Role of smooth muscle cells on endothelial cell cytosolic free calcium in porcine coronary arteries

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Bu`del, Stéphane, Alexander Schuster, Nikos Stergiopoulos, Jean-Jacques Meister, and Jean-Louis Bény. Role of smooth muscle cells on endothelial cell cytosolic free calcium in porcine coronary arteries. Am J Physiol Heart Circ Physiol 281: H1156–H1162, 2001.—We tested the hypothesis that the cytosolic free calcium concentration in endothelial cells is under the influence of the smooth muscle cells in the coronary circulation. In the left descending branch of porcine coronary arteries, cytosolic free calcium concentration ([Ca2+]i) was estimated by determining the fluorescence ratio of two calcium probes, fluo 4 and fura red, in smooth muscle and endothelial cells using confocal microscopy. Acetylcholine and potassium, which act directly on smooth muscle cells to increase [Ca2+]i, were found to indirectly elevate [Ca2+]i in endothelial cells; in primary cultures of endothelial cells, neither stimulus affected [Ca2+]i, yet substance P increased the fluorescence ratio twofold. In response to acetylcholine and potassium, isometric tension developed by arterial strips with intact endothelium was attenuated by up to 22% (P < 0.05) compared with strips without endothelium. These findings suggest that stimuli that increase smooth muscle [Ca2+]i, can indirectly influence endothelial cell function in porcine coronary arteries. Such a pathway for negative feedback can moderate vasoconstriction and diminish the potential for vasospasm in the coronary circulation.

METHODS

Endothelial cells lining the lumen of blood vessels are separated from the surrounding layers of smooth muscle cells by the internal elastic lamina. Nevertheless, endothelial cells and smooth muscle cells each extend projections through fenestrations in this lamina to establish heterocellular contacts, known as myoendothelial bridges. While the presence of heterocellular gap junctions has been established using electron microscopy (20, 23), these morphological observations are limited to relatively few vessels. Nevertheless, complimentary studies have demonstrated heterocellular coupling based on functional evidence. In the porcine coronary artery, for example, changes in membrane potential of smooth muscle cells are electrotonically transmitted to adjacent endothelial cells (5, 27). Furthermore, endothelial cell hyperpolarization in response to acetylcholine is transmitted to smooth muscle cells in rabbit arteries (8), guinea pig mesenteric arterioles (28), and feed arteries of hamster skeletal muscle (11). This electrotonic mechanism of cell-to-cell coupling has also been proposed to explain the actions of endothelium-derived hyperpolarizing factor (EDHF) (8, 28).

Cytosolic free calcium concentration ([Ca2+]i) regulates contractile activity in vascular smooth muscle cells and the production of vasodilators in endothelial cells. In arterioles isolated from peripheral vascular beds of the hamster, recent studies (10, 29) have indicated that Ca2+ can diffuse from arteriolar smooth muscle into endothelial cells and thereby modulate vasomotor activity. Because the nature of heterologous coupling may differ between vascular beds and across species (25), we questioned whether a corresponding mechanism may be present in the coronary circulation.

In the present study, we used confocal microscopy to test the hypothesis that [Ca2+]i in endothelial cells can be influenced by [Ca2+]i of smooth muscle cells in coronary arteries. Our strategy was to use stimuli known to specifically modulate [Ca2+]i in smooth muscle cells and to examine whether corresponding changes occurred in the [Ca2+]i of endothelial cells. The consequence of such an effect was evaluated in complimentary studies of isometric force production.

Experimental Approach

Experiments were performed using the left anterior descending coronary branches of the freshly killed domestic pig (Sus scrofa) obtained from the local slaughterhouse. The tissue was prepared for experiments in three configurations. First, intact and endothelium-denuded vessel strips were studied using calcium imaging. Second, similarly prepared
strips were used in organ bath studies to evaluate isometric force production. Third, primary cultures of endothelial cells were used to ascertain whether respective experimental stimuli had direct effects on endothelial cell \([Ca^{2+}]_i\).

**Endothelial Cell Primary Cultures**

The lumen of the freshly excised vessel was rinsed by injection of cold DMEM (GIBCO-BRL) supplemented with streptomycin (0.6 g/l) and penicillin (600,000 U/l) and transported in this solution to the laboratory. The endothelial cells were collected by gentle rubbing of the internal face of the vessel with a scalpel and centrifuged (8 min at 800 g) in M199 culture medium (GIBCO-BRL) supplemented with 10% fetal calf serum, 2 mM glutamine, nonessential amino acids, MEM vitamin solution, and 50 mg/l gentamycin. The cell pellet was resuspended in Medium 199 culture medium and plated on gelatin (0.1%)-coated plastic culture petri dishes with a center well. Cells were cultured at 37°C under 5% CO₂. The culture medium was changed three times a week. Endothelial cells were identified by their ability to take up labeled low-density lipoprotein and their morphology (5). At 2–5 days, cells were fusiform and forming islets; confluent monolayers of polygonal cobblestone-like cells developed at 5–8 days (5). Experiments were performed at 3 days of culture.

