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Calcium oscillations in human mesenteric vascular smooth muscle

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ABSTRACT

Phenylephrine (PE)-induced oscillatory fluctuations in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of vascular smooth muscle have been observed in many blood vessels isolated from a wide variety of mammals. Paradoxically, until recently similar observations in humans have proven elusive. In this study, we report for the first time observations of adrenergically-stimulated $[Ca^{2+}]_i$ oscillations in human mesenteric artery smooth muscle. In arterial segments preloaded with Fluo-4 AM and mounted on a myograph on the stage of a confocal microscope, we observed PE-induced oscillations in $[Ca^{2+}]_i$, which initiated and maintained vasoconstriction. These oscillations present some variability, possibly due to compromised health of the tissue. This view is corroborated by our ultrastructural analysis of the cells, in which we found only (5 ± 2)% plasma membrane-sarcoplasmic reticulum apposition, markedly less than measured in healthy tissue from laboratory animals. We also partially characterized the oscillations by using the inhibitory drugs 2-aminoethoxydiphenyl borate (2-APB), cyclopiazonic acid (CPA) and nifedipine. After PE contraction, all drugs provoked relaxation of the vessel segments, sometimes only partial, and reduced or inhibited oscillations, except CPA, which rarely caused relaxation. These preliminary results point to a potential involvement of the sarcoplasmic reticulum Ca^{2+} and inositol 1,4,5-trisphosphate receptor (IP3R) in the maintenance of the Ca^{2+} oscillations observed in human blood vessels.

1. Introduction

Calcium oscillations and waves have been shown to represent the major Ca^{2+} signal for excitation–contraction coupling in smooth muscle of blood vessels in healthy experimental animals [1–7]. The mechanism underlying these phenomena appears complex and has been subject to numerous investigations in the past two decades. The basic mechanism has been shown to be a propagated wave of calcium induced calcium release (CICR) at the inositol 1,4,5-trisphosphate (IP3) receptors (IP_3R) [8]. While repetitive asynchronous Ca^{2+} wave-like oscillations are responsible for vascular tone and determine the size of the blood vessel lumen [8], synchronized Ca^{2+} oscillations have been associated with vasomotion [5,9]. Moreover, we have shown that refilling of the sarcoplasmic reticulum (SR) via plasma membrane (PM)-SR junctions is required for the maintenance of both the repetitive Ca^{2+} waves and vascular tone [8,10].

Unfortunately, the phenomenon of myoplasmic Ca^{2+} oscillations has thus far not been reported for intact smooth muscle tissue from human blood vessels (although the first report of intracellular Ca^{2+} waves was based on measurements done in smooth muscle cells cultured from humans [11]). In a recent publication, we documented this functional difference between vascular smooth muscles of human and murine origin, while demonstrating a striking difference in the membranous ultrastructure of the smooth muscle derived from the two different sources [12]. The human smooth muscle, which lacked Ca^{2+} oscillations, had very little peripheral SR and practically no PM-SR junctions, while the mice in accordance with previous reports responded to adrenergic activation with asynchronous repetitive Ca^{2+} waves and exhibited abundant peripheral SR and numerous PM-SR junctions. In addition, we noted that while the human blood vessels lacked Ca^{2+} oscillations the Rho-kinase component of their myofilament activation was enhanced [12]. Since the human patients supplying the blood vessels were generally elderly and of compromised health, we hypothesized that in human vascular smooth muscle the degradation of membranous ultrastructure leading to loss of Ca^{2+} wave-like oscillations could be causally linked to the development of vascular dysfunction and disease. Considering the prevalence of human vascular disease and the amount of

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suffering it inflicts, it is clear that the above putative link between aberrant Ca^{2+} signaling and vascular disease should be further investigated. However, without clear evidence of Ca^{2+} oscillations or waves in human vascular smooth muscle it could be argued that human blood vessels are different from those of experimental animals.

In this communication, we show for the first time that adrenergic stimulation of human mesenteric arteries induces Ca^{2+} oscillations in the medial smooth muscle cells. Since the challenges facing online living human blood vessel research are considerably greater than encountered in the laboratory animal, most of the mechanistic details are usually first established in the latter. The present demonstration that vascular Ca^{2+} signaling in humans involves repetitive Ca^{2+} oscillations provides the clinical relevance for their study in health and disease.

2. Materials and methods

This study was conducted according to the World Medical Association Declaration of Helsinki and informed consent was obtained from all subjects before sampling. Approval was granted by the institutional Ethics Review Board of the Universidad Complutense, Madrid, Spain, for the use of human specimen.

2.1. Tissue collection and preparation

Patients were recruited from those undergoing abdominal surgery at the General Surgery Service (Hospital Clínico Universitario San Carlos, Madrid, Spain). Samples of the mesenteric artery were collected during the surgical procedures, kept in RPMI 1640 (Gibco) medium at 4 °C and used within 30 min after the operations. Only tissues devoid of any obvious lesions were used. The tissue was transferred to physiological saline solution (PSS) and mesenteric arteries 0.3–1.5 mm in diameter were isolated, cleaned from connective tissue and cut into multiple segments that were about 2 mm in length.

