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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)

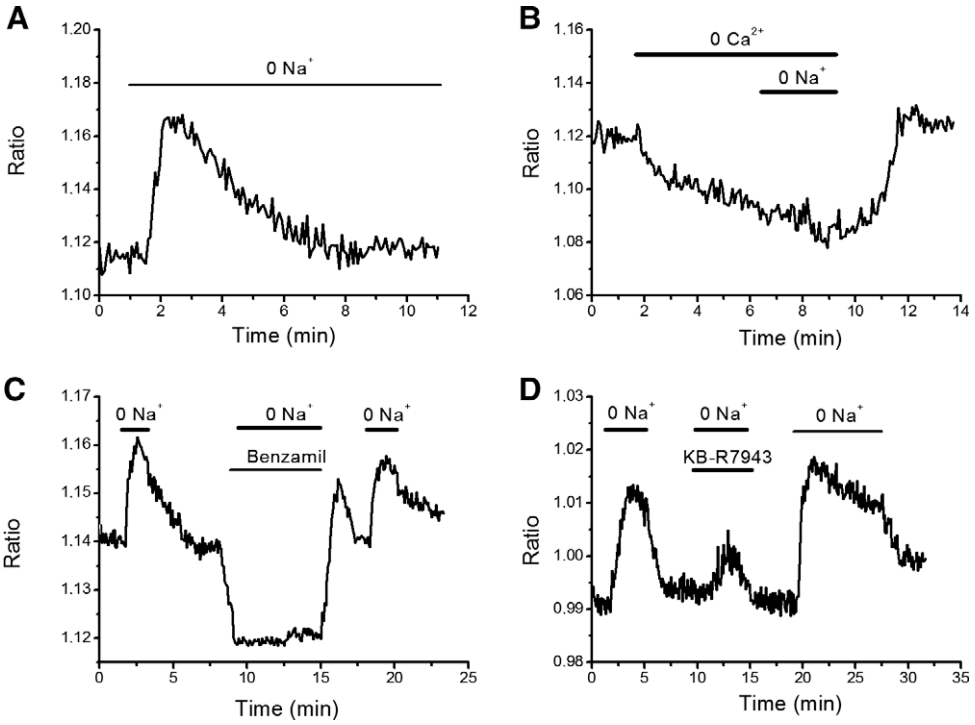

Fig. 1. Effect of the incubation in 0 Na^+ on the $[Ca^{2+}]_i$ in RAECs. Removal of external Na^+ ($0Na^+$) causes an increase in $[Ca^{2+}]_i$ in presence (A), but not absence (B), of extracellular Ca^{2+}. Benzamil (100 μM) (C), and KB-R 7943 (30 μM) (D) dampened the $0Na^+$-induced Ca^{2+} transient.

(Fig. 1D), a selective blocker of the reverse-mode at this dose [15,19]. Although benzamil is a fluorescent molecule which causes a drop in the background signal, we have previously demonstrated its usefulness in studying NCX role in the Ca^{2+} response to ATP in rat CMECs [15]. Indeed, background fuorescence remains constant during the recording period preceding and following ATP superfusion [15], a feature which enables kinetic analyses. Altogether, these data confirm the molecular, electrophysiological and functional (i.e. eNOS activation) evidence that NCX1 is expressed in the intact endothelium of rat aorta [19,20,26]. The Ca^{2+} response to $0Na^+$ in resting (i.e. non-stimulated) cells concurs with previous findings obtained in rat CMECs [15], rabbit aortic ECs [17] and intact endothelium of rabbit cardiac valve [27]. Conversely, the lack of Ca^{2+} signals in bovine pulmonary artery ECs bathed in $0Na^+$ suggests that the intracellular Na^+ concentration is too low to turn NCX into the reverse-mode [28]. It follows that NCX has the potential to shape agonist-induced Ca^{2+} signals and regulate Ca^{2+}-sensitive endothelial functions in a wide range of vascular beds.