**Vessel Preparation for Calcium Imaging and Organ Bath Studies**

The arterial lumen was rinsed with Krebs solution containing (in mM) 118.7 NaCl, 2.5 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 24.8 NaHCO₃, and 10.1 glucose and gassed with 95% O₂-5% CO₂ (pH 7.4). Vessels were transported to the laboratory at ambient temperature (~20°C) in this solution. With the use of a stereo microscope, a 15-mm segment of the artery was cut immediately below the bifurcation from the circumflex artery, cleaned of fat and connective tissue, and opened with care taken to preserve the integrity of endothelial cells.

**Dyes and Dye Loading**

The use of dual indicators to study intracellular calcium dynamics minimizes the contribution of artifactual changes in the fluorescence signal not related to changes in \([Ca^{2+}]_i\). (16, 30), e.g., the slight tissue movement in response to contractile agonists and nonuniform dye loading. We applied this principle here to study \([Ca^{2+}]_i\) dynamics in the endothelium and smooth muscle of the arterial wall using the combination of fluo 4 and fura red. In this manner, an increase in \([Ca^{2+}]_i\) simultaneously increases the fluorescence of fluo 4 and reduces that of fura red.

Dye loading was performed in a HEPES-buffered solution, which contained (in mM) 145 NaCl, 5 KCl, 1 CaCl₂, 0.5 MgSO₄, 1 NaH₂PO₄, 20 HEPES, and 10.1 glucose (pH 7.4); the solution also contained fura red (40 μM), fluo 4 (20 μM), and pluronic F-127 (2% vol/vol) to improve loading. All incubations were at 37°C using 1 ml of dye-loading solution; 1 h was used for vessel preparations and 30 min for cultured cells. After dye loading was completed, preparations were washed with HEPES-buffered solution that did not contain either dye or pluronic F-127.

Preliminary experiments indicated that several contiguous smooth muscle cells were loaded with dye by lateral diffusion from damaged edges of the tissue; however, the dye did not diffuse into smooth muscle cells either through the adventitia or through the intact intima. Therefore, to obtain endothelial cell dye loading without dye uptake into underlying smooth muscle, a piece of vessel (~8 × 15 mm) was excised and placed into the loading solution. After incubation, ~2-mm-wide strips were cut from the midregion of the tissue. This precaution ensured that the central strips contained dye only in the endothelial cells. To load the underlying smooth muscle cells, the intimal surface of the vessel segment was gently rubbed with a blunted scalpel blade to remove the endothelium before placement into the dye solution.

Each tissue was observed for ≤1 h after dye loading. Within this period, there was no evidence of preferential leakage or photobleaching of either dye, as confirmed by the stability of the baseline fluorescence ratio. In addition, when endothelial cells were selectively loaded, no cross-contamination from underlying smooth muscle cells was detectable within 1 h (based on the morphology of the labeled cells). Beyond 1 h, however, smooth muscle cell labeling was occasionally apparent.

**Calcium Imaging**

Vessel preparations were placed in a custom-made transparent Plexiglas chamber (volume: 1 ml); a coverslip served as the bottom. A small ring (internal diameter, 5 mm; thickness, 0.5 mm) was positioned on the coverslip, and the vessel strip was clamped to the ring at each end with the intimal surface facing the microscope objective. This configuration provided a ~0.5-mm gap between the tissue surface and the coverslip, which ensured perfusion of the intimal surface. The preparation was then equilibrated for at least 15 min before observation. Cultured endothelial cells were observed in the same petri dish in which they were cultured.

Preparations were placed on the stage of an Axiovert 135M inverted microscope coupled to a LSM 410 laser scanning unit (Zeiss; Oberkochen, Germany) and perfused with Krebs solution gassed with 75% N₂-20% O₂-5% CO₂ (pH 7.4); higher P0₂ diminishes the intensity of the probe fluorescence (2). The chamber was perfused continuously (1.5 ml/min); inflow was through a four-port Manifold (World Precision Instruments; Sarasota, FL) with continuous aspiration of effluent. This arrangement removed any free dye and ensured delivery of agonists at desired concentrations.

