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Propagation of fast and slow intercellular Ca^{2+} waves in primary cultured arterial smooth muscle cells

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ABSTRACT

Smooth muscle contraction is regulated by changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). In response to stimulation, Ca^{2+} increase in a single cell can propagate to neighbouring cells through gap junctions, as intercellular Ca^{2+} waves. To investigate the mechanisms underlying Ca^{2+} wave propagation between smooth muscle cells, we used primary cultured rat mesenteric smooth muscle cells (pSMCs). Cells were aligned with the microcontact printing technique and a single pSMC was locally stimulated by mechanical stimulation or by microejection of KCl. Mechanical stimulation evoked two distinct Ca^{2+} waves: (1) a fast wave (2 mm/s) that propagated to all neighbouring cells, and (2) a slow wave (20 μm/s) that was spatially limited in propagation. KCl induced only fast Ca^{2+} waves of the same velocity as the mechanically induced fast waves. Inhibition of gap junctions, voltage-operated calcium channels, inositol 1,4,5-trisphosphate (IP_3) and ryanodine receptors, shows that the fast wave was due to gap junction mediated membrane depolarization and subsequent Ca^{2+} influx through voltage-operated Ca^{2+} channels, whereas, the slow wave was due to Ca^{2+} release primarily through IP_3 receptors. Altogether, these results indicate that temporally and spatially distinct mechanisms allow intercellular communication between SMCs. In intact arteries this may allow fine tuning of vessel tone.

1. Introduction

Intercellular communication between smooth muscle cells (SMCs) of arteries allow coordinated vasomotor responses, control of vascular resistance and blood flow. Changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is a key step in several activities performed by SMCs, including contraction. An increase in $[Ca^{2+}]_i$ in one cell, in response to chemical, electrical or mechanical stimuli, can propagate to neighbouring SMCs along the arterial wall as an intercellular Ca^{2+} wave [1].

At the single cell level, cytoplasmic Ca^{2+} increase is a result of either Ca^{2+} influx from the outside of the cell and/or Ca^{2+} release from the sarcoplasmic reticulum (SR). Voltage-operated calcium channels (VOCCs) mediate Ca^{2+} influx across the plasma membrane, whereas Ca^{2+} release from the SR occurs through the activation of two families of Ca^{2+} channels: inositol 1,4,5-trisphosphate (IP_3) and ryanodine (Ry) receptors. IP_3 receptors (IP_3Rs) are activated by Ca^{2+} itself and IP_3 [2], a second messenger generated by the activation of phospholipase C (PLC) in response to stimuli, whereas Ry receptors (RyRs) are activated by Ca^{2+} [3,4]. A small increase in $[Ca^{2+}]_i$ can then induce further Ca^{2+} release from the SR, a process known as Ca^{2+}-induced Ca^{2+}-release (CICR), which results in a Ca^{2+} wave that progresses throughout the cell. Depending on cell type, IP_3 and Ry receptors can cooperate to propagate Ca^{2+} waves [5–13].

In arteries, Ca^{2+} waves arising in a single SMC can propagate to neighbouring cells through gap junction channels [14,15]. These channels allow direct exchange of ions, nucleotides and other small molecules (<1 kDa), thereby couple the cells both electrically and metabolically [16]. Previous studies have provided evidence of electrical coupling between SMCs in different arteries, but the diffusion of Ca^{2+}, IP_3, cyclic ADP-ribose and other second messengers has also been shown [14,17,18]. A considerable amount of theoretical work has been conducted on the propagation of intercellular Ca^{2+} waves (for e.g. [19–22]). However, the relative contribution of electrical coupling and messenger diffusion in the propagation of intercellular Ca^{2+} waves remain elusive.

To investigate the propagation mechanisms of Ca^{2+} waves at the cellular level, we used primary cultured rat mesenteric SMCs (pSMCs) loaded with the fluorescent Ca^{2+} dye Fluo-4. These cells were derived from the same tissue in which Ca^{2+} wave propagation was observed in arterial segments [15]. We applied the

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microcontact printing technique to culture pSMCs on collagen lines. The aligned arrangement of the cells facilitates local stimulation of a single cell at a restricted site, away from neighbouring cells and the observation of Ca^{2+} wave progression from one cell to another. To induce an intercellular Ca^{2+} wave, a single pSMC was locally stimulated with a micropipette or by microejection of KCl. Our results show that the propagation of intercellular Ca^{2+} waves depend on membrane depolarization-induced Ca^{2+} entry and require a SR with functional IP_3 receptors. Moreover, we show the effects of subsequent stimuli on the appearance of Ca^{2+} waves. Our results demonstrate that mechanical stimulation triggered a fast Ca^{2+} wave (2 mm/s) that propagated to all neighbouring cells and a slow Ca^{2+} wave (20 μm/s) that was spatially limited in propagation. Inhibitors of gap junctions, VOCCs, IP_3 and Ry receptors where applied to assess their involvement in the propagation of Ca^{2+} waves.

