Ca$^{2+}$-Images of Smooth Muscle Cells and Endothelial Cells in One Confocal Plane in Femoral Artery Segments of the Rat

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ABSTRACT—Simultaneous recording of Ca$^{2+}$-images in one confocal plane from vascular smooth muscle cells (SMCs) and endothelial cells (ECs) of an intact rat femoral artery segment was performed using indo-1 and a confocal microscope. During application of 10$^{-6}$M acetylcholine (ACh), elevation and oscillation of intracellular Ca$^{2+}$ concentration ($[\text{Ca}^{2+}]_i$) were observed in ECs but not in SMCs. Sequential conduction of Ca$^{2+}$ oscillation from an EC to the neighboring ECs in one longitudinal direction was often observed in the presence of ACh. On the other hand, the activation of voltage-dependent Ca$^{2+}$ channels by external 30 mM K$^+$ resulted in the elevation of $[\text{Ca}^{2+}]_i$ only in SMCs. When 10$^{-6}$M ACh was added in the presence of 30 mM K$^+$, it was observed in one confocal plane that $[\text{Ca}^{2+}]_i$ in ECs and SMCs was almost simultaneously increased and decreased, respectively. The simultaneous recording method in this intact preparation will provide a line of valuable information about the interactions between SMCs and ECs, based on spatio-temporal analyses of absolute values of $[\text{Ca}^{2+}]_i$ in individual cells.

Keywords: Ca$^{2+}$-imaging, Rat femoral artery, Endothelium, Confocal microscope

Vascular endothelial cells release many vasoactive substances such as nitric oxide (NO), endothelin and prostaglandins, which modulate physiological activity of vascular smooth muscle cells (SMCs) and endothelial cells (ECs) per se as well. In particular, NO is a multi-functional modulator that mediates apoptosis, neurotransmission, immune response and vasodilation (1–4). Production of NO is regulated by activity of endothelial NO synthase, which is dependent on endothelial intracellular Ca$^{2+}$ concentration ($[\text{Ca}^{2+}]_i$) (1, 5). Of importance is, therefore, understanding of mechanisms involved in regulation of endothelial $[\text{Ca}^{2+}]_i$. On the other hand, physiological significance of interactions between ECs and SMCs via hormonal, mechanical and electrical couplings has been proposed (6–8). Since these factors affect $[\text{Ca}^{2+}]_i$ in ECs as well as SMCs and substantially change the amount of NO released from ECs, the integral examination in intact tissue preparation allows us to understand the regulatory mechanisms under physiological and/or patho-physiological conditions. The recent development of confocal microscope enabled us to visualize individual SMCs and ECs in artery segments (9–12). The spatio-temporal coupling between SMCs and ECs was, however, not fully understood because of the technical difficulty to measure $[\text{Ca}^{2+}]_i$ simultaneously in ECs and SMCs, whereas both the endothelial and smooth muscle layers were alternatively focused using novel equipment (11). Since ratiometric indicators have not been used yet in this aim, the absolute $[\text{Ca}^{2+}]_i$ of individual SMCs and ECs in intact vascular segments remains to be determined. In the present study, Ca$^{2+}$-images of SMCs and ECs from an intact rat femoral artery segment were simultaneously measured in one confocal plane. The ratiometric Ca$^{2+}$-images were obtained by use of indo-1 as an indicator.

MATERIALS AND METHODS

Male Wistar rats, weighing 190–240 g, were anesthetized with ether, stunned and killed by bleeding. All experiments were carried out in accordance with guiding principles for the care and use of laboratory animals (the Science and International Affairs Bureau of the Japanese Ministry of Education, Science, Sports and Culture) and also with the approval of the ethics committee in Nagoya City University. Femoral artery and aorta were dissected from rats and cut into segments of 5 mm in length and opened along the vessel axis. Arterial diameter in femoral arterial segments was approximately 0.6 mm. The tissue was loaded

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Images of Femoral Artery Segments

with Ca\textsuperscript{2+} indicator, indo-1 AM, by incubation in the normal HEPES solution containing 25 \(\mu\)M indo-1 AM and 0.05% cremophor EL for 1 h at room temperature. After washing for 0.5 h, the segment was placed on a chamber (0.5 ml in volume) with the endothelial layer down (Fig. 1). The chamber was placed on the stage of a confocal microscope. As shown in Fig. 1A, the rat femoral artery segment has undulation of membrane elastica interna, which exists between smooth muscle and endothelial layers. The segment was loaded with a weight of 0.3 g to make a flat confocal plane and to minimize the movement of the tissue.