An argon ion laser was used to excite both probes at 488 nm. The light emitted was split with a dichroic beam splitter (560 nm) and simultaneously directed onto respective photomultiplier tubes (PMT). One PMT was equipped with a 590-nm long-pass filter to record the fura red emission (peak, 516 nm); the other PMT was equipped with a 510- to 525-nm band-pass filter to record fluo 4 emission (peak, 516 nm). Images were acquired using a ×10 objective (numerical aperture, 0.3) for vessel preparations and a ×40 objective (numerical aperture, 0.75) for cultured cells. The pinhole was set to 20 Zeiss arbitrary units.

**Data Acquisition and Analysis**

Fluorescent signals were collected during scans that were performed every 3 s for up to 2 min (40 images total). Each scan lasted 547 ms and yielded a digitized image (256 × 256 pixels) for the region of analysis, which was stored on a Macintosh G3 computer for subsequent analysis. Light intensity values for pixels in each image ranged from 0 to 255. With the use of an RGB format, the eight-bit signal associated with fura red fluorescence was stored in the red memory, and that for fluo 4 fluorescence was stored in the green memory; the blue memory was not used. Fluorescence intensities were then extracted from each image using IP-LAB Spectrum software (Signal Analytics; Vienna, VA). A
macro was developed that assigned a fluorescence ratio to each pixel of an image by dividing the intensity level stored in G with that stored in R. Regions for analysis were then chosen, and changes in fluorescence were monitored over the sequence of up to 40 images. The output enabled changes in the fluorescence ratio to be plotted over time.

For cultured cells, a rectangular region of interest that included ~10 cells was used to monitor fluorescence change upon drug application. For vessel preparations, a region of analysis that encompassed ~300 cells was selected in the middle of the strip to avoid damaged edges. For data collection, regions were selected (according to responses evoked by respective agonists) that included ~50 cells. In addition to qualitative changes in fluorescence, the spatial pattern of the response was analysed to determine whether only certain cells showed an increase in [Ca\textsuperscript{2+}].

The results obtained from endothelial cells in vessel preparations were expressed as a percentage of the response of the same cells to substance P delivered at the beginning of the experiment. Control experiments indicated that two successive applications of substance P (separated by up to 1 h) evoked fluorescence responses of similar intensity (data not shown); therefore, the response to substance P was used as an internal reference. In cultured endothelial cells obtained from these vessels, we (12) have shown that resting [Ca\textsuperscript{2+}]

was taken here to represent a change in [Ca\textsuperscript{2+}].

Thus a 1% change in the fluorescence ratio for [Ca\textsuperscript{2+}]

was taken per animal. Student’s t-test was used to compare results; P < 0.05 was accepted as statically significant.

RESULTS

Calcium Probe Cell Loading in Intact Tissue

When observed with either the 590-nm long-pass filter (fura red) or the 510- to 525-nm band-pass filter (fluo 4), smooth muscle cells in tissues appeared as long fibers readily distinguishable from the endothelial cells, which are round shaped (Fig. 1). The smooth muscle cells appeared brighter with fluo 4, whereas endothelial cells appeared brighter with fura red. Thus both cell shape and the properties of dye loading readily enabled identification of the respective cell types and provided a reliable index for where the fluorescence originated.

Stimulation of Endothelial Cell Receptors

We (1, 7, 9) have previously reported the effects of bradykinin and substance P on cultured endothelial cells obtained from porcine coronary arteries. In the present study, we investigated the effects of these peptides on [Ca\textsuperscript{2+}]; in endothelial cells of intact strips of coronary arteries. Complimentary experiments on cultured cells provided a reference for agonist selectivity. Compared with resting levels, exposure to bradykinin (100 nM) transiently increased endothelial cell [Ca\textsuperscript{2+}], increasing the fluorescence ratio by 38 ± 5% (n = 15; Fig. 2A). In a complimentary manner, substance P (10 nM) transiently increased the fluorescence ratio by 45 ± 2% (n = 24, P = 0.12 vs. bradykinin; Fig. 2B).

Stimuli Acting on Smooth Muscle Cells

High potassium. Superfusion of vessel strips with high potassium depolarizes smooth muscle cells from...
The fluorescence ratio increased by 65 ± 6% (n = 4) above baseline in smooth muscle cells in endothelium-denuded preparations. In the endothelial cells on intact arterial strips (n = 16), the fluorescence ratio increased by 7 ± 1% above baseline, corresponding to a 14 ± 2% increase (i.e., of ~84 nM) in [Ca²⁺]ᵢ, relative to the substance P-induced response on the same cells (Fig. 3A). This response occurred in one to three regions of >30 cells in the region of analysis (~300 cells). In contrast, high potassium had no effect on [Ca²⁺]ᵢ of cultured endothelial cells (n = 5; Fig. 4) despite its depolarizing action (7).

