Cell Calcium 66 (2017) 33–47

Contents lists available at ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

Acetylcholine induces intracellular Ca^{2+} oscillations and nitric oxide release in mouse brain endothelial cells

Estella Zuccolo[a], Dmitry Lim[b], Dlzar Ali Kheder[a,c], Angelica Perna[d], Paolo Catarsi[e], Laura Botta[a], Vittorio Rosti[e], Laura Riboni[f], Giulio Sancini[g], Franco Tanzi[a], Egidio D'Angelo[h,i], Germano Guerra[d,**,1], Francesco Moccia[a,*,1]

[a] Laboratory of General Physiology, Department of Biology and Biotechnology "Lazzaro Spallanzani", University of Pavia, Pavia, Italy
[b] Department of Pharmaceutical Sciences, University of Eastern Piedmont "Amedeo Avogadro", Novara, Italy
[c] Department of Biology, University of Zakho, Kurdistan-Region of Iraq, Iraq
[d] Department of Medicine and Health Sciences "Vincenzo Tiberio", University of Molise, Via F. De Santis, 86100 Campobasso, Italy
[e] Center for the Study of Myelofibrosis, Research Laboratory of Biotechnology, Foundation IRCCS Policlinico San Matteo, Pavia, Italy
[f] Department of Medical Biotechnology and Translational Medicine, LITA-Segrate, University of Milan, Segrate, 20090 Milan, Italy
[g] Department of Experimental Medicine, University of Milano-Bicocca, 20900 Monza, Italy
[h] Department of Brain and Behavioral Sciences, University of Pavia, 27100 Pavia, Italy
[i] Brain Connectivity Center, C. Mondino National Neurological Institute, 27100 Pavia, Italy

ARTICLE INFO

Article history:
Received 16 February 2017
Received in revised form 5 June 2017
Accepted 10 June 2017
Available online 12 June 2017

Keywords:
Mouse brain microvascular endothelial cells
Acetylcholine
Nitric oxide
Ca^{2+} signaling
Intracellular Ca^{2+} oscillations

ABSTRACT

Basal forebrain neurons increase cortical blood flow by releasing acetylcholine (Ach), which stimulates endothelial cells (ECs) to produce the vasodilating gasotransmitter, nitric oxide (NO). Surprisingly, the mechanism whereby Ach induces NO synthesis in brain microvascular ECs is unknown. An increase in intracellular Ca^{2+} concentration recruits a multitude of endothelial Ca^{2+}-dependent pathways, such as Ca^{2+}/calmodulin endothelial NO synthase (eNOS). The present investigation sought to investigate the role of intracellular Ca^{2+} signaling in Ach-induced NO production in bEND5 cells, an established model of mouse brain microvascular ECs, by conventional imaging of cells loaded with the Ca^{2+}-sensitive dye, Fura-2/AM, and the NO-sensitive fluorophore, DAF-DM diacetate. Ach induced dose-dependent Ca^{2+} oscillations in bEND5 cells, 300 μM being the most effective dose to generate a prolonged Ca^{2+} burst. Pharmacological manipulation revealed that Ach-evoked Ca^{2+} oscillations required metabotropic muscarinic receptor (mAchR) activation and were patterned by a complex interplay between repetitive ER Ca^{2+} release via inositol-1,4,5-trisphosphate receptors ($InsP_3Rs$) and store-operated Ca^{2+} entry (SOCE). A comprehensive real time-polymerase chain reaction analysis demonstrated the expression of the transcripts encoding for M3-mAChRs, $InsP_3R1$ and $InsP_3R3$, Stim1-2 and Orai2. Next, we found that Ach-induced NO production was hindered by L-NAME, a selective NOS inhibitor, and BAPTA, a membrane permeable intracellular Ca^{2+} buffer. Moreover, Ach-elicited NO synthesis was blocked by the pharmacological abrogation of the accompanying Ca^{2+} spikes. Overall, these data shed novel light on the molecular mechanisms whereby neuronally-released Ach controls neurovascular coupling in blood microvessels.

1. Introduction

The brain consumes ≈20% of the total energy produced by the body and therefore requires an adequate supply of oxygen and nutrients to maintain its structural and functional integrity [1,2]. The mechanism by which an increase in neural activity leads to a corresponding elevation in cerebral blood flow (CBF) has been termed neurovascular coupling (NVC) or functional hyperaemia [1,2]. The stimulation of basal forebrain cholinergic neurons has long been known to increase cortical perfusion by inducing the vasodilation of intraparenchymal microvessels [3–6].