2. Methods

2.1. Tissue preparation and cell culture

Male Wistar rats (300 ± 50 g) were anesthetized with isoflurane (4%) and then decapitated in agreement with the Care of Animals (edited by the Swiss Academy of Medical Sciences and the Helvetic Society of Natural Sciences). All experiments performed on rat mesenteric arteries were approved by the Cantonal Veterinary Office (authorization number 1799.1) in agreement with the law on animal protection in Switzerland. The mesenteric arcade was excised and placed in a physiological solution (in mM: 145 NaCl, 5 KCl, 1 $CaCl_2$, 0.5 $MgSO_4$, 1 Na_2HPO_4, 20 HEPES, 23 Tris base, 10.1 glucose, pH 7.4). The methods used for isolation and culture are similar to the protocol described by Golovina and Blaustein [23]. Briefly, the superior mesenteric artery was removed and placed in isolation solution (in mM: 137 NaCl, 5.4 KCl, 0.44 KH_2PO_4, 0.42 NaH_2PO_4, 0.2 $MgCl_2$, 4.2 $NaHCO_3$, 0.2 $CaCl_2$, 0.05 EGTA, and 10 HEPES, 10.1 glucose, pH 7.4). First- and second-order arteries were extensively cleaned of adventitial fat and connective tissue using fine forceps and vannas scissors. Blood was removed by injecting isolation solution into the lumen using a syringe with a gauge needle. Arteries were cut to 2–3 mm length and prepared for enzymatic digestion during 20 min at 37 °C in isolation solution containing collagenase (2 mg/ml) and elastase (1 mg/ml) (both from Sigma–Aldrich, Buchs, Switzerland). Arteries were then rinsed with isolation solution during 10 min at room temperature and transferred to 60 mm diameter culture dishes containing 5 ml culture medium (Dulbecco's modified Eagle's medium (DMEM)) (Invitrogen, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS) (BioConcept, Allschwil, Switzerland), 20 mM L-glutamine and 1000 U/ml penicillin/streptomycin (Invitrogen). Explants were cultured for 10–12 days. The purity of the primary cultured mesenteric artery smooth muscle cell cultures was verified by positive staining with smooth muscle specific alpha-actin and desmin (data not shown).

2.2. Microcontact patterning

To facilitate local stimulation of one cell and the observation of Ca^{2+} wave propagation between pSMCs, cells were grown on ibidi coverslips (Ibidi GmbH, Martinsried, Germany) patterned with collagen lines of 50 μm width created by the microcontact printing (μCP) technique, described previously [24]. Briefly, optical lithography was used to etch the topography of interest on a silicon wafer, serving as a mould to produce silicone stamps (Dow Corning, Wiesbaden, Germany). Silicon stamps were cleaned with ethanol, treated for 30 s with plasma oxygen (Plasmaline, Tegal Corporation, Petaluma, CA) and incubated for 1 h with collagen type I. (50 μg/ml in serum free culture medium) (Sigma–Aldrich, Buchs, Switzerland). Contact was made for 4 min with the coverslip surface that was also activated for 30 s with plasma oxygen. The remaining gaps on the coverslip were blocked with poly(L-lysine)(20)-graft(2)-poly(ethylene glycol)(3.5) (PLL-g-PEG; SuSoS AG, Dübendorf, Switzerland) to form non-adhesive areas. PSMCs adhered only to the collagen lines and were cultured for 1 day before experiments.

2.3. Calcium measurements and image analysis

PSMCs were loaded for 40–50 min at room temperature in physiological solution (in mM: 145 NaCl, 5 KCl, 2 $CaCl_2$, 0.5 $MgSO_4$, 1 Na_2HPO_4, 20 HEPES, 23 Tris base, 10.1 glucose, pH 7.4) containing 10 μM Fluo-4 AM in the presence of 2.5% pluronic F-127 (both from Molecular Probes) and subsequently washed for 30 min with physiological solution for deesterification. Experiments were performed with a Nikon (Tokyo, Japan) Eclipse TE300 inverted microscope. Data were acquired with a Micromax PB1300 and a Micromax 512FT cooled charge-coupled device cameras (Roper Scientific, Trenton, NJ) controlled by Metamorph software. Fluo-4 was excited at 488 nm with a high pressure mercury arc lamp (excitation filters: 490/20 nm, dichroic mirror: 510 nm). Light intensity was regulated by neutral density filters. Light exposure time was 50 ms and acquisition rate was 20 Hz. Increase in $[Ca^{2+}]_i$ due to local mechanical stimulation leads to an increase in the emitted fluorescence. Increases in $[Ca^{2+}]_i$ over time are presented as F/F_0, where F is the average fluorescence intensity in the region of interest (ROI) at each time point during time lapse imaging, and F_0 is the fluorescence intensity in the ROI at time = 0. Changes in F/F_0 are presented as $\Delta F/F_0 = (F - F_0)/F_0$. All experiments were performed at room temperature. Image and kymograph analysis was performed with ImageJ software (NIH Image, Bethesda, MD) and data processing was performed with MatLab software (MathWorks, MA, USA).

2.4. Mechanical stimulation

Local mechanical stimulus was applied by gently and transiently touching a single cell with a micropipette (1 μm tip diameter, Eppendorf Femtotips I.). Under visible light the micropipette was initially positioned just above the target cell. Then, during fluorescence image capture, the pipette was lowered to transiently (<1 s) contact the cell with a transjector (transjector 5246, Eppendorf, Schönenbuch, Switzerland) attached to a micromanipulator (Leica Mikroskopie und Systeme GmbH). All experiments were performed in a homemade flow chamber. Perfusion flow (2 ml/min) was provided with a syringe pump (SP 210 IW, World Precision Instruments, Stevenage, UK). To exclude the possibility that paracrine signals diffuse to neighbouring cells, the direction of the perfusion flow was opposite to the pipette axis. Without perfusion of the extracellular medium, the onset Ca^{2+} signal could propagate from the target cell to all neighbouring cells in both directions. At the end of each experiment the stimulated cell was observed under transmitted light to verify that the membrane depression was slight and had not visibly damaged the cell. Control experiments showed that the stimulated cell could be repetitively and reproducibly stimulated without change in the rate of Ca^{2+} increase for subsequent stimuli (see Supp. Fig. S1).

2.5. Chemical stimulation

Chemicals were administered with local microejection of the drug. Microejection was performed by filling the micropipette (0.5 μm inner diameter, Eppendorf Femtotips I.) with KCl, placing