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# Mechanical stimulation induces $Ca_i^{2+}$ transients and membrane depolarization in cultured endothelial cells

## Effects on $Ca_i^{2+}$ in co-perfused smooth muscle cells

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Cytosolic $Ca^{2+}$ concentration and membrane potential were monitored in individual cultured enothelial cells mechanically stimulated with a micropipette attached to the stage of a microscope. Both dimpling and poking of endothelial cells resulted in $Ca_i^{2+}$ transients (from $63 \pm 12$ to $397 \pm 52$ nM, characterized by a refractory period of approx. 2 min) and cell depolarization. $Ca_i^{2+}$ transients of the reduced amplitude ($201 \pm 41$ nM) were evoked by mechanical stimulation of endothelial cells incubated in a $Ca^{2+}$-free medium. Dimpling-induced $Ca_i^{2+}$ transients were refractory to the pretreatments with pertussis toxin, colchicine, or cytochalasin B, and were not mimicked by an increase in the hydrodynamic pressure. In a co-perfusion system (endothelium: smooth muscle), both the KCl-induced depolarization and ionomycin-induced increase in $Ca_i^{2+}$ in the endothelial cells resulted in the reduction of $Ca_i^{2+}$ in the smooth muscle cells. The data reported are consistent with the phenomenon of vascular relaxation in response to the increased blood flow. We hypothesize that the mechanical interaction of the formed elements with the microvascular endothelium can serve as a pacemaker for the sustained relaxation of vascular smooth muscle.

Mechanoreceptor; cytosolic $Ca^{2+}$; Fura-2; (Endothelium, Smooth muscle)

## 1. INTRODUCTION

During the last few years, chemical interactions between the formed elements and the vascular endothelium have been the focus of numerous studies (review [1]). Release of ADP and serotonin by aggregating platelets has been recognized as a stimulus for the endothelial cells to respond with calcium transients which, in turn, trigger the secretion of endothelium-derived relaxing factor(s) [2–6]. The possibility of mechanical interaction between the formed elements and the vascular endothelium has been largely overshadowed by these discoveries. There is emerging evidence that mechanical forces (turbulent fluid shear stress or changes in blood flow) acting upon endothelial cells can modulate their topography and turnover [7,8]. Furthermore, the existence of stretch-activated channels in the endothelial cells has been recently demonstrated using the patch-clamp technique [9]. Here, we attempted to test the hypothesis that mechanical interaction of the formed elements with the vascular endothelium induces calcium transients, representing a pacemaker drive for an endothelial cell to secrete chemical principles mediating vascular relaxation.

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## 2. MATERIALS AND METHODS

Pulmonary artery endothelium CCL 209, from *Bos taurus*, was obtained from ATCC (Rockville, MD) at passage 16. Cells were grown on glass coverslips in Eagle's MEM supplemented with 20% fetal bovine serum. A7R5 embryonic rat aorta smooth muscle cells (ATCC) were grown in Dulbecco's MEM supplemented with 10% fetal bovine serum. Cells were loaded with 10 $\mu$M fura-2/AM for 45 min, washed, and cover slips were placed into a Sykes-Moore chamber in Eagle's MEM

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(without phenol red) buffered with 20 mM Hepes (pH 7.4). Cells were allowed to equilibrate for 30 min at room temperature, then mounted on the stage of a Nikon Diaphot microscope with quartz epiillumination optics, equipped for photon counting (R374 Hamamatsu Photonics, Japan). A micromanipulator was attached to the stage of the microscope (Zeiss, FRG) stationed on a vibration-free table. Light from a 100 W mercury bulb passed through 340 and 380 nm interference filters (Ditric Optics, Hudson, MA) shuttling mechanically at a rate of ~1 rotation/s, BV dichroic cube, and UV 40 × oil-immersion objective (Nikon). Emitted light was collected at 480–530 nm. The photomultiplier was interfaced with a chart recorder. After subtraction of background fluorescence, 340:380 ratios were obtained and converted to the respective concentrations of $Ca_i^{2+}$ using fura-2 microstandards dissolved in the intracellular-like medium at various free calcium concentrations, as described [10,11]. For measurement of the membrane potential, cells were loaded with 50–100 nM bisoxonol [$DiOC_5(3)$] for 15 min, and fluorescence signals were monitored using a B-dichroic cube, as detailed in [12].