3.2. NCX contributes to Ca^{2+} extrusion in the lumenal face of rat aorta stimulated by ATP

The role served by NCX in ATP-induced Ca^{2+} signaling was first studied by exposing rat aortic rings to ATP (20 μM) in presence and absence of extracellular Na^+. In this preparation, ATP mainly stimulates P_{2Y1}, P_{2Y2}, and P_{2Y12} receptors to recruit PLC-β and cause a rapid Ca^{2+} release from $InsP_3Rs$ [24]. The Ca^{2+} peak is followed by a slower decay to the baseline due to Ca^{2+} entry mediated by SOCE [24–25]. As depicted in Fig. 2A and C, removal of extracellular Na^+ reduced the rate of decline to the baseline, as indicated by a significantly longer τ_{80-20} (τ_{80-20} = 34.03 ± 2.55 s, n = 466, PSS; τ_{80-20} = 69.74 ± 7.63 s, n = 466, $0Na^+$; $p < 0.05$). A similar result was obtained when the experiment was repeated in absence of external Ca^{2+} (τ_{80-20} = 15.72 ± 1.25 s, n = 202, $0Ca^{2+}$; τ_{80-20} = 30.37 ± 2.20 s, n = 202, $0Ca^{2+}$ $0Na^+$; $p < 0.05$) (Fig. 2B and C). Consistent with SOCE-mediated Ca^{2+} entry during the decay of the Ca^{2+} response to ATP [24], τ_{80-20} was significantly ($p < 0.05$) faster in $0Ca^{2+}$ than in PSS. Altogether, these experiments suggest that NCX operate in the forward-mode to remove Ca^{2+} out of the cytoplasm in ATP-stimulated rat aortic endothelium, as also observed in rat cardiac microvascular ECs [15] and rabbit aortic ECs [17]. Accordingly, with NCX working in the reverse-mode, inhibition of NCX activity by Na^+ removal would dampen, rather than prolong, agonist-induced Ca^{2+} signals, as reported in EA.hy926 cells [18]. To confirm the aforementioned findings, the Ca^{2+} response to ATP was then measured in presence of either benzamil (100 μM) or 3′,4′-dichlorobenzamil (DCB; 30 μM), which block both the forward- and the reverse-mode. These drugs significantly ($p < 0.05$) prolonged the descending phase of the Ca^{2+} transient evoked by ATP (Fig. 3A, B, and D). In addition, loading the cells with Na^+ by the cation ionophore monensin (100 μM) resulted in a Ca^{2+} transient with significantly ($p < 0.05$) increased τ_{80-20}. Finally, we studied the effect of KB-R 7943 (30 μM), which selectively impairs the Ca^{2+} entry mode, on the decay phase of the Ca^{2+} signal. KB-R 7943 may curtail the Ca^{2+} response to a number of agonists, including ATP [11] and phenylephrine [29], in vascular smooth muscle cells (VSMCs), where NCX mediates Na^+-dependent Ca^{2+} inflow. Nevertheless, the data so far obtained indicate that NCX contributes to Ca^{2+} clearance when the endothelium of intact rat aorta is exposed to ATP. However, KB-R 7943, which is ineffective on the forward-mode at this dose, did not significantly affect the decay of the Ca^{2+} response to ATP in such preparation (Fig. 3C and D). This finding confirms that, unlike VSMCs, purinergic stimulation may commit NCX to extrude Ca^{2+} out of the cytosol in vascular ECs. This feature provides the first demonstration that NCX role in intact endothelium may depend on the activating agonist. In this view, NCX provides a pathway for Ach-induced Ca^{2+} entry in *in situ* ECs of rat aorta via the reverse-mode [19], whereas it contributes to Ca^{2+} extrusion through the forward-mode in presence of ATP.

