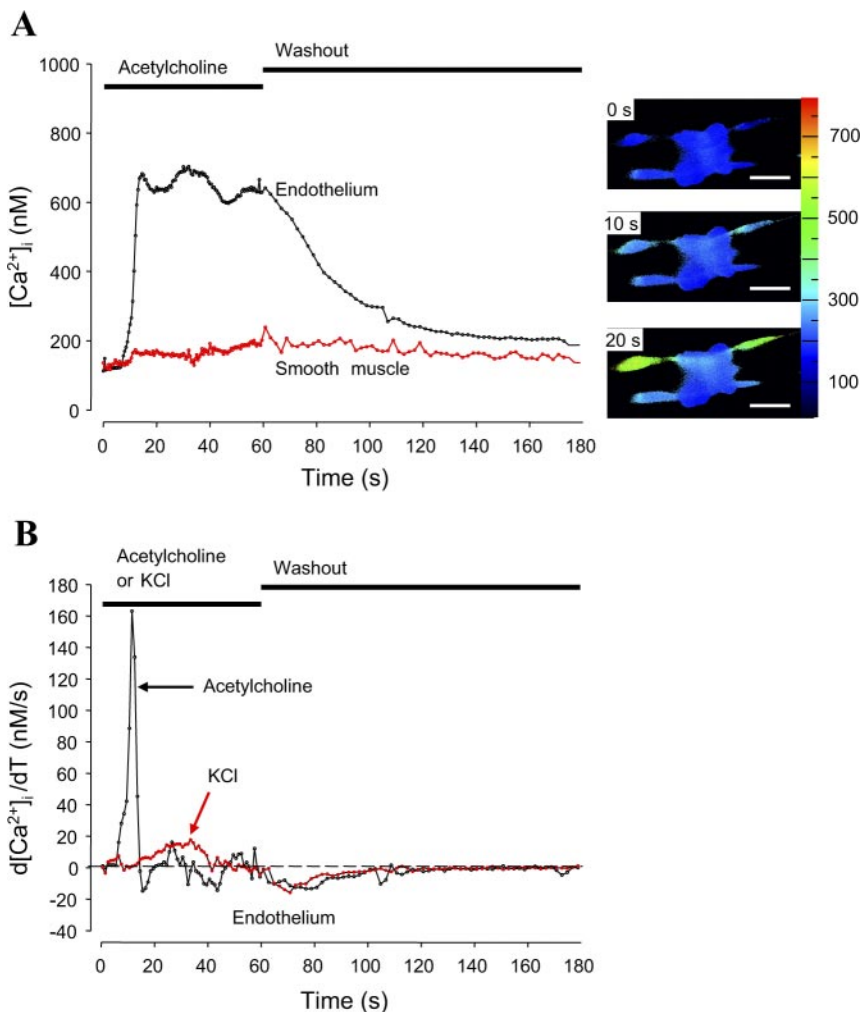


Fig. 5. *A*: captured images of  $[NO]_i$  in perfused afferent arteriole in a single experiment. The arteriole was exposed to 100 mmol/l KCl. The images represent the time at 10, 25, 30, and 40 s. Arrow indicates endothelial cell. *B*: summarized data from 5 experiments. The arterioles were exposed to 100 mmol/l KCl (gray) and compared with control (white) and arterioles pretreated with L-NAME ( $10^{-4}$  mol/l;  $n = 5$ ) before exposure to KCl (black). White bars represent 20  $\mu$ m. Data are shown as means  $\pm$  SE. \*\* $P < 0.01$ .