The design and description of a co-perfusion system are detailed in section 3. Endothelial cells were grown on collagen-coated (vitrogen) polycarbonate membranes (0.8 $\mu$m pore size; Nucleopore, Pleasanton, CA) glued to rubber rings with Millipore cement no.1 formulation (Millipore, Bedford, MA). Confluent cultures were used in co-perfusion experiments. Fluorescent indicators were obtained from Molecular Probes (Eugene, OR), other chemicals being from Sigma (St. Louis, MO).

## 3. RESULTS

A micropipette tip rounded over a flame to form an approx. 5 $\mu$m diameter bulb was driven by a micromanipulator over fura-2-loaded endothelial cells placed on the stage of a microscope equipped for microfluorometric measurements. Micropipette movements were operated in two modes: (i) delicate 'brushing' movements of the micropipette while its distance from the endothelial cell surface was progressively shortened (fig.1) and (ii) 'poking' movements of the micropipette. In the instances when visible changes in endothelial cell appearance were produced the results were discarded. Both techniques of mechanical stimulation of the endothelial cells yielded similar results. Fig.2A represents a typical recording of $Ca^{2+}$ transients induced by a delicate dimpling. Both the brushing and poking of endothelial cells resulted in $Ca^{2+}$ transients from baseline of 63 ± 12 to 397 ± 52 nM ($n$ = 12). By repetitive application of these forces, it was possible to elicit a 'train' of calcium transients not affecting cell morphology. The duration of $Ca^{2+}$ upstroke averaged 21.3 ± 9.8 s. Following the initiation of $Ca^{2+}$ transients, cells remained refractory to a subsequent stimulation for about 1–2 min (fig.2A). Similar, but less consistent responses were evoked by the addition of Sephadex beads (not shown).

Mechanical stimulation of the endothelial cells resulted in a brisk membrane depolarization (fig.2B). It was characterized by oscillations of bisoxonol fluorescence. The observed $Ca^{2+}$ transients, however, were not induced by membrane depolarization, since these cells do not express voltage-operated calcium channels ([13], and author's data, not shown). If this effect was secondary to an activation of stretch receptors with a high sodium and calcium conductance, as has been reported previously [9], one would expect that omission of calcium from the medium could result in the abolishment of $Ca_i^{2+}$ transients in response to mechanical stimulation. This, however, was not the case. The endothelial cells incubated in a calcium-free medium were able to produce a train of $Ca^{2+}$ transients when stimulated repetitively by a touching micropipette (fig.2C). Increases in $Ca_i^{2+}$ averaged 201 ± 41 nM ($n$ = 7), a value significantly lower ($p < 0.01$) than $Ca^{2+}$ transients in the complete medium. Thus, in addition to $Ca^{2+}$ influx via stretch-activated ion channels with high $Ca^{2+}$ conductance [9], calcium can be mobilized from intracellular stores. Since GTP-binding proteins could participate in this coupling, endothelial cells were next pretreated with pertussis toxin (100 ng/ml for 5–6 h). The application of mechanical stimuli elicited $Ca^{2+}$ transients which were not different from those evoked in the untreated cells (fig.2D). Pretreatment of the cells with either colchicine (up to 50 $\mu$M) or cytochalasin B (10–50 $\mu$M), although reducing the amplitude of $Ca_i^{2+}$ transients to 243 ± 31 and 193 ± 17 nM, respectively, did not abolish the response of endothelial cells to mechanical stimulation (fig.2E,F). Furthermore, the increased perfusion rate (from 1 to 10 ml/min) did not mimic the observed effect of mechanical stimulation, but resulted in an initial transient elevation followed (1–2 min) by a steady, reversible decrease of $Ca_i^{2+}$ in endothelial cells (not shown). At present, the mode of coupling of mechanoreceptors to trigger mechanism(s) of $Ca^{2+}$ mobilization (extra- and intracellular) remains obscure.