Ca\textsuperscript{2+}-imaging of cells in an intact femoral artery segment

Ca\textsuperscript{2+}-images were obtained using a confocal microscope (RCM8000; Nikon, Tokyo) and Ratio3 software (Nikon) (13, 14). Laser excitation light of 355 nm was applied from the bottom of the chamber through a water-immersion objective (Fluor 40, 1.15 NA, Nikon). The emission light was split into two ranges by filters centered at 405 and 485 nm and detected by photomultipliers. The Ca\textsuperscript{2+}-images were exhibited as ratio of fluorescence intensity, \(F_{405}/F_{485}\). The resolution of the microscope was approximately 0.34 \(\mu\)m \(\times\) 0.27 \(\mu\)m \(\times\) 0.5 \(\mu\)m (x, y and z direction). Eight Ca\textsuperscript{2+}-images taken sequentially once every 33 ms were averaged and stored on optical disk cartridges (LM-A410; Panasonic, Osaka) by a rewritable optical disk recorder (LQ-4100A, Panasonic). Stored data were replayed later and analyzed by Ratio3 software. The averaged ratio Ca\textsuperscript{2+}-image of 171 \(\times\) 138 \(\mu\)m was obtained every 2 s. The fluorescence signal used to calculate \([\text{Ca}^{2+}]_i\) was measured in a whole-cell area and averaged. The fluorescence signal of SMCs was sometimes week because the endothelial layer probably prevented sufficient loading of SMCs with indo-1AM. Therefore, to calculate \([\text{Ca}^{2+}]_i\) in SMCs, we chose SMCs whose fluorescence signals of 405 and 485 nm were greater than 60 out of 256 gray levels. For the analysis of the reduction in \([\text{Ca}^{2+}]_i\), we chose SMCs whose fluorescence signals were greater than 80 gray levels. The experiments were performed at 25 \(\pm\) 1°C. In some experiments, we applied 0.3 \(\mu\)M phentolamine to block \(\alpha\) adrenoceptors in the artery segments, but nevertheless phentolamine did not affect any Ca\textsuperscript{2+} response of ECs and SMCs.

Solutions and drugs

Normal HEPES-buffered solution contained: 137 mM NaCl, 5.9 mM KCl, 2.2 mM CaCl\textsubscript{2}, 1.2 mM MgCl\textsubscript{2}, 14 mM glucose and 10 mM HEPES. High K\textsuperscript{+} HEPES solution contained: 112.9 mM NaCl, 30 mM KCl, 2.2 mM CaCl\textsubscript{2}, 1.2 mM MgCl\textsubscript{2}, 14 mM glucose and 10 mM HEPES. The pH of these solutions was adjusted to 7.4 with 10 N NaOH. The following chemicals and drugs were used in the present study: indo-1 AM (Dojindo, Kumamoto), cremorhor EL (Sigma, St. Louis, MO, USA), acetylcholine chloride (ACh) and phentolamine (Wako Pure Chemical Industries, Osaka).

