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The mechanism of agonist induced Ca^{2+} signalling in intact endothelial cells studied confocally in *in situ* arteries

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ABSTRACT

In endothelial cells there remain uncertainties in the details of how Ca^{2+} signals are generated and maintained, especially in intact preparations. In particular the role of the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA), in contributing to the components of agonist-induced signals is unclear.

The aim of this work was to increase understanding of the detailed mechanism of Ca^{2+} signalling in endothelial cells using real time confocal imaging of Fluo-4 loaded intact rat tail arteries in response to muscarinic stimulation. In particular we have focused on the role of SERCA, and its interplay with capacitative Ca^{2+} entry (CCE) and ER Ca^{2+} release and uptake. We have determined its contribution to the Ca^{2+} signal and how it varies with different physiological stimuli, including single and repeated carbachol applications and brief and prolonged exposures.

In agreement with previous work, carbachol stimulated a rise in intracellular Ca^{2+} in the endothelial cells, consisting of a rapid initial phase, then a plateau upon which oscillations of Ca^{2+} were superimposed, followed by a decline to basal Ca^{2+} levels upon carbachol removal. Our data support the following conclusions: (i) the size (amplitude and duration) of the Ca^{2+} spike and early oscillations are limited by SERCA activity, thus both are increased if SERCA is inhibited. (ii) SERCA activity is such that brief applications of carbachol do not trigger CCE, presumably because the fall in luminal Ca^{2+} is not sufficient to trigger it. However, longer applications sufficient to deplete the ER or even partial SERCA inhibition stimulate CCE. (iii) Ca^{2+} entry occurs via STIM-mediated CCE and SERCA contributes to the cessation of CCE. In conclusion our data show how SERCA function is crucial to shaping endothelial cell Ca signals and its dynamic interplay with both CCE and ER Ca releases.

1. Introduction

Endothelial cells play a variety of important physiological roles, including influencing vascular tone, permeability, inflammation and platelet aggregation [1–5]. There is structural and functional heterogeneity in the endothelium depending on the size and location of the blood vessel it lines. However elevation of intracellular Ca^{2+} in endothelial cells is key to their physiological functions, including regulation of vascular tone, inter-endothelial cell gap formation, angiogenesis, immune defense, gene expression and cell growth [6,7]. In endothelial cells Ca^{2+} can be released from the intracellular store in the ER. Depletion of the ER Ca^{2+} store induces Ca^{2+} entry across the plasma membrane, in a process known as capacitative or store operated Ca^{2+} entry (CCE or SOCE) [8–11]. Coupling of agonists to receptors can open receptor operated channels, through which Ca^{2+} can also enter the cell [12–15]. Details of the interplay between these elements and the characteristics of the resulting Ca^{2+} signals are still being elucidated. For example cytosolic calcium signalling plays an important role in the generation of endothelium derived relaxing factors (NO, EDHF), which contributes to vascular smooth muscle relaxation, and in turn, reduces blood pressure. However, the mechanisms controlling the spatial and temporal characteristics of the Ca^{2+} signalling involved in the activation of eNOS and other processes, are still poorly understood. Recently, the proteins STIM-1 and Orai 1 have emerged as candidate components mediating CCE [16–20]. Stim-1 responds to the depletion of Ca^{2+} stores activating CCE via interaction with ORAI. However, it has also been reported that unlike macrovessels, CCE is not important in microvascular endothelial cell [21,22]. We have recently shown that in ureteric precapillary arterioles (microvessels), Ca^{2+} signalling in endothelial cells does not require Ca^{2+} entry and consists mainly of asynchronous, localized, high frequency Ca^{2+} oscillations [23].

From the above it can be appreciated that the SR/ER Ca-ATPase (SERCA) will be intimately involved in governing the characteristics and kinetics of agonist-induced Ca signals, through binding Ca, influencing Ca releases through IP_3-dependent Ca^{2+} channels and instigating and terminating CCE via effects on luminal Ca levels [24].

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However the information concerning SERCA's role in the endothelium is limited. Fierro and Parekh found that SERCA pumps were remarkably effective in RBL-1 cells and could prevent I_{CRAC} from activating, despite significant Ca^{2+} leakage from the stores [25]. Patch clamp studies in other types of cells [26,27] suggest however that SERCA should be able to terminate CCE as it refills the store.

Thus there is a need to systematically examine all these components in one cell type and endothelium from a single type of blood vessel. Furthermore there is also a lack of clarity around the roles of SERCA and CCE in the Ca^{2+} signals occurring in *intact* endothelium. While there may be advantages to studying cultured endothelial cells, recent research has highlighted the importance of studying the intact endothelium e.g. [28,29]. In the current study therefore we have determined the temporal and spatial organization of Ca^{2+} signals induced by muscarinic stimulation *in situ* in Fluo-4 loaded endothelial cells of rat tail artery using confocal microscopy, to better define the mechanisms controlling the characteristics of the Ca signals in response to muscarinic stimulation and the exact role of SERCA during different parameters of stimulations.

