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CaMKII regulates intracellular Ca^{2+} dynamics in native endothelial cells

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ABSTRACT

Localized endothelial Ca^{2+} signalling, such as Ca^{2+} pulsars, can modulate the contractile state of the underlying vascular smooth muscle cell through specific endothelial targets. In addition to $K_{Ca}3.1$ as a target, Ca^{2+} pulsars, an IP_3R-dependent pulsatile Ca^{2+} release from the endoplasmic reticulum (ER) could activate a frequency-sensitive Ca^{2+}-dependent kinase such as CaMKII. In the absence of extracellular Ca^{2+}, acetylcholine increased endothelial CaMKII phosphorylation and activation, thereby suggesting CaMKII activation independently of Ca^{2+} influx. Herein, a reciprocal relation where CaMKII controls endothelial Ca^{2+} dynamics has been investigated in mesenteric arteries. Both CaMKIIα and β isoforms have been identified in endothelial cells and close proximity (<40 nm) suggests their association in heteromultimers. Intracellular Ca^{2+} monitoring with high speed confocal microscopy then showed that inhibition of CaMKII with KN-93 significantly increased the population of Ca^{2+} pulsars active sites (+89%), suggesting CaMKII as a major regulator of Ca^{2+} pulsars in native endothelium. Mechanistic insights were then sought through the elucidation of the impact of CaMKII on ER Ca^{2+} store. ER Ca^{2+} emptying was accelerated by CaMKII inhibition and ER Ca^{2+} content was assessed using ionomycin. Exposure to KN-93 strongly diminished ER Ca^{2+} content (−61%) by relieving CaMKII-dependent inhibition of IP_3 receptors (IP_3R). Moreover, *in situ* proximity ligation assay suggested CaMKII-IP_3R promiscuity, essential condition for a protein–protein interaction. Interestingly, segregation of IP_3R within myoendothelial projection (MEP) appears to be isoform-specific. Hence, only IP_3R type 1 and type 2 are detected within fenestrations of the internal elastic lamina, sites of MEP, whilst type 3 is absent from these structures. In summary, CaMKII seems to act as a Ca^{2+}-sensitive switch of a negative feedback loop regulating endothelial Ca^{2+} homeostasis, including Ca^{2+} pulsars.

1. Introduction

Strategically positioned between the blood and vascular smooth muscle cells (SMCs), endothelial cells (ECs) are keepers of vascular homeostasis. Intracellular Ca^{2+} is a powerful and versatile signalling molecule, essential to numerous regulatory mechanisms of endothelial functions. For example, thrombin-induced rise in global Ca^{2+} activates Ca^{2+}-sensitive PKC, leading to increased endothelial permeability [1,2]. Ca^{2+} is also involved in the regulation of endothelial gene transcription. ATP- or bradykinin-evoked increase in endothelial Ca^{2+} level stimulates nuclear translocation of NFAT (Nuclear Factor of Activated T cells), a transcription factor [3]. Fine-tuning of vascular tone is also a crucial Ca^{2+}-driven function of vascular endothelium. Indeed, generation of nitric oxide (NO), a potent vasodilator by endothelial nitric oxide synthase (NOS3) involves direct binding of Ca^{2+}/Calmodulin complex

Abbreviations: K_{Ca}, calcium-activated potassium channel; NO, nitric oxide; NOS3, endothelial nitric oxide synthase; CaMKII, Ca^{2+}/calmodulin kinase II; ER, endoplasmic reticulum; KN-93, (2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl) amino-N-(4-chlorocinnamyl)-N-methylbenzylamine); IP_3R, inositol 1*4*5-trisphosphate receptor; MEP, myoendothelial projection; SMC, smooth muscle cell; EC, endothelial cell; NFAT, nuclear factor of activated T cells; Ca^{2+}/CaM, Ca^{2+}/calmodulin complex; TRPV, transient receptor potential vanilloid channel; TRPA, transient receptor potential ankyrin channel; PLB, phospholamban; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; P_0, opening probability; MA, mesenteric artery; IEL, internal elastic lamina; ACh, acetylcholine; ROIs, regions of interest; PLA, proximity ligation assay; EDHF, endothelium-derived hyperpolarizing factor; SOCE, store-operated Ca^{2+} entry.