To examine the possible effects of changes in $Ca_i^{2+}$ and membrane potential of the endothelial

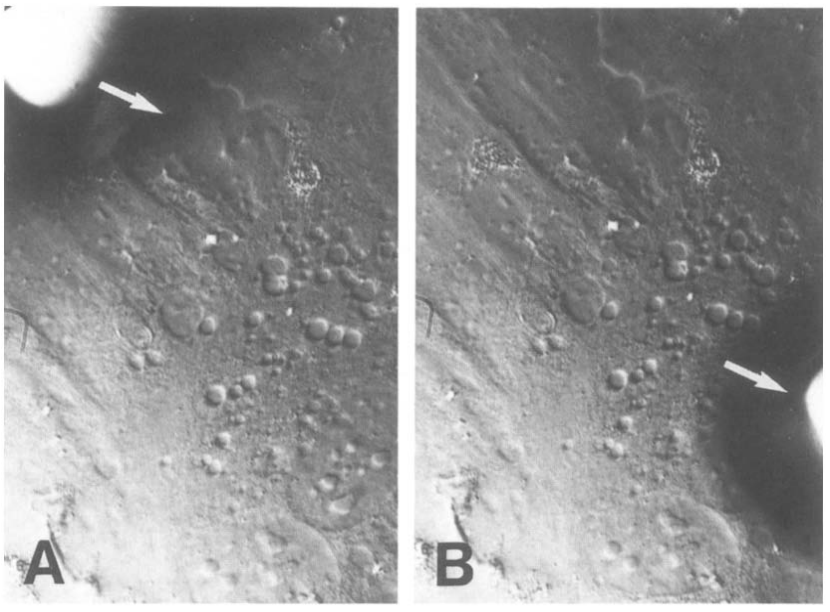


Fig.1. Movement of a micropipette over monolayer of endothelial cells. The rounded tip of the pipette is moving through the upper left field (A) to the lower right field (B). No changes in morphology of the monolayer are seen. When morphological alterations were observed following dimpling of the cells, experimental results were discarded. Hoffman modulation optics; × 400.

cells on cytosolic calcium concentration in smooth muscle cells, a chamber for a separate perfusion of both cell types was designed (fig.3). Communication between cell types could be achieved only by the diffusion through a polycarbonate membrane. Endothelial cells were subjected to a calcium ionophore or to $K^+$ depolarization and $Ca_i^{2+}$ of smooth muscle cells was monitored microfluorimetrically. When the integrity of endothelial monolayers was compromised, perfusion of the endothelial cell compartment with ionomycin resulted in an increase of $Ca_i^{2+}$ in the smooth muscle cells (not shown). In contrast, perfusion of confluent endothelial monolayers with 0.5–1 $\mu$M ionomycin produced a consistent decline in $Ca_i^{2+}$ in A7r5 smooth muscle cells (fig.4A). This effect required the discontinuation of perfusion of smooth muscle cell compartment, a maneuver which per se produced no changes in $Ca_i^{2+}$. Similarly, perfusion of the endothelial cell compartment with the medium containing 30 mM KCl was accompanied by a decrease in $Ca_i^{2+}$ of the smooth muscle cells (fig.4B) from basal level of 108 ± 19 to 21 ± 2.5 nM ($n$ = 7). This endothelial conditioning of $Ca_i^{2+}$ in the smooth muscle cells did not affect their responses to the direct stimulation with ionomycin or KCl (fig.4A,B, arrow 2), and was completely reversible upon reinstitution of perfusion with the regular medium (not shown).