We demonstrate that the initial Ca^{2+} wave and the early Ca^{2+} oscillations originated from the ER by the interplay of repetitive releases and re-uptake of Ca^{2+} by IP_3-dependent Ca^{2+} channels and SERCA pump activity, respectively. In contrast, interplay between Ca^{2+} release, Ca^{2+} influx through CCE channels and SERCA was required to sustain the late Ca^{2+} oscillations and plateau.

2. Materials and methods

2.1. Ca^{2+} measurements

Tail was removed from rat humanely killed by cervical dislocation under CO_2 anesthesia in accordance with Home Office legislation Artery was dissected from the ventral grove, cleaned of fat and kept in physiological saline before use. They were loaded with Fluo-4 acetoxymethyl ester (Invitrogen, UK, 15 μmol/L; dissolved in DMSO with pluronic acid) for 2–3 h at 20 °C and then transferred to indicator-free solution for 30 min. Small segments of Fluo-4 loaded tail artery (3–4 mm in length) were cut open and fixed to the bottom of the chamber by aluminum foil clips, to minimize movement, with endothelium facing down. All experiments were performed at 30 °C. We used a Nipkow disc based, confocal microscope (Perkin Elmer), connected to a sensitive iXon cooled charge-coupled device camera (Andor). Images were collected at 30 frames per second using a 60× water objective (NA 1.20) for best spatial resolution or dry (20×, 0.70 NA) for a larger field of view.

2.2. Solutions

Physiological saline of the following composition was used (mmol/L): NaCl 120.4, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 2.0, glucose 8, and HEPES 11. In some experiments, Ca^{2+}-free solution (2 mmol/L EGTA) was used. 2-Aminoethoxydiphenyl borate (2-APB), ryanodine, and U-73122 (to inhibit phospholipase C), were from Calbiochem (Nottingham, UK), all other chemicals were from Sigma (UK). Phenylephrine, endothelin-1 (ET-1), ryanodine, and carbachol were dissolved in water; 2-APB, cyclopiazonic acid (CPA, to inhibit SERCA), U-73122 in DMSO.

2.3. Immunohistochemistry

Specimens of dissected tail artery in the absence of stimulation (control) or following two consecutive applications of carbachol in the presence of CPA to deplete Ca^{2+} stores and maximally activate CCE (see Fig. 11 for details), were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4 for 1.5 h at 23 °C, and then in 25% sucrose in 0.1 M PBS overnight at 4 °C, cut on a cryostat at 10 μm and mounted onto poly-lysine coated microscope slides. Cryostat sections were rinsed in 0.1 M PBS, permeabilized in alcohol and processed for single labelling immunofluorescence. In brief, tissue sections were pre-blocked in 10% normal donkey serum in 0.1 M PBS and incubated with rabbit polyclonal STIM1 (C-terminal) antibody (ProSci Incorporated, Nottingham, UK; 1:300) overnight at 4 °C. The secondary antibody was Texas red-conjugated (AffiniPure) donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories; West Grove, PA, USA) and sections of tail artery were mounted under Vectashield containing 4′6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) to preserve fluorescence and stain cell nuclei. Specificity of immunostaining was determined by omitting the primary antibody. Sections were examined using an Axioplan Universal microscope, and images were processed using the Axio Vision 3.0 Imaging system with deconvolution options (Carl Zeiss Vision, Jena, Germany).

2.4. Statistics

All analysis was performed using Microcal Origin 8.0 (Massachusetts, USA). Quantification of calcium records from confocal imaging were made by measuring the peak amplitude, frequency and duration of calcium transients. The results are given as percentage of control value unless otherwise stated. Values are means ± SEM, and n is number of vessels or cells. Between 3 and 5 animals were used for each series of experiments. Differences were taken as significant at $P < 0.05$ in Student's *t*-test.

3. Results

3.1. Live morphology of the ECs in situ

By taking a series of optical sections in the *Z* direction it was possible to recreate a three-dimensional image of the endothelium of intact rat tail artery loaded with Fluo 4 (Fig. 1, Video 1). The endothelial cells (EC) appeared as monolayer of cells lining the luminal surface of the elastic lamina with variable length and width (Fig. 1, Video 1).

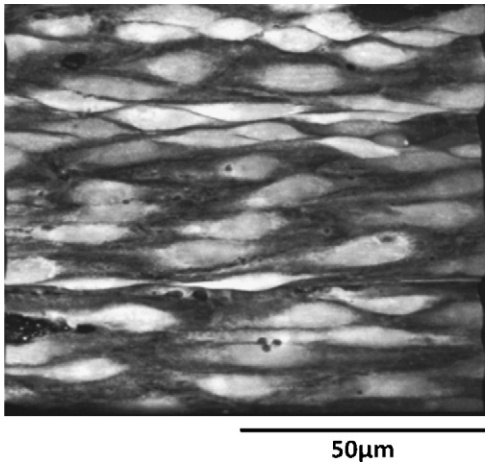

Fig. 1. Live morphology of endothelial cells (ECs) of intact rat tail artery *in situ*. 3-Dimensional confocal image of intact ECs seen in *x*–*y* projection observed using 60× objective (Video 1).