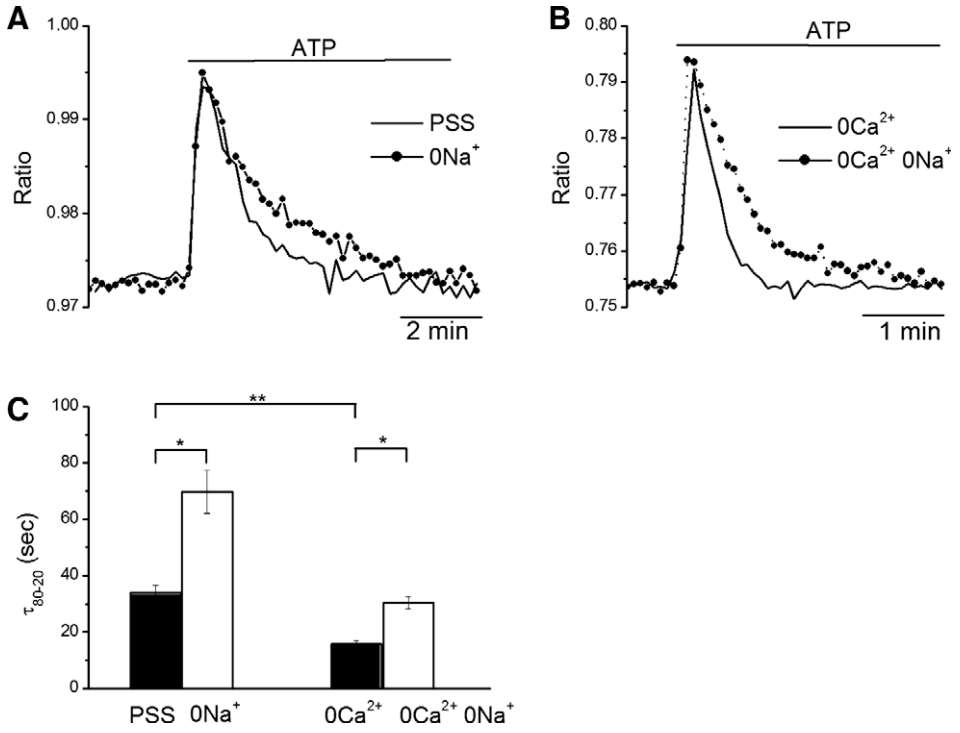

Fig. 2. Effect of Na^+ removal on the decay of the Ca^{2+} response to ATP. Na^+ removal ($0Na^+$) makes the 20 μM ATP-induced Ca^{2+} signal slower both in PSS (A) and in $0Ca^{2+}$ (B). C, τ_{80-20} values of ATP-induced Ca^{2+} transients measured under the conditions depicted in Panels A and B. N is equal to 466 and 202 for the experiments conducted in the presence and absence of extracellular Ca^{2+}, respectively. Single and double asterisk indicate a $p < 0.05$.