These observations allow certain inferences to be made. Mechanical stimulation of the endothelial cells results in membrane depolarization and $Ca^{2+}$ transients. These phenomena are independent as endothelial cells do not seem to express voltage-operated calcium channels [13]. Most likely, both events are evoked by an activation of stretch mechanoreceptors with high sodium and calcium conductances [9]. An increase in $Ca_i^{2+}$ is in part due to release of $Ca^{2+}$ from intracellular stores, a process which appears to be pertussis toxin-insensitive. Mechanical stimulation of the cytochalasin B- and colchicine-pretreated endothelial cells evoked $Ca_i^{2+}$ transients of the reduced amplitude. In view of recent observations on the direct effect of cytochalasins on cytosolic calcium [14], it is difficult to attribute this reduction of the amplitude of $Ca_i^{2+}$ transients to the specific effect on the mechanoreceptor. It has been reported that neither cytochalasin B nor colchicine affected mechanoreceptor function as judged by

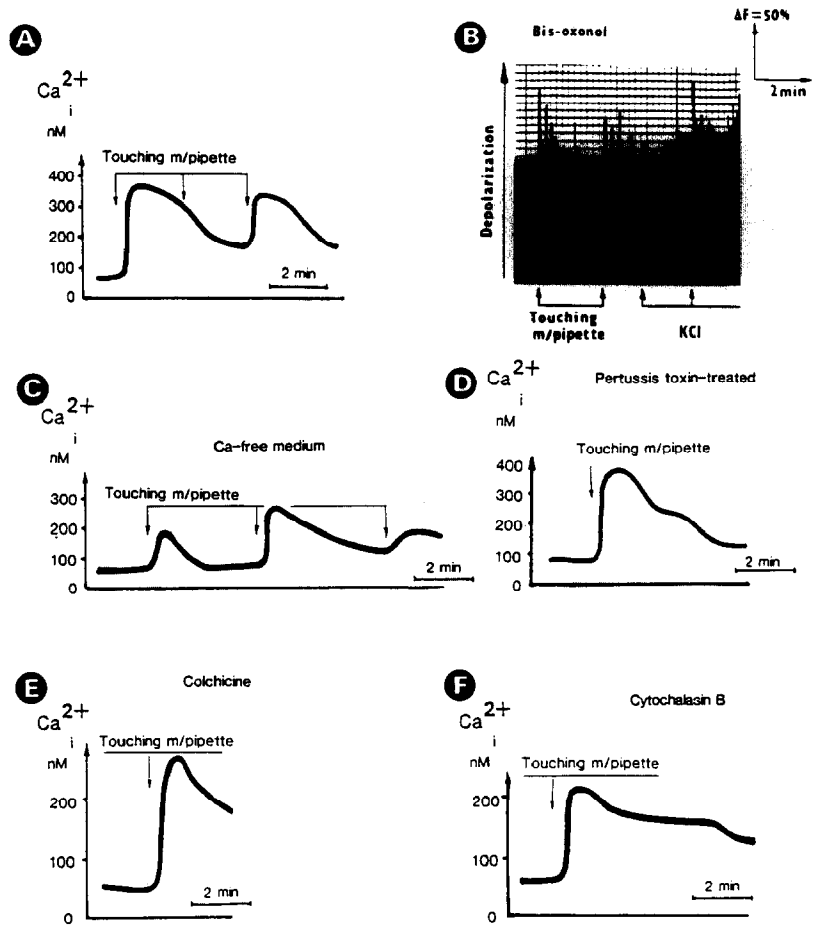


Fig.2. Effects of mechanical stimulation on $Ca_i^{2+}$ and membrane potential of endothelial cells. (A) Repetitive stimulation of an endothelial cell with a micropipette moving in the brushing mode. Note that the cell was refractory to subsequent stimulation falling within 2.1 min after the initiation of a $Ca_i^{2+}$ transient. (B) Mechanical stimulation of an endothelial cell results in a burst-like oscillating depolarization. For comparison, effects of KCl (15 and 50 mM final concentrations) on bisoxonol fluorescence are presented. (C) A train of $Ca_i^{2+}$ transients elicited by a series of mechanical stimuli. Endothelial cells were incubated in a calcium-free medium. (D) 5 h incubation of endothelial cells with pertussis toxin (100 ng/ml) did not affect $Ca_i^{2+}$ transients induced by mechanical stimulation. (E) Pretreatment of endothelial cells with colchicine (50 $\mu$M) did not abolish $Ca_i^{2+}$ transients induced by mechanical stimulation. (F) Pretreatment of endothelial cells with 50 $\mu$M cytochalasin B did not abolish $Ca_i^{2+}$ transients induced by mechanical stimulation.

the patch-clamp currents [15]; however, their interference with the release of $Ca^{2+}$ from the intracellular pool(s) is not excluded. $K^+$ depolarization of the endothelial cells, as well as the application of ionomycin, results in the generation of diffusible substance(s) which decrease(s) $Ca_i^{2+}$ in the smooth muscle cells. It has been demonstrated that calcium ionophores stimulate production of endothelium-derived relaxing factor(s) and $PGI_2$ [5,6,16]. It is possible that endothelial cell membrane depolarization also stimulates production of these vasodilatory principles (cessation of production of vasoconstrictors, although less likely, remains a distinct possibility). If the described phenomena take place in the microvasculature, they can provide an explanation for the relaxation of the vasculature in response to the increased blood flow [17]. Based upon the above findings, one can speculate that the passage of the formed elements and their 'collision' with the endothelial cells may serve as a pacemaker for repetitive $Ca^{2+}$ transients causing tonic secretion to