Statistical analyses

Data are shown as the mean ± S.E.M. in the text and figures. Statistical significance was evaluated using the paired or unpaired Student’s t-test. The symbol * and ** indicate statistical significance at \(P\) values of 0.05 and 0.01, respectively.
RESULTS

Figure 2 shows two dimensional fluorescence images of an intact rat femoral arterial (A) and aortic segment (B) visualized with Indo-1. The fluorescence images at 485 nm were taken at confocal planes adjusted at nearly 0 and 10 μm from the bottom of the tissue. In these images, the vessel axis was the vertical direction and the cells along the vertical direction were, therefore, ECs. On the other hand, the cells along the horizontal direction in the plane at 10 μm in the femoral artery and aorta were SMCs. It is notable that, in the image of femoral artery in the plane at 10 μm, both ECs and SMCs were observed and can be readily distinguished from each other by their shapes and running directions (the upper panel in Fig. 2A). Based on the image of the femoral artery at near 0 μm (the lower panel in Fig. 2A), it can be assumed that the ECs located in the inner parts of the undulation in membrane elastic intima remained without being peeled off during the experimental procedure. In contrast, ECs in the aortic segment were homogeneously distributed in the inner wall (the lower panel in Fig. 2B). A major advantage of using the rat femoral artery segment was, therefore, that Ca²⁺-images of both SMCs and ECs were obtained simultaneously at one confocal plane.

Figure 3 shows Ca²⁺-images of ECs in rat femoral artery segment during the response to ACh. Ca²⁺-images were taken at a confocal plane adjusted at 10 μm from the bottom of the tissue. In Fig. 3B, Ca²⁺-images of five ECs before and after the application of 10 μM ACh were selected and shown in pseudocolor. Changes in [Ca²⁺] in two representative ECs (red and blue lines) and the averaged values from all ECs (black line) were plotted against time in Fig. 3A.

![Figure 2](image-url)

**Fig. 2.** Fluorescence images of segments from a rat femoral artery (A) and aorta (B). Images obtained as the 485-nm emission signal were taken in the confocal planes at near 0 (lower panels) and 10 μm (upper panels) from the bottom of the segment as shown in the illustration at the top. Vessel axis is the vertical direction in these images.
and compared with the control (broken lines). During the application of 10 μM ACh, Ca^{2+} oscillations were detected in individual ECs. Frequency and amplitude of Ca^{2+} oscillations in ECs varied from cell to cell (0.14 ± 0.01 Hz and 129 ± 37 nM, respectively, n = 7 in 3 arterial segments). Moreover, Ca^{2+} oscillations were propagated from an EC to neighboring ECs in one longitudinal direction. When ACh was removed, Ca^{2+} oscillations in ECs disappeared (not shown). ACh did not affect [Ca^{2+}]_i in SMCs (n>20).

Figure 4 shows Ca^{2+}-images of SMCs in rat femoral artery segments that were stimulated with 30 mM K^+. Ca^{2+}-images were taken at a confocal plane adjusted at 10 μm from the bottom of the tissue. Ca^{2+}-images of eight to ten SMCs before and after the application of 30 mM K^+ are shown in pseudocolor in Fig. 4B, and the changes in [Ca^{2+}]_i in two representative SMCs (red and blue lines)
and the averaged values from all SMCs (black line) are plotted against time. The [Ca^{2+}]_i before the application of 30 mM K^+ are shown as broken lines in Fig. 4A. During the application of 30 mM K^+, tonic elevation of [Ca^{2+}]_i was observed in 95% SMCs, while Ca^{2+} oscillations were detected in only 5% SMCs (6 out of 120 cells in 6 arterial segments). In SMCs whose fluorescence signals of 405 and 485 nm were greater than 80 out of 256 gray levels, [Ca^{2+}]_i was tonically elevated from 133 ± 66 nM by 30 mM K^+ (n = 12 in 6 arterial segments, P<0.01). Pretreatment of the artery segment with nifedipine abolished the 30 mM K^+-induced Ca^{2+} response of SMCs, suggesting that activation of voltage-dependent Ca^{2+} channels (VDCCs) is responsible for the elevation of [Ca^{2+}]_i. The application of
30 mM K⁺, however, did not affect [Ca²⁺]ᵢ in ECs (185 ± 14 and 236 ± 31 nM in the absence and presence of 30 mM K⁺, respectively; n = 6 in 5 arterial segments; P > 0.05).