* Corresponding author at: Laboratory of General Physiology, Department of Biology and Biotechnology "Lazzaro Spallanzani", University of Pavia, Via Forlanini 6, 27100, Pavia, Italy.
** Corresponding author.
E-mail addresses: germano.guerra@unimol.it (G. Guerra), francesco.moccia@unipv.it (F. Moccia).
[1] These authors share senior authorship.

http://dx.doi.org/10.1016/j.ceca.2017.06.003
0143-4160/

Acetylcholine (Ach) released by post-synaptic terminals binds to endothelial muscarinic receptors (mAchRs), thereby leading to nitric oxide (NO) production and microvessel vasodilation [3,7,8]. Although a wealth of information is available about the molecular mechanisms that underpin NO release in response to neural activity in the central nervous system [9,10], the signaling pathways that drive NO synthesis in brain microvascular endothelial cells remain largely obscure [11].

In other vascular beds [12,13], Ach stimulates NO production by initiating an oscillatory increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The stimulation of Gq/11-coupled mAchRs recruits phospholipase Cβ (PLCβ) to synthesize the second messengers diacylglycerol (DAG) and inositol-1,4,5-trisphosphate ($InsP_3$), which triggers brief pulses of Ca^{2+} release from the endoplasmic reticulum (ER) [14]. The ensuing depletion of the endogenous Ca^{2+} reservoir activates the ER Ca^{2+} sensor Stromal Interaction Molecule 1 (Stim1), which oligomerizes and relocates in proximity of the plasma membrane to trap and gate the store-operated Ca^{2+}-permeable channel, Orai [15,16]. Store-operated Ca^{2+} entry (SOCE) is indispensable to maintain Ach-evoked intracellular Ca^{2+} oscillations over time by replenishing the ER Ca^{2+} pool [12,15,17]. The interaction between the cyclical $InsP_3$-dependent Ca^{2+} discharge and the sustained SOCE supports Ach-dependent NO release in several vascular districts, such as mouse and rat tail artery endothelial cells [15,17,18], rat mesenteric artery endothelial cells [19], and mouse aortic endothelium [20,21]. The Ca^{2+}-dependent step in the enzymatic reaction leading to NO synthesis is provided by calmodulin (CaM) activation, which relieves the constitutive inhibitory effect of caveolin on endothelial NO synthase (eNOS) and enables NO formation [22,23]. Moreover, intracellular Ca^{2+} signals may stimulate cyclooxygenase-2 (COX-2) to synthesize the vasorelaxing agent, prostaglandin E2 (PGE2) [24], and intermediate- and small-conductance Ca^{2+}-activated K^+ channels, which represent another powerful vasodilator in microvessels [25]. As mentioned earlier, however, the waveform of Ach-induced Ca^{2+} signaling in brain microvascular endothelial cells and its relationship with NO release are still unclear.

Herein, we used bEND5 cells, an established model of immortalized mouse brain microvascular endothelial cells [26–28], to investigate the mechanistic link between Ach-induced increase in $[Ca^{2+}]_i$ and NO production. By using a combination of Ca^{2+} and NO imaging and real-time polymerase chain reaction (qRT-PCR), we demonstrated that Ach triggers repetitive intracellular Ca^{2+} oscillations in bEND5 cells by inducing a complex interplay between SOCE and ER-dependent Ca^{2+} mobilization. The spiking response to Ach, in turn, leads to a robust NO release, which requires the concomitant activation of all the Ca^{2+} signaling pathways involved. These findings provide significant insights on the subtle mechanisms of CBF regulation by brain microvascular endothelial cells.

2. Materials and methods

2.1. Cell culture

Mouse endothelial cell bEND5 cells were used to investigate the Ca^{2+} and NO response to Ach in mouse brain microvascular endothelial cells. The bEND5 cell line (American Type Culture Collection, Manassas, VA, USA) is an immortalized mouse cell line from brain capillary endothelial cells. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum, 4 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 50 Units/mL penicillin, and 50 mg/mL streptomycin, 1% minimal essential medium nonessential amino acids exactly, as originally described previously [28]. Cells were cultured in a humidified cell culture incubator at 37 °C and an atmosphere of 5% CO_2/95% air. The bEND5 cells used in this study were passaged between 17 to 21 times.

