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Na^+–Ca^{2+} exchanger contributes to Ca^{2+}extrusion in ATP-stimulated endothelium of intact rat aorta

Roberto Berra-Romani [a], Abdul Raqeeb [b,1], Alejandro Guzman-Silva [a], Julián Torres-Jácome [c], Franco Tanzi [d], Francesco Moccia [d,*]

[a] School of Medicine, Department of Biomedicine, 13 Sur 2702, Colonia Volcanes, 72000 Puebla, Mexico
[b] Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada
[c] Instituto de Fisiología, Benemérita Universidad Autónoma de Puebla, 13 Sur 2702, Colonia Volcanes, 72000 Puebla, Mexico
[d] Department of Physiology, University of Pavia, via Forlanini 6, 27100 Pavia, Italy

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ABSTRACT

The role of Na^+–Ca^{2+} exchanger (NCX) in vascular endothelium is still matter of debate. Depending on both the endothelial cell (EC) type and the extracellular ligand, NCX has been shown to operate in either the forward (Ca^{2+} out)- or the reverse (Ca^{2+} in)-mode. In particular, acetylcholine (Ach) has been shown to promote Ca^{2+} inflow in the intact endothelium of excised rat aorta. Herein, we assessed the involvement of NCX into the Ca^{2+} signals elicited by ATP in such preparation. Removal of extracellular Na^+ ($0Na^+$) causes the NCX to switch into the reverse-mode and induced an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), which disappeared in the absence of extracellular Ca^{2+}, and in the presence of benzamil, which blocks both modes of NCX, and KB-R 7943, a selective inhibitor of the reverse-mode. ATP induced a transient Ca^{2+} signal, whose decay was significantly prolonged by $0Na^+$, benzamil, DCB, and monensin while it was unaffected by KB-R 7943. Notably, lowering extracellular Na^+ concentration increased the sensibility to lower doses of ATP. These date suggest that, unlike Ach-stimulated ECs, NCX promotes Ca^{2+} extrusion when the stimulus is provided by ATP in intact endothelium of rat aorta. These data show that, within the same preparation, NCX operates in both modes, depending on the chemical nature of the extracellular stimulus.

1. Introduction

An increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is a key signal in the regulation of endothelial function by extracellular stimuli [1]. Therefore, alterations in Ca^{2+} handling may lead to dysregulation of endothelial proliferation, migration, and permeability, which in turn result in severe cardiovascular disease [2]. Autacoids-induced Ca^{2+} signals are mainly shaped by the interplay between Ca^{2+} release from inositol-1,4,5-trisphosphate ($InsP_3$) sensitive stores and external Ca^{2+} influx through the so-called store-operated Ca^{2+} entry (SOCE) pathway [1]. The molecular identity of the latter in endothelial cells (ECs) is still controversial [3]. Evidence has been provided about the involvement of both the STIM1/Orai1 complex [4], which mediate an inwardly-rectifying, Ca^{2+}-selective current, and members of the non-selective canonical transient receptor potential (TRPC) family [5], which are permeable to both Ca^{2+} and Na^+. A tight control of the spatio-temporal profile of the intracellular Ca^{2+} elevation enables Ca^{2+} to subserve a wide range of functions in vascular endothelium [6]. For instance, the establishment of a Ca^{2+} gradient between the sub-plasmalemmal space and the bulk cytoplasm results in the selective recruitment of Ca^{2+}-sensitive effectors targeted to the plasma membrane (PM), such as the endothelial nitric oxide synthase (eNOS) [7]. Nitric oxide (NO) release from vascular endothelium, in turn, plays a key role in regulating both vascular tone and permeability [8]. Sub-plasmalemmal Ca^{2+} levels depend on the activity of the Na^+/Ca^{2+} exchanger (NCX) which may either extrude or import calcium ions across PM depending on membrane potential and transmembrane Na^+ and Ca^{2+} gradients [9,10]. In resting cells, NCX removes Ca^{2+} from the cell by operating in forward mode ($3Na^+$ in:1 Ca^{2+} out), whereas, in activated cells, TRPC-dependent Na^+ accumulation beneath the PM cause the NCX to switch to the reverse-mode ($3Na^+$ out:1 Ca^{2+} in) and contribute to Ca^{2+} entry [11–13]. This process relies on the close proximity between NCX and the TRPC engaged by extracellular stimuli, which may either physically interact [14] or be clustered in limited membrane nanodomains [12]. The role of NCX in intracellular Ca^{2+} homeostasis in ECs is still matter of debate. NCX has been shown to extrude Ca^{2+} upon agonist stimulation in rat cardiac microvascular ECs (CMECs) [15], rat brain microvascular ECs [16] and rabbit aortic ECs [17]. Conversely, NCX mediates autacoid-

* Corresponding author. Fax: +39 0382 987527.
E-mail address: francesco.moccia@unipv.it (F. Moccia).
[1] Present address.

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induced Ca^{2+} inflow in the umbilical vein EC-derived endothelial cell line EA.hy926 [18] and in intact endothelium of rat aorta [19]. Unraveling the mode whereby NCX regulates intracellular Ca^{2+} signaling in vascular endothelium has a physiopathological relevance. It has been reported that the reverse-mode (i.e. Ca^{2+} entry) of NCX sustains NO production and results in endothelium-dependent relaxation [20]. In line with these observations, a recent study suggested that NCX augments neovascularization in mice with hindlimb ischemia by promoting eNOS-dependent angiogenesis [21]. Therefore, NCX has been proposed as a suitable target to design novel therapeutic interventions to treat cardiovascular disease [22].