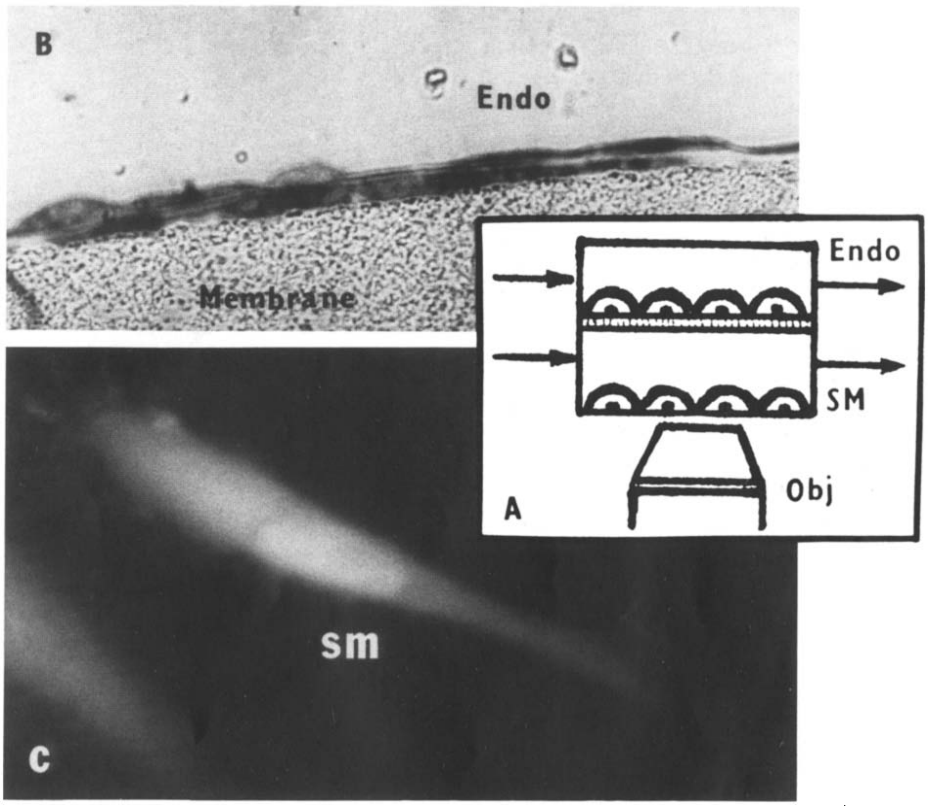


Fig.3. A chamber for compartmentalized co-perfusion studies of interactions between the endothelial (Endo) and smooth muscle (SM) cells. (A) General design. Endothelial cells were densely seeded on collagen-coated polycarbonate membranes (pore size 0.8 $\mu$m, diameter 13 mm; Nucleopore) glued to rubber gaskets to prevent edge damage. Cells were maintained as described previously. A7r5 smooth muscle cells derived from embryonic rat aorta (ATCC) were grown on glass coverslips in Dulbecco's MEM supplemented with 10% fetal bovine serum. The smooth muscle cells were loaded with fura-2/AM as described in section 2. Both cell types were placed into separately perfused compartments (200 $\mu$M) of the chamber, mounted on the stage of a microscope (Obj, objective), and fura-2 fluorescence was monitored, as detailed previously. When the endothelial cell compartment was perfused with ionomycin or KCl-containing medium, perfusion of the smooth muscle cells was temporarily interrupted. (B) Endothelial cell monolayer on the polycarbonate membrane; × 100. (C) Fluorescence micrograph of fura-2-loaded smooth muscle cells illuminated at 380 nm. × 400.