Acetylcholine. In the porcine coronary artery, acetylcholine has a direct effect on smooth muscle cells to cause contraction, not by depolarization but through the activation of phospholipase C (15). The response is triggered by a transient increase in [Ca²⁺]ᵢ followed by a plateau phase (24). Our findings here confirm that, in the smooth muscle cells of an endothelium-denuded strip, acetylcholine (10 μM) transiently increased the fluorescence ratio by 87 ± 9% above baseline (n = 14). When endothelial cells were observed, the fluorescence ratio increased by 10 ± 2% above baseline (n = 10), corresponding to a 21 ± 3% increase (i.e., of ~126 nM) in [Ca²⁺]ᵢ relative to the response to substance P in the same cells (Fig. 3B). This response involved one to three regions of >30 cells within the region of analysis (~300 cells). In contrast, acetylcholine had no effect on [Ca²⁺]ᵢ of cultured endothelial cells (n = 5; Fig. 4).

Isoproterenol. As summarized in Fig. 5, each of the preceding stimuli increased [Ca²⁺]ᵢ. We therefore investigated the effect of isoproterenol, a stimulus that hyperpolarizes smooth muscle cells of porcine coronary arteries (5, 15) and should therefore reduce [Ca²⁺]ᵢ. In smooth muscle cells of vessel strips without endothelium, isoproterenol (10 μM) decreased the fluorescence ratio by 9 ± 3% below baseline (n = 4); however, no change in the fluorescence ratio was detected in endothelial cells of intact strips (n = 4). The fall in [Ca²⁺]ᵢ in smooth muscle was less than that needed to observe a corresponding change in the endothelium with our detection system.

Physiological Effects

In porcine coronary arteries, endothelium-dependent vasodilatation to nitric oxide and to EDHF are triggered by an increase of [Ca²⁺]ᵢ in endothelial cells (14, 19, 26). We therefore reasoned that an indirect elevation of endothelial cell [Ca²⁺]ᵢ originating from a rise in smooth muscle cell [Ca²⁺]ᵢ should lead to endothelium-dependent relaxation. To test this hypothesis, concen-

![Figure 2](http://ajpheart.physiology.org/)

![Figure 3](http://ajpheart.physiology.org/)
In addition to calcium and other ions, small (<1 kDa) water-soluble molecules, e.g., D-myo-inositol (1,4,5)-trisphosphate [Ins(1,4,5)P₃], can diffuse through the gap junctions (6). In such manner, after stimulation of phospholipase C and the production of Ins(1,4,5)P₃ in response to acetylcholine (15), Ins(1,4,5)P₃ as well as calcium may have diffused through gap junctions to influence endothelial cell [Ca²⁺]i. Whereas the elevation of Ins(1,4,5)P₃ would elevate [Ca²⁺]i in both endothelial cells and smooth muscle cells, electrical coupling would have the opposite effect in respective cell layers (3). For example, in endothelial cells (which lack voltage-operated calcium channels) (1, 12), membrane depolarization and hyperpolarization would passively decrease and increase calcium entry, respectively, through changing the electrochemical gradient (21). In contrast, the presence of voltage-operated calcium channels in “excitable” smooth muscle cells (17) would lead to the activation of calcium entry with depolarization and inhibition of calcium entry with hyperpolarization. Experiments in which tension production was measured revealed the consequence of this interaction on smooth muscle function. With a high-potassium (i.e., nonselective depolarizing) solution, the contribution of endothelium-derived vasodilator production appeared smaller compared with when the smooth muscle cell [Ca²⁺]i was increased by acetylcholine, which elevated endothelial cell [Ca²⁺]i (and vasodilator production) independent of changes in membrane potential (15).

The present experiments enabled [Ca²⁺]i to be imaged in multiple homologous cells simultaneously. Although responses in smooth muscle cells were homo-

**Fig. 4.** Representative fluorescence ratio of fluo 4 to fura red from primary cultured endothelial cells. In successive treatments, neither ACh (10 μM) nor high-potassium solution (75 mM) had an effect on cytosolic free calcium concentration; in contrast, substance P (10 nM) promptly elicited a twofold increase in the fluorescence ratio.