To analyze the spatio-temporal interactions between SMCs and ECs, Ca²⁺-images of both SMCs and ECs were simultaneously obtained in a confocal plane adjusted at 10 μm from the bottom of the segment. Effects of ACh on both SMCs and ECs were examined in the presence of 30 mM K⁺ (Fig. 5). Changes in [Ca²⁺]ᵢ in two representative ECs and SMCs were plotted against time in Fig. 5A. Corresponding Ca²⁺-images in pseudocolor are shown in Fig. 5B. In the presence of 30 mM K⁺, tonic elevation and/or oscillatory changes in [Ca²⁺]ᵢ in SMCs were observed (black and blue lines in Fig. 5A, Ca²⁺-images at 0 and 8 s in Fig. 5B). Thereafter, addition of 10 μM ACh induced the elevation and the oscillation of [Ca²⁺]ᵢ in individual ECs.
(Fig. 5A, red and green lines, Ca\textsuperscript{2+}-images at 100, 150 and 200 s), whereas [Ca\textsuperscript{2+}], in SMCs was substantially reduced in a synchronous manner with the increase in [Ca\textsuperscript{2+}] in ECs (Fig. 5A, black and blue lines). When [Ca\textsuperscript{2+}], in ECs was elevated by ACh from 236 ± 31 to 447 ± 57 nM (n = 6 in 5 arterial segments, P<0.05), that in SMCs was significantly reduced from 403 ± 66 to 283 ± 44 nM (n = 12 in 6 arterial segments, P<0.01). On the other hand, the rate of elevation of [Ca\textsuperscript{2+}], in ECs was similar to that of the reduction in SMCs (r: 11 ± 3 vs 18 ± 6 s, respectively, P>0.05).

**DISCUSSION**

The experiments described here demonstrate that simultaneous Ca\textsuperscript{2+}-imaging of SMCs and ECs in one confocal plane is possible in an intact artery segment. It is particularly noteworthy that in the present study, individual cellular [Ca\textsuperscript{2+}], in the intact artery segments was estimated with a ratiometric Ca\textsuperscript{2+} indicator, indo-1. By use of this spatio-temporal analyses of Ca\textsuperscript{2+}-images, we showed that ACh-induced elevation of Ca\textsuperscript{2+} in ECs is tightly associated with decrease in [Ca\textsuperscript{2+}] in neighboring SMCs in the intact femoral artery segment.

It is well known that vasoactive substances such as ACh, bradykinin and substance P elevate [Ca\textsuperscript{2+}], in ECs to facilitate NO release (1). In the presence of these stimuli, the global Ca\textsuperscript{2+} in individual ECs, which was detected as averaged [Ca\textsuperscript{2+}], was tonically increased (15, 16). In single dispersed ECs, oscillatory and/or sustained increase in [Ca\textsuperscript{2+}], are observed when these stimulants are present. Although observations of changes in endothelial [Ca\textsuperscript{2+}], in intact artery segments are quite limited (10, 12), it has been shown that individual ECs elicit Ca\textsuperscript{2+} waves and oscillations during the application of ACh in intact rat tail artery segments. Consistently, we detected similar Ca\textsuperscript{2+} oscillations and the intercellular Ca\textsuperscript{2+} waves in intact femoral artery segments during the application of ACh, while the frequency of Ca\textsuperscript{2+} oscillations was slightly different (the present study: 0.14 Hz, Kasai et al. (10): 0.3 Hz). Preliminary observations indicate that ECs in intact rat aortic and guinea pig cerebral artery segments also showed Ca\textsuperscript{2+} oscillations during the application of 10 μM UTP and 0.1 μM bradykinin, respectively (Y. Ohi and K. Muraki, unpublished observation), suggesting that the oscillatory changes in [Ca\textsuperscript{2+}], during the response to agonists are common phenomena in intact vascular ECs.