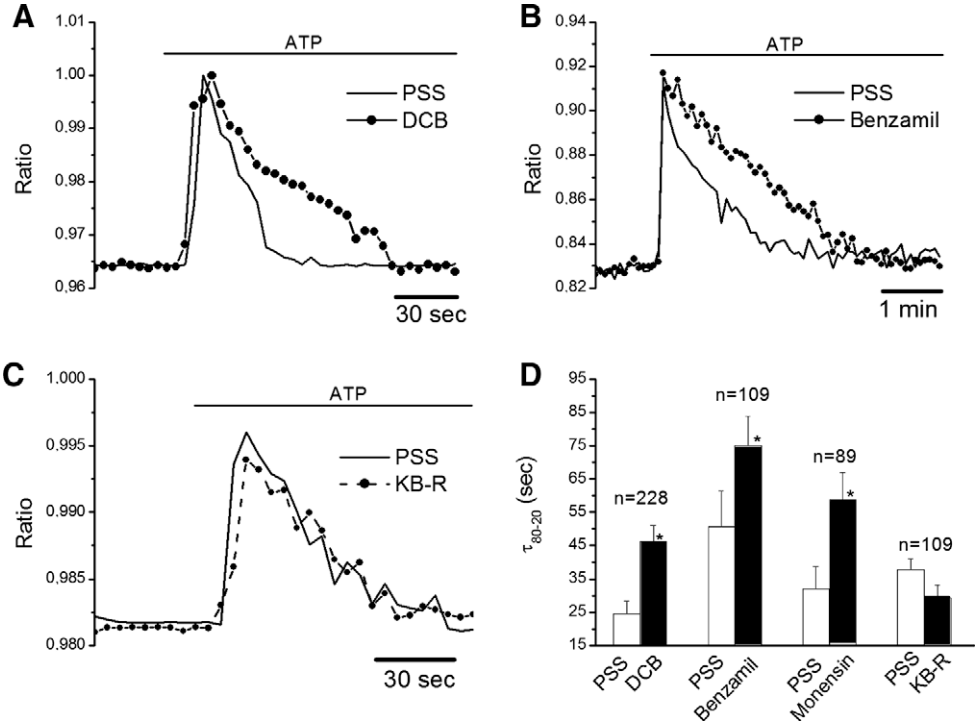

Fig. 3. Effect of NCX inhibition on ATP-evoked increase in $[Ca^{2+}]_i$. DCB (30 M) (A) and benzamil (100 M) (B), which impair both the forward and the reverse-mode of NCX, significantly prolong the decay of the Ca^{2+} response to ATP. (C) KB-R 7943 (30 M), which selectively blocks the reverse-mode of the exchanger, does not affect ATP-elicited $[Ca^{2+}]_i$ elevation. (D) τ_{80-20} values of ATP-induced Ca^{2+} transients measured under the conditions depicted in Panels A–C. The number of cells analyzed is shown above the bars. Single asterisk indicates $p < 0.05$.

3.3. NCX tunes endothelial sensitivity to low ATP concentrations

Local Ca^{2+} signals are gaining increasing attention as key determinants of endothelial physiology [30]. More specifically, we have recently shown that inhibiting NCX by lowering external Na^+ unmasked the Ca^{2+} response to an increase in extracellular Ca^{2+} levels signaled by the Ca^{2+}-sensing receptor [31]. In ECs of intact rat aorta, low doses of ATP (5 μM) elicited a detectable Ca^{2+} transient only in 10 out of 114 cells (8.8%). However, such percentage augmented to 46.2% (54 responsive cells out of 117) when 5 μM ATP was added in absence of extracellular Na^+. The same result was obtained when rat aortic rings were challenged with 10 μM ATP in presence (28 out of 76 cells, 36.8%) and absence (32 out of 76 cells, 50%) of external Na^+. This finding strongly suggests that, low doses of ATP may induce a Ca^{2+} elevation occurring in close proximity to the plasma membrane, where NCX is localized (see also [31]). Under physiological conditions, the resulting NCX activation prevents the Ca^{2+} response from extending to the bulk cytosol and spatially limits the Ca^{2+} signal to the sub-membranal domain, where a number of endothelial effectors, such as eNOS and PLA2, are present [7].

4. Conclusions

Our data provide the evidence that NCX operates in the forward-mode in *in situ* ECs of isolated rat aorta, exposed to ATP and contributes to restoring the Ca^{2+} transient to the baseline. Conversely, in the same preparation, the exchanger may be switched by Ach into the reverse-mode and sustain Ca^{2+} inflow [19]. To the best of our knowledge, this is the first report of NCX ability to operate in both modes in the same cells depending on the extracellular ligand. We speculate that, in the intact endothelium of rat aorta, the plasmalemmal channels recruited by ATP and Ach, respectively, display either different cation permeability or a different spatial relationship with NCX. Indeed, it might be hypothesized that ATP-dependent store-operated Ca^{2+} entry is gated by Orai1, which enables Ca^{2+}, but not Na^+, entry into the cells. Consequently, the sub-plasmalemmal Ca^