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(Ca^{2+}/CaM) [4,5]. Furthermore, intracellular Ca^{2+} has been shown to modulate endothelial membrane potential by activating Ca^{2+}-activated potassium channel ($K_{Ca}2.3$ and $K_{Ca}3.1$) [6,7]. Although endothelial functions governed by Ca^{2+} have been extensively studied, endothelial Ca^{2+} homeostasis has mainly been investigated from a global cytoplasmic perspective [8–11]. Interestingly, an increasing body of evidence suggests that localized Ca^{2+} dynamics might actually be a critical player in the modulation of endothelial functions [12–14].

Tight modulation of intracellular Ca^{2+} levels from a spatiotemporal standpoint has major functional impact. Thus, localized Ca^{2+} events such as Ca^{2+} pulsars, Ca^{2+} wavelets, TRPV4- and TRPA1-sparklets have recently been characterized and are suggested to regulate vascular tone [12–16]. Early work on Ca^{2+} pulsars reported a spontaneous Ca^{2+} release from IP_3-sensitive stores within myoendothelial projections (MEP) in mesenteric resistance arteries [15]. Similar to Ca^{2+} pulsars, Ca^{2+} wavelets have been reported in skeletal muscle arteries [16]. More recently, a spatially restricted Ca^{2+} influx through plasma membrane ion channels, TRPV4- and TRPA1-sparklets, has been reported with an elegant demonstration of the requirement of intracellular scaffolding for cellular function [12,14,16]. These investigations emphasize the need for a better understanding of the regulatory mechanisms of confined Ca^{2+} dynamics.

Ca^{2+} pulsar results from the spontaneous and oscillatory Ca^{2+} release through IP_3 receptors (IP_3R) in a definite subcellular region, the MEP. A critical aspect of this localized endothelial Ca^{2+} signal is its occurrence within MEPs. This anatomical structure forms a spatially restricted microenvironment allowing intimate communication between the endothelium and the underlying SMCs [17]. Ca^{2+} pulsars can indeed modulate SMCs contractile state by promoting endothelial MEP-$K_{Ca}3.1$ channels opening and therefore smooth muscle hyperpolarization. This signalling cascade can lead to SMCs relaxation. CaMKII, a Ca^{2+}-dependent protein kinase, might represent a novel target of Ca^{2+} pulsars and extend the scope of Ca^{2+} pulsar outcomes. However, in depth characterization of Ca^{2+} events like Ca^{2+} pulsars is yet missing. For example, the mechanisms regulating the stochasticity or spatial dispersion of the events or the relative role of IP_3R subtypes remain to be determined.

CaMKII is characterized by its unique ability to decode and integrate oscillatory Ca^{2+} signals into specific outcomes [18,19] as previously shown in neurons [20]. Interestingly, CaMKII is a key modulator of Ca^{2+} homeostasis in cardiomyocytes mainly through two pathways. First, CaMKII phosphorylates phospholamban (PLB), hence removing its inhibitory influence on SERCA2a, accelerating cytoplasmic Ca^{2+} clearance [21]. On the other hand, CaMKII phosphorylates nuclear IP_3R type 2 (IP_3R-2) resulting in a decreased channel P_0 [22]. CaMKII-associated inhibition of IP_3R has also been reported in HeLa cell [23]. Although well characterized in cardiomyocytes, putative role for CaMKII in the control of Ca^{2+} dynamics in native endothelium has yet to be established. Moreover, while expression of all three IP_3R isoforms have been reported in native endothelium [15], it is unclear whether CaMKII regulation of IP_3R is restricted to IP_3R-2. Similarly, it is unknown if IP_3R modulation by CaMKII is restricted to the cardiac isoform. Although CaMKII heteromultimers have been reported in neurons, it is unknown if they are present in native ECs.

The present study was then undertaken to elucidate a CaMKII-Ca^{2+} pulsars relationship and the pathways involved. Interestingly, CaMKII modulates Ca^{2+} pulsars through management of the population of Ca^{2+} pulsar active sites. This regulation appears to occur through IP_3R inhibition interaction and accordingly, CaMKII regulates endothelial ER Ca^{2+} store. Therefore, the findings from this study strongly suggest that endothelial CaMKII is a Ca^{2+} sensor switch involved in a negative feedback loop modulating Ca^{2+} pulsars.

2. Material and methods

2.1. Animals

Animal manipulations were approved by the Montreal Heart Institute Animal Care Committee. Mice (3–4 months old, both genders) were sacrificed by intraperitoneal injection of a lethal dose of pentobarbital (150 mg/kg). Transgenic mice expressing GCaMP2 [24], a fluorescent Ca^{2+}-sensor under connexin 40 promotor, were used for Ca^{2+} pulsars experiments. C57/BL6 mice (Charles River) were used for endoplasmic reticulum Ca^{2+} content experiments, immunohistochemistry and *in situ* proximity ligation assay. Every experimental series were reproduced with tissues from a minimum of two individual mice ($N \geq 2$).

2.2. Tissue preparation

Third- or fourth-order mesenteric arteries (MA) (diameters $\approx$ 60–100 μm) were isolated from C57/BL6 mice or transgenic mice. For all type of experiments, MA were cleaned of adipose and connective tissue in cooled HEPES solution (134 mM NaCl, 6 mM KCl, 10 mM glucose, 10 mM HEPES, 1 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.4), cut longitudinally, and pinned with the endothelium facing up (*en face* configuration) on Sylgard blocks. Sample size for each experiment ("*n*") represents the number of individual arterial preparation.

2.3. Endothelial Ca^{2+} imaging

Experiments were performed at 37 °C in a physiological salt solution (PSS) containing: 119 mM NaCl, 4.7 mM KCl, 24 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 11 mM Glucose and 1.5 mM $CaCl_2$. Experiments with extracellular Ca^{2+}-free solutions were performed in PSS with 5 mM EGTA and where $CaCl_2$ was omitted. All solutions were oxygenated (12% O_2, 5% CO_2) throughout the experiment.

Ca^{2+} dynamics were monitored in endothelium of MA from GCaMP2 mice (Ca^{2+} pulsars) or C57BL/6 mice loaded with Fluo-4 (endoplasmic reticulum Ca^{2+} content). For Fluo-4 experiments, MA were incubated with the dye (10 μM) and 2.5 μg/ml of pluronic acid for 45 min at 37 °C. Extracellular Ca^{2+} contribution to recorded fluorescence was eliminated with the incubation of MA for 120 s in a Ca^{2+}-free PSS solution prior exposure to cyclopiazonic acid (30 μM; Fig. 5) or before addition of ionomycin (10 μM; Fig. 6).

Ca^{2+} imaging was performed with an Andor Revolution confocal system and images were acquired with Andor iQ 2.8 software (Andor Technology) using an electron-multiplying CCD camera (iXon) on an upright Nikon Eclipse FN-1 microscope. Full frame (512×512) images were acquired at ≈15 frames/s. Ca^{2+} binding to GCaMP2 or Fluo-4 was recorded at ex/em 488/510 nm. Autofluorescence of the internal elastic lamina (IEL) was also assessed at ex/em 488/510 nm. Ca^{2+} pulsars and global Ca^{2+} were analyzed using SparkAn software (A. Bonev, UVM).

Analysis of Ca^{2+} pulsars imaging was carried out offline using regions of interest (ROIs) defined by a 5×5 pixels box manually positioned at the site corresponding to the pulsar's peak amplitude location ("Ca^{2+} pulsar sites"). "Total Ca^{2+} pulsars frequency" is defined as the number of Ca^{2+} pulsar events in a field of view per time. "Ca^{2+} pulsars frequency/active sites" is defined as the mean of individual Ca^{2+} pulsar frequencies for each active site. "Ca^{2+} pulsars active sites" is defined as the number of Ca^{2+} pulsar sites in a field of view which had a minimum of one Ca^{2+} event in the experimental condition. Substantial intervessel variability was circumvented by normalizing the values recorded to the basal values (control; absence of any drug) from the same preparation. Basal values were then set as 100%. Global Ca^{2+} increase was assessed by measuring