**Fig. 5.** Summary data (means ± SE) for the change in the fluorescence ratio of fluo 4 to fura red in strips of porcine coronary arteries. Responses are shown to BK (100 nM), substance P (10 nM), ACh (10 μM), and high-potassium solution (75 mM). Open bars, data from endothelial cells of intact strips; solid bars, data from smooth muscle cells from endothelium-denuded strips. Numbers in parentheses are the numbers of experiments.
geneous in the areas observed, only clusters of ~30 endothelial cells exhibited the indirect rise in \([\text{Ca}^{2+}]_i\). The simplest explanation is that the heterocellular coupling between the endothelium and smooth muscle is nonuniform along the wall of porcine coronary arteries. The small decrease in \([\text{Ca}^{2+}]_i\), observed in the smooth muscle cells in response to isoproterenol was not reflected in the endothelial cells. This is probably because possible changes in \([\text{Ca}^{2+}]_i\) reflected in the endothelial cells are too small, in that case, to be detected compared with the larger variation induced by acetylcholine or potassium.

We tested the effects of three gap junction uncouplers in this study (data not shown). With the use of either palmitoleic acid (10 μM) or halothane (1.74 mM), we found a suppression of heterocellular calcium coupling. However, control experiments revealed that each of these agents initially elevate smooth muscle \([\text{Ca}^{2+}]_i\), and then reduce subsequent responses to acetylcholine. In organ bath studies, carbexonolone (100 μM) relaxed precontracted (0.3 μM U-46619) intact strips by 94 ± 5% (n = 4). We conclude that the actions of these agents are nonspecific in our preparation.

Methodological Considerations

The ratiometric calcium probe fura 2 has been applied extensively to the study of \([\text{Ca}^{2+}]_i\) dynamics in vascular cells (22). A distinct advantage of ratiometric measurements is the elimination of artifacts associated with tissue movement and changes in the focal plane (13). However, the use of fura 2 requires ultraviolet excitation, and such illumination may often not be available with confocal microscopy. We therefore sought to develop a ratiometric method that did require ultraviolet light. For this purpose, we loaded fura red and fluo 4 into the endothelium and smooth muscle. These respective dyes have opposite response characteristics to changes in \([\text{Ca}^{2+}]_i\), such that the ratio of their emitted light provides a sensitive measure of \([\text{Ca}^{2+}]_i\) dynamics independent of tissue movement.

Our method was constrained to observations of one cell layer at a time and used a relatively slow frequency of sampling (20 image frames/min) to prevent excessive photobleaching. Although we were unable to study the actual temporal relationship between the increase in \([\text{Ca}^{2+}]_i\) in smooth muscle and that in endothelium, we speculate that this interaction is quite rapid (10, 22). In observations of either the endothelium or smooth muscle on vessel strips, the fluorescence increase preceded the slight movement of tissue by the same brief interval (1–2 image frames).

Appropriate calibration enables results to be expressed in terms of calcium concentration instead of an increase in the fluorescence ratio. However, in these experiments, no calibration was performed, mainly because in order for calibration to be consistent it should have been performed at the end of each experiment, because each tissue slightly differs in its dye-loading proprieties and autofluorescence. However, because of the photobleaching phenomenon, the recording of the maximal fluorescence for both dyes at the end of the experiment would be smaller than the fluorescence induced by direct stimulation of the cells (18). Therefore, to estimate the actual changes in endothelial cell \([\text{Ca}^{2+}]_i\), we expressed our results as a percentage of the response to substance P and related these to our previous measurements of substance P-induced changes in \([\text{Ca}^{2+}]_i\) in cultured endothelium. The reproducibility of consecutive responses to substance P supports this estimation.

The effect of bradykinin and substance P on \([\text{Ca}^{2+}]_i\) of the endothelial cells in an intact segment of coronary arteries was not qualitatively different than that of cultured endothelial cells. This finding substantiates...
that our method was indeed suitable for effective observations of \([\text{Ca}^{2+}]\), dynamics in endothelial cells of intact vessel preparations. However, this relationship is likely to hold only for relatively young primary cultures because, after \(\sim 3\) days in culture, substance \(P\) no longer has an effect on \([\text{Ca}^{2+}]\), whereas the transient \([\text{Ca}^{2+}]\) response to bradykinin becomes markedly prolonged (12).

**Physiological Significance**

This study illustrates the subtle interplay between endothelial cells and smooth muscle cells working as a functional unit through heterologous coupling (3) in coronary arteries. Whereas a pronounced response to endothelium-dependent vasodilators (e.g., substance \(P\)) can be readily demonstrated, the indirect elevation of \([\text{Ca}^{2+}]\); in endothelial cells in response to direct elevation of \([\text{Ca}^{2+}]\); in smooth muscle cells is more subtle and may provide an intrinsically active signal-pathway for tempering vasoconstriction. Under physiological conditions, such a mechanism would act continuously to reduce the potential for vasospasm. With coronary artery disease or disruption of the endothelium, such feedback would be lost, with adverse effects on coronary blood flow.

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