The different contribution of the exchanger to intracellular Ca^{2+} homeostasis in ECs has been ascribed to changes in NCX properties in cultured cells as compared to *in situ* ECs [19]. An alternative explanation involves the widespread concept of compartmentalization of the Ca^{2+} signaling toolkit [23]. When considering that NCX integrates local Ca^{2+} and Na^+ changes beneath the PM, the operation mode of the exchanger may be selectively governed by its close proximity to a specific agonist-activated channel [14]. For instance, tight spatial coupling to Na^+-dependent TRPC channels will favor the reverse-mode, while local accumulation of Ca^{2+} as a consequence of Ca^{2+} release from $InsP_3$-sensitive receptors ($InsP_3Rs$) or Ca^{2+} entry through Orai1 is expected to favor the forward-mode.

In order to assess whether NCX role in endothelial Ca^{2+} signaling is related to the activating agonist, we exposed the ECs lining excised rat aorta, where NCX promotes Ca^{2+} entry on Ach stimulation, to ATP. We provided the evidence that, under such conditions, NCX promotes Ca^{2+} clearance and masks the response to low agonist concentrations. Our results indicate therefore that NCX may drive either Ca^{2+} inflow or removal depending on the chemical nature of the extracellular stimulus.

2. Materials and methods

2.1. Dissection of the aorta

Wistar rats aged 2–3 months were sacrificed with an overdose of diethyl ether. The thoracic and abdominal aortas were dissected out and perfused with physiological salt solution (PSS). The vessel was cleaned of the surrounding connective tissue, cut in ~5 mm long rings, stored in PSS at room temperature (22–24 °C) and used within 5 h.

2.2. Solutions

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 PSS ($0Ca^{2+}$), 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 Na^+-free PSS ($0Na^+$), extracellular Na^+ was replaced by an equimolar amount of *N*-methyl-D-glucamine (NMDG) and HCl, as previously shown [15]. Aortic rings were bathed in $0Ca^{2+}$ for no longer than 90 s min before applying ATP [24].

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

The technique used to evaluate changes in $[Ca^{2+}]_i$ in intact endothelium has been previously described [25]. Briefly, the aortic ring was opened and loaded with 16 μmol Fura-2/AM for 60 min at room temperature, washed and fixed by small pins with the luminal face up. *In situ* ECs were visualized by an upright epifluorescence Axiolab microscope (Carl Zeiss, Oberkochen, Germany), usually equipped with a Zeiss × 63 Achroplan objective (water-immersion, 2.0 mm working distance, 0.9 numerical aperture). ECs 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 exciting 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 exciting filters were mounted on a filter wheel (Lambda 10, Sutter Instrument, Novato, CA, USA). Custom software, working in the LINUX environment, was used to drive the camera (Extended-ISIS Camera, Photonic Science, Millham, UK) and the filter wheel, and to measure and plot on-line the fluorescence from 20 to 30 rectangular "regions of interest" (ROI) enclosing 20–30 single cells. Due to EC geometry and since cell borders were not clearly identifiable, a ROI may not include the whole EC or may include part of an adjacent EC. Adjacent ROIs never superimposed. $[Ca^{2+}]_i$ was monitored by measuring, for each ROI, the ratio of the mean fluorescence emitted at 510 nm when exciting alternatively at 340 and 380 nm (shortly termed "Ratio"). An increase in $[Ca^{2+}]_i$ causes an increase in the Ratio. Ratio measurements were performed and plotted on-line every 3–5 s. Off-line analysis could also be performed by recording images of the entire field of cells and using custom-made macros developed by Scion Corporation software (www.scioncorp.com). The experiments were performed at room temperature.

2.4. Data analysis

For each protocol, data were collected from at least three rats. Every tracing is the average of about 20–30 cells recorded from the same visual field. NCX involvement in the Ca^{2+} response to ATP was evaluated by measuring the time required by $[Ca^{2+}]_i$ to decay from 80% to 20% of its peak amplitude (τ_{80-20}), as shown elsewhere [15]. Repetitive stimulation could result in a reduced response to ATP even after 20 min of washout between the two applications (unpublished data). Therefore, every experimental maneuver was repeated on two different rings obtained from the same aorta by varying its order of execution as respect to the control. However, the temporal order of the experiments did not significantly influence the values of τ_{80-20}, which were thus pooled together as previously shown (Moccia et al. [15]). Statistical comparisons were made by Student's *t*-test for paired observations. $p < 0.05$ was considered significant.

2.5. Chemicals

Fura-2/AM was purchased from Molecular Probes (Molecular Probes Europe BV, Leiden, The Netherlands). All other chemicals were obtained from Sigma.

3. Results and discussion

3.1. NCX is sensitive to benzamil and KB-R 7943 in rat aortic ECs

In order to ascertain NCX activity in *in situ* ECs of rat aorta, we investigated the Ca^{2+} response to Na^+ removal ($0Na^+$). Such maneuver is expected to cause NCX to work in the Ca^{2+} influx mode and, therefore, to increase $[Ca^{2+}]_i$ and elevate the intracellular Na^+ concentration. Due to the progressive intracellular Na^+ depletion, NCX becomes less and less active and the PMCA pump can restore the basal $[Ca^{2+}]_i$ [15]. Accordingly, replacement of external Na^+ with an equimolar amount of NMDG induced a transient increase in $[Ca^{2+}]_i$ in 190 out of 331 cells (Fig. 1A). Reversal of the Na^+ gradient did not evoke a detectable Ca^{2+} signal in absence of extracellular Ca^{2+} in 123 out of 123 cells (Fig. 1B). Notably, the $0Na^+$-induced elevation in intracellular Ca^{2+} levels was reversibly impaired by benzamil (100 μM) (Fig. 1C), which inhibits both the forward- and the reverse-mode of NCX, and by KB-R 7943 (30 μM)