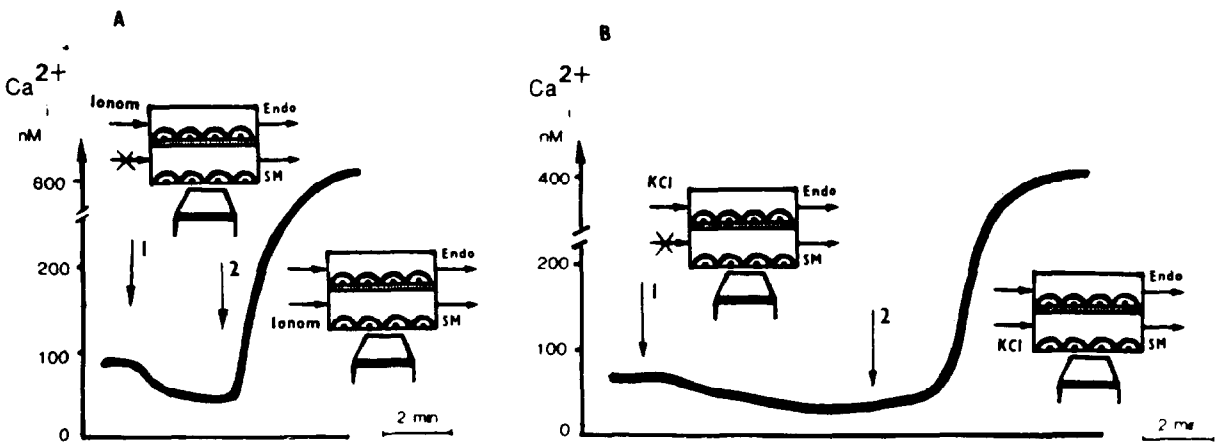


Fig.4. Consecutive perfusion of the endothelial cell compartment (arrow 1) and smooth muscle cell compartment (arrow 2) with 1 $\mu$M ionomycin (A) or 30 mM KCl (B).

the microenvironment of the products which decrease $Ca_i^{2+}$ in the smooth muscle cells. Such a mechanism could serve purposes of spatial adjustment of the microvascular bed to the passing formed elements and shed light on the pathophysiology of microcirculation in the disorders of size and shape of the formed elements. The described model of the compartmentalized co-perfusion of the endothelial and smooth muscle cells may be useful in studies of cellular interactions mediated by secretory products.

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## NOTE ADDED IN PROOF

G.M. Rubanyi and P.M. Vanhoutte have recently published their data supporting the possible role of $K^+$-depolarization in the induction of the secretion of endothelium-derived relaxing factor [(1988) Circ. Res. 62 1098–1103].