2.2. Confocal $[Ca^{2+}]_i$ imaging and myography

The segments of human mesenteric arteries were loaded in PSS with Fluo-4AM (5 μM, Invitrogen, and 5 μM Pluronic F-127, Sigma–Aldrich) for 45 min at 37 °C and then left to equilibrate for 30 min (three 10-min washes) in normal PSS. Depending on the diameter of the vessel, the arterial segments were inverted for easier access to the muscular layer (to avoid rupture smaller segments were left un-inverted) and isometrically mounted on a myograph set on the stage of a confocal microscope for $[Ca^{2+}]_i$ measurements.

For vascular reactivity studies, we employed a modification of the methodology described by Mulvany and Halpern [13], using a 310A Danish Myo Technologies individual isometric myograph. The changes in $[Ca^{2+}]_i$ were measured using an inverted BioRad MRC-1024 laser scanning confocal microscope with a 20× air lens (Nikon Eclipse TE300).

The smooth muscle layer was illuminated using the 488-nm line of an argon-krypton laser, and a high-gain photomultiplier tube collected the emission at wavelengths between 505 and 550 nm. The measured changes in Fluo-4AM fluorescence level are proportional to the relative changes in $[Ca^{2+}]_i$. Laser intensity and gain were maintained constant during the experiments, which were all performed at 37 °C. Video capture was set to 900 cycles (approximately 1.25 s/cycle) for each experiment. Phenilephrine (PE) (10 μM, Sigma–Aldrich) was added to the bath 150 s after the start of the video capture and after 300 s the inhibitory drugs (nifedipine, Sigma–Aldrich; cyclopiazonic acid, Sigma–Aldrich; 2-Aminoethoxydiphenyl borate, Tocris) were added. At the same time vascular reactivity was measured with the myograph.

2.2.1. Image and contractility analysis

All data used for the Ca^{2+} traces were analyzed by ImageJ, using the built-in regions-of-interest (ROI) function to select the areas of interest, which were then line-scanned to produce the actual traces. Pseudo-colour visualization was performed by ImageJ, using customized lookup tables to assign colour for each pixel intensity value. Results were graphed using Gnuplot 4.6. Signal amplitude and contractile force attenuation measurements are reported as (mean ± SEM).

2.3. Electron microscopy imaging

Human mesenteric arteries from abdominal surgery were fixed with 4% paraformaldehyde + 2.5% glutaraldehyde in cold (4 °C) 0.1 M Na cacodylate buffer immediately after surgery. Arteries were then dissected, cleaned of the connective and fat tissue, and then cut into 1–2-mm-long segments. Arterial segments were fixed in the same fixative solution for 6 h. The segments were washed five times with cacodylate buffer every 30 min, and left overnight at 4 °C. Post-fixation with 1% osmium tetroxide and potassium ferricyanide in distilled water was done for 1.5 h, after which the segments were washed with distilled water (three 10-min washes) and dehydrated in increasing concentrations of acetone 50, 60, 70, 80, 90, 95 (15 min each) and 100% (three 10-min washes). The segments were then left in a gradual resin infiltration (1:3, 1:1, 3:1 acetone + pure resin) and finally left in pure resin (TAAB 812 mix) at 60 °C overnight. The resin embedded samples were sectioned by diamond knife, and the 80-nm sections were collected onto copper grids and post-stained with 1% uranyl acetate and Reynolds lead citrate for 4 and 3 min, respectively. Smooth muscle cell electron micrographs were obtained using a Jeol JEM-1010 high resolution transmission electronic microscope (JEOL, Tokyo, Japan).

2.3.1. Quantification of the PM-SR reticulum junctions

Electron micrographs of the human mesenteric arteries were converted into digital images, which were analyzed using the freeware inkscape 0.47 (inkscape.org) and an in-house modification of its 'measure.py' script to allow direct output of line measurements into a text file that could be used for quantitative analysis. To measure the extent of linear PM and SR apposition, superficial SR membrane segments and the PM in the individual vascular smooth muscle cell cross-sections were manually outlined and measured in units of pixels in inkscape. The length in pixels of the scale bar in the images provided a calibration gauge. For this analysis, we considered any peripheral SR as part of a PM-SR junction if its membrane was localized at a distance of 30 nm or less from the PM. High magnification whole cell montages were obtained from 11 cells and the entire PM was outlined and measured. Similarly, we measured the segments of junctional SR (Fig. 2A) and calculated the SR/PM ratio to obtain the data in the histogram of Fig. 2B. Measurements are reported as (mean ± SEM).

3. Results

3.1. Phenylephrine (PE)-induced $[Ca^{2+}]_i$ oscillations and contractile response

Calcium oscillations and subsequent contraction were observed in human mesenteric artery smooth muscle after addition of 10 μM PE (Fig. 1). The data for this part of the study were collected from samples from 9 patients and between 5 and 22 cells in each