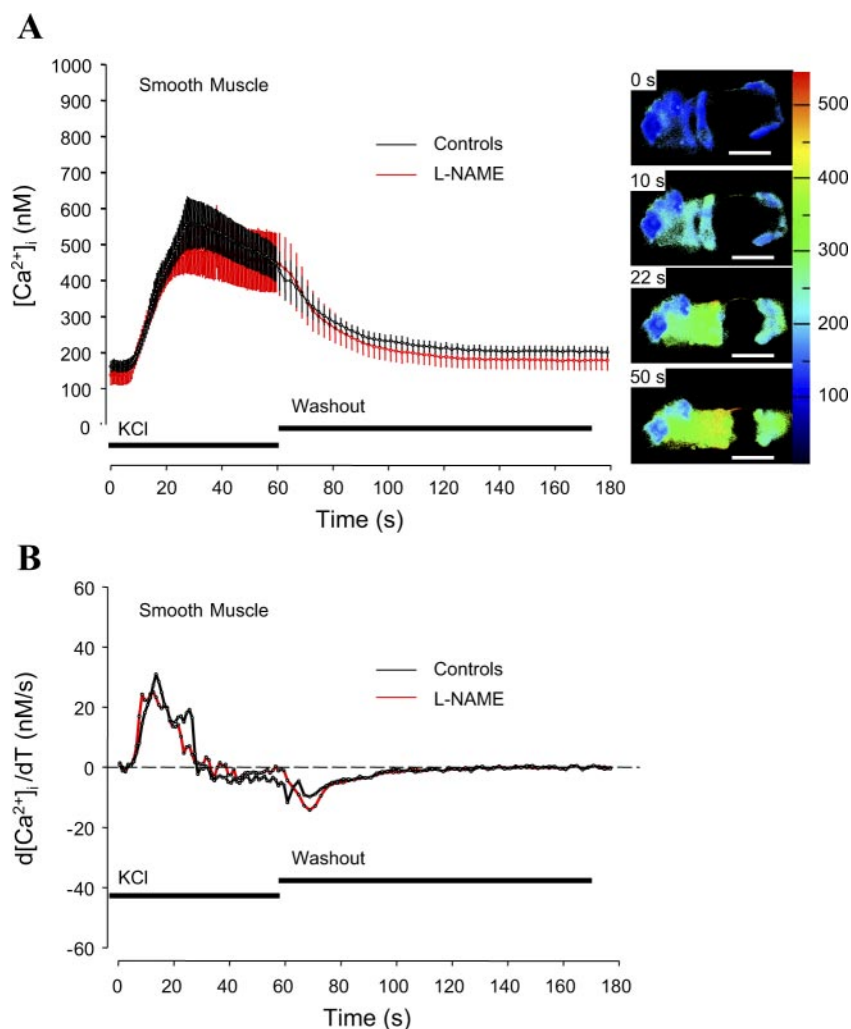


Fig. 6. Time course of  $[Ca^{2+}]_i$  changes in smooth muscle cells in perfused arterioles during depolarization-induced vasoconstriction. *A*: KCl induced  $[Ca^{2+}]_i$  changes in smooth muscle cells in perfused arterioles ( $n = 7$ ) and arterioles treated with L-NAME (red;  $10^{-4}$  mol/l;  $n = 5$ ). Data are shown as means  $\pm$  SE. *Right*: selected ratiometric images of changes in smooth muscle  $[Ca^{2+}]_i$  at different time points in an untreated perfused afferent arteriole during contraction. *Right* scale bar represents pseudocolor translation in nmol/l  $Ca^{2+}$ . For a movie of this experiment, please see online supplement. *B*: differentiation of the data from *A* shows identical changes in calcium rates in untreated (black) and arterioles treated with L-NAME treated (red). White bars represent 20  $\mu$ m.

(see online supplement for details) of a true resistance vessel. Smooth muscle  $[Ca^{2+}]_i$  was measured continuously at the edge of midplane sections of the arterioles, thereby avoiding inclusion of emission light from the endothelial cells, and endothelial cell  $[Ca^{2+}]_i$  was measured in “windows” between dye-loaded smooth muscle cells.

Depolarization with potassium was chosen as the standard vasoconstrictor stimulus to avoid direct activation of endothelial cell membrane receptors by various hormones. Depolarization of afferent arterioles leads to activation of voltage-dependent calcium channels of L-, P-, and Q-type (2, 15, 16), and accordingly an L-type calcium channel blocker inhibited 80% of the depolarization-induced increase in smooth muscle cell  $[Ca^{2+}]_i$ . Voltage-dependent calcium channels ( $Ca_v$ ) have been described in endothelial cells (2, 4, 15), but most of these are of small conductance and have little functional importance (17). Indeed, agonist-induced  $Ca^{2+}$  entry into endothelial cells is diminished by depolarization of the plasma membrane with high potassium solutions (17, 19) and inhibitors of voltage-dependent calcium channels do not affect agonist-induced  $Ca^{2+}$  entry into endothelial cells (20, 30). A lack of involvement of endothelial  $Ca_v$  in the present vascular responses to depolarization is also supported by the observation that there was a delay of 10–15 s between the calcium increase in the

smooth muscle and the endothelium. Furthermore, the endothelial calcium waves originated only at the sites where the smooth muscle cells were activated. Using a high potassium concentration as a stimulus also excludes endothelial hyperpolarizing factor as a contributor to the observed responses.