When concentration of external K\textsuperscript{+} was increased to 30 mM, the elevation of global [Ca\textsuperscript{2+}], via the activation of VDCCs in SMCs and sustained contraction were exhibited. It has been reported that, in the presence of 30 mM K\textsuperscript{+}, the frequency of Ca\textsuperscript{2+} sparks, which are rarely observed in intact artery segments under the control conditions where cells are superfused with solution containing 5.9 mM K\textsuperscript{+}, is also increased (9). Low sampling frequency of Ca\textsuperscript{2+}-images did not allow us to detect any Ca\textsuperscript{2+} sparks in SMCs in femoral artery segments that were exposed to 30 mM K\textsuperscript{+}. However, 5% of SMCs had rhythmic oscillatory changes in [Ca\textsuperscript{2+}] under these conditions, hence implying that Ca\textsuperscript{2+} sparks-like events occurred in certain SMCs and were responsible for initiation of the oscillation (9, 17, 18). On the other hand, against the previous observation that endothelial [Ca\textsuperscript{2+}], was reduced by high K\textsuperscript{+} (19), it was not the case in the present study. Since 30 mM K\textsuperscript{+} depolarizes ECs only to approximately ~30 mV, the reduction in [Ca\textsuperscript{2+}], may not be sufficient to be detected. Consistently, 61 mM K\textsuperscript{+} significantly decreased [Ca\textsuperscript{2+}], in rat coronary arterial ECs in situ, whereas 16 mM K\textsuperscript{+} did not (19).

In the present study, the temporal coupling of Ca\textsuperscript{2+} signaling between SMCs and ECs was visualized in intact femoral artery segments. The increase in endothelial [Ca\textsuperscript{2+}], by ACh was associated with the decrease in SMCs and the rate of their responses was similar (r: approx. 10 s), suggesting a tight coupling of Ca\textsuperscript{2+} response between ECs and SMCs. In co-culture of SMCs with ECs, application of ATP increased [Ca\textsuperscript{2+}], in ECs, while this reduced it in SMCs (20). Since the treatment with NO synthase inhibitors abolished the ATP-induced Ca\textsuperscript{2+} response of SMCs, it is postulated that NO released from ECs affects SMCs as a paracrine factor. Although comparative studies about Ca\textsuperscript{2+}-imaging of ECs and SMCs in intact vascular segments are quite limited, Kasai et al. have clearly shown that the Ca\textsuperscript{2+} response of rat tail arterial cells to perivascular nerve stimulation is effectively inhibited by ACh where the oscillatory Ca\textsuperscript{2+} response of ECs occurs (10). When rat femoral artery segments were pre-contracted by 60 – 90 mM K\textsuperscript{+}, ACh-induced vasodilation was completely inhibited in the presence of NO synthase inhibitors, suggesting that NO released from ECs is involved in the response (21). However, we cannot rule out the possibility that endothelial derived hyperpolarization factors were additionally involved in the vasodilation by ACh in our preparation where 30 mM K\textsuperscript{+} was used as the stimulus (22).

A ratiometric dye, indo-1, is more advantageous than fluo-3 and -4 to estimate [Ca\textsuperscript{2+}], and visualize Ca\textsuperscript{2+}-images in ECs and SMCs in intact vascular segments. In previous studies, Ca\textsuperscript{2+}-images of ECs and SMCs in intact vascular segments were visualized with fluo-3, and therefore the absolute changes in [Ca\textsuperscript{2+}], were totally unclear (9 – 12). In the present study, we demonstrated that [Ca\textsuperscript{2+}], in SMCs did not completely return to the resting level in the presence of ACh (280 nM vs 150 nM). In contrast, preliminary experiments showed that 10 μM ACh abolished contraction induced by 30 mM K\textsuperscript{+} in rat femoral artery segments (100.6 ± 11.0% reduction, n = 7). These suggest that the reduction in [Ca\textsuperscript{2+}], in SMCs may not play the central role in vasodilation by ACh in rat femoral artery. Further experiments
are needed to elucidate the physiological significance of the partial reduction in \([Ca^{2+}]\), in SMCs.

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