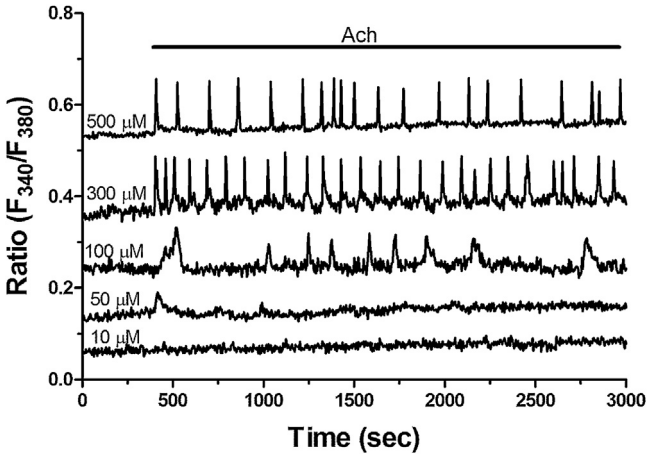

Fig. 1. Acetylcholine evokes repetitive Ca^{2+} transients in bEND5 cells. Acetylcholine causes the immediate initiation of an oscillatory Ca^{2+} response whose duration, amplitude of the 1st spike and interspike interval (ISI) was a function of agonist concentration. In this and the following figures, Ach was added at the time indicated by the horizontal bar drawn around the Ca^{2+} tracings. The baseline of Ca^{2+} tracings has been shifted to avoid their overlapping for representation purposes.

Human brain endothelial cells (hCMEC/D3) were obtained from Institut National de la Santé et de la Recherche Médicale (INSERM, Paris, France). hCMEC/D3 cells cultured between passage 25 and 35 were used. As described in [29], the cells were seeded at a concentration of 27,000 cells/cm^2 and grown in tissue culture flasks coated with 0.1 mg/mL rat tail collagen type 1, in the following medium: EBM-2 medium (Lonza, Basel, Switzerland) supplemented with 5% fetal bovine serum (FBS), 1% Penicillin–Streptomycin, 1.4 μM hydrocortisone, 5 μg/mL ascorbic acid, 1/100 chemically defined lipid concentrate (Invitrogen), 10 mM HEPES and 1 ng/mL basic FGF (bFGF). The cells were cultured at 37 °C, 5% CO_2 saturated humidity.

2.2. Solutions

Physiological salt solution (PSS) had the following composition (in mM): 150 NaCl, 6 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 Glucose, 10 Hepes. In Ca^{2+}-free solution ($0Ca^{2+}$), Ca^{2+} was substituted with 2 mM NaCl, and 0.5 mM EGTA was added. Solutions were titrated to pH 7.4 with NaOH. In Mn^{2+}-quenching experiments, 200 μM $MnCl_2$ was added to the $0Ca^{2+}$ external solution. The osmolality of PSS as measured with an osmometer (Wescor 5500, Logan, UT) was 338 mmol/kg.

2.3. $[Ca^{2+}]_i$ measurements

We utilized the Ca^{2+} imaging set-up we have described elsewhere [30]. bEND5 or hCMEC/D3 cells were loaded with 4 μM fura-2 acetoxymethyl ester (Fura-2/AM; 1 mM stock in dimethyl sulfoxide) in PSS for 1 h min at room temperature. After washing in PSS, the coverslip was fixed to the bottom of a Petri dish and the cells observed by an upright epifluorescence Axiolab microscope (Carl Zeiss, Oberkochen, Germany), usually equipped with a Zeiss × 40 Achroplan objective (water-immersion, 2.0 mm working distance, 0.9 numerical aperture). The cells were excited alternately at 340 and 380 nm, and the emitted light was detected at 510 nm. A first neutral density filter (1 or 0.3 optical density) reduced the overall intensity of the excitation light and a second neutral density filter (optical density = 0.3) was coupled to the 380 nm filter to approach the intensity of the 340 nm light. A round diaphragm was used to increase the contrast. The excitation filters were mounted on a filter wheel (Lambda 10, Sutter Instrument, Novato, CA, USA). Custom