In the microperfused renal afferent arteriole, inhibition of NO formation had a profound effect on the vasoconstrictor effect of depolarization. Thus 65 mmol/l potassium only caused half-maximal contraction in the afferent arterioles while this potassium concentration is associated with full contraction in other vascular preparations (9). Because of the low potassium sensitivity, exposure to a  $K^+$  concentration of 100 mmol/l for 1 min was used as the standard stimulus. The vasoconstriction induced by  $K^+$  was transient with relaxation after  $\sim 40$  s. Experiments with prolonged exposure to high  $K^+$  (3 min) showed that release of the vasoconstriction was not complete, but stabilized at a level of  $\sim 23\%$  of maximal constriction. Both the low sensitivity and transient contraction in response to  $K^+$  were reversed after inhibition of NO synthase with L-NAME, which suggests that NO synthesis is crucial for the blunting of  $K^+$  sensitivity.

Simultaneous measurements of  $[Ca^{2+}]_i$  in smooth muscle and endothelium showed that depolarization led to a rapid increase in  $[Ca^{2+}]_i$  in the smooth muscle. This was followed by

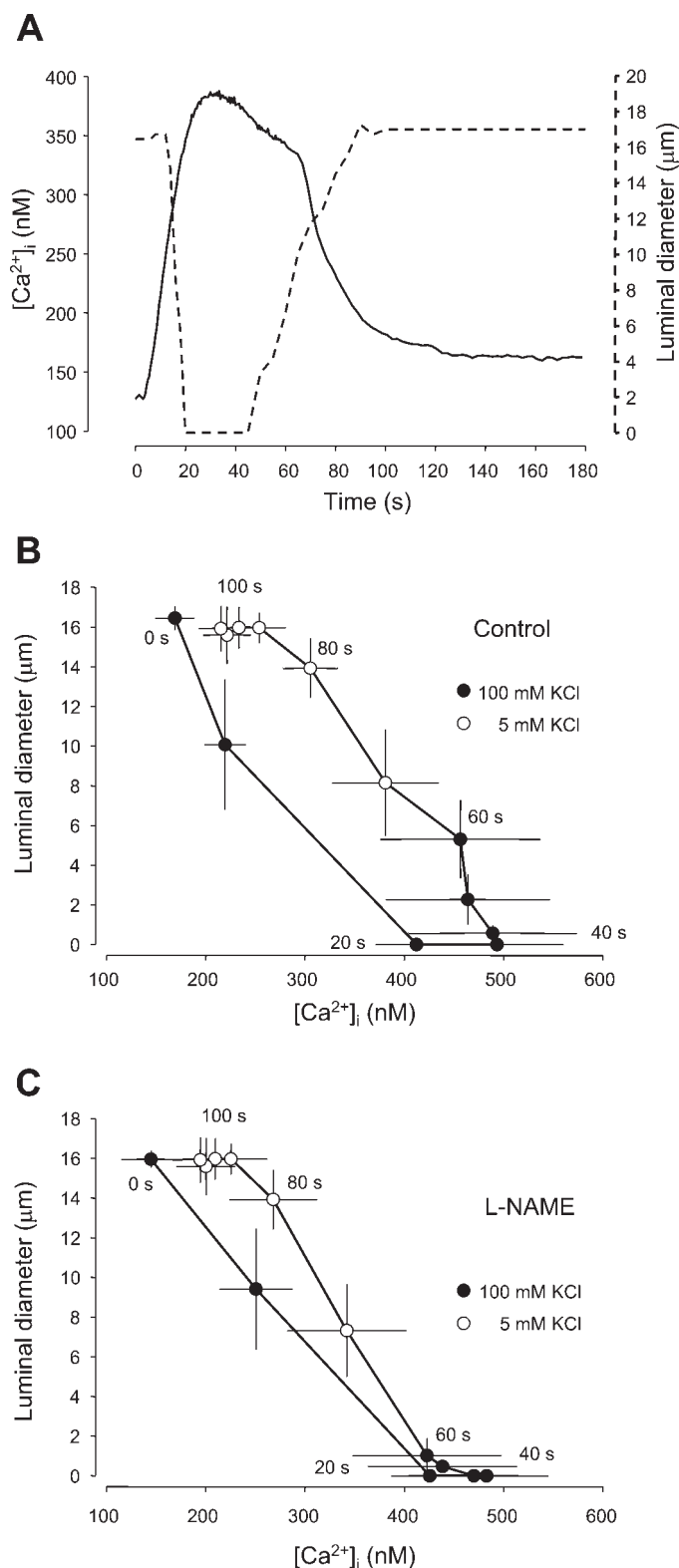


Fig. 7. *A*: simultaneous measurements of  $[Ca^{2+}]_i$  and luminal diameter of a perfused afferent arteriole in a single experiment.  $F_{380}$  images were used to determine luminal changes in diameter and the  $F_{340}/F_{380}$  ratios were used to calculate the simultaneous changes in calcium concentration. *B*: relationship between luminal diameter and  $[Ca^{2+}]_i$  during a full contraction-relaxation cycle induced by high  $K^+$  in perfused arterioles ( $n = 7$ ). The arterioles were exposed to 100 mmol/l KCl from 0 to 60 s (filled symbols). Washout took place from 70 to 120 s (open symbols). *C* as in *B*, but arterioles were treated with L-NAME ( $10^{-4}$  mol/l;  $n = 5$ ) before exposure to KCl. Data are means  $\pm$  SE.

vasoconstriction and an increase in  $[Ca^{2+}]_i$  in the adjacent endothelial cell. In the vasoconstricted phase, the geometry of the blood vessel was constant, and this allowed use of the cumulative nonratiometric dye DAF-FM to estimate production of NO. Consistent with the tested hypothesis, these measurements demonstrated that NO production increased during this time and coincided with the increase in endothelial calcium concentration. The increase in NO production was prevented by L-NAME. There was a striking temporal coincidence between peak endothelial  $[Ca^{2+}]_i$  and NO accumulation after 40 s of exposure to elevated  $K^+$ , exactly at a time when the arteriole relaxed despite the presence of  $K^+$ . This is compatible with a causal and sequential coupling between the rise in endothelial  $[Ca^{2+}]_i$  and calmodulin-dependent activation of eNOS and release and diffusion of NO. The mechanism by which the increase in smooth muscle  $[Ca^{2+}]_i$  leads to increase in endothelial  $[Ca^{2+}]_i$  is not addressed in the present study. Myoendothelial coupling in afferent arterioles may allow diffusion of  $Ca^{2+}$  or  $IP_3$  through gap junction (3, 14, 25, 26), and release of extracellular messengers may occur or opening of mechano-sensitive endothelial cation channels may be initiated by the vasoconstriction (8, 20, 22–24, 32). The latter mechanism may provide one explanation of why calcium is transferred from smooth muscle to endothelium during vasoconstriction, while an increase in endothelial  $[Ca^{2+}]_i$  induced by acetylcholine was not transferred to the smooth muscle cells.

Treatment of the afferent arterioles with L-NAME to block production of NO led to a significant enhancement of vasoconstriction, while it had no effect of the changes in the intracellular calcium concentrations in the smooth muscle cells after depolarization with  $K^+$ . This demonstrates that NO feedback in afferent arterioles did not cause vasodilatation by reducing the  $[Ca^{2+}]_i$ . In the arterioles with intact NO production, a vasodilatation began despite a continuous high SMC  $[Ca^{2+}]_i$ . Thus, in isolated perfused renal afferent arterioles, NO formation must cause vasodilatation by reducing the calcium sensitivity of the contractile apparatus. The most likely explanation is cyclic GMP-dependent stimulation of the myosin light chain phosphatase resulting in a rightward shift of the  $[Ca^{2+}]_i$  sensitivity curve (5, 28).

In contrast to the present findings, the  $EC_{50}$  for  $K^+$ -induced contraction in rat renal arcuate arteries with an internal diameter  $\sim 170 \mu\text{m}$  is 27 mmol/l and is unchanged by L-NAME (29). The segmental heterogeneity in responsiveness is consistent with the view that the ability of NO to influence vascular function is optimal in resistance microvessels like afferent arterioles that have only one layer of smooth muscle.

In addition to providing evidence for the NO feedback mechanism between smooth muscle and endothelium, the study also demonstrates a calcium wave in the endothelium initiated beneath contracting smooth muscle cells. The velocity of the calcium wave was slow,  $\sim 1.2 \mu\text{m/s}$ , and it decreased in amplitude when spreading from one cell to the next, suggesting that the mechanism was not self-regenerative. The distance over which this slow endothelial calcium wave is likely to mediate efficient signal transfer is therefore short (50–100  $\mu\text{m}$ ). However, the renal afferent arteriole is also short and therefore an endothelial calcium wave appears suited to provide a local signaling mechanism within this important resistance vessel. Only little is known about  $Ca^{2+}$  movements between endothelial cells (12, 21). An endothelial calcium

wave has been reported to occur in lung capillaries (31) and in cultured endothelial cells (10), but it has never before been demonstrated in perfused resistance vessels and as the result of a signal coming from the smooth muscle.

The present study provides direct evidence for the hypothesis that depolarization-induced vasoconstriction of the afferent arteriole is followed by an increase in calcium concentration in the endothelium, spreading of a calcium wave along the endothelium with stimulation of NO formation and reduction in calcium sensitivity of the contractile apparatus in vascular smooth muscle. This mechanism may functionally reduce the effect of any vasoconstrictor and thereby contribute to the increased susceptibility to vasoconstrictors in conditions with endothelial dysfunction, such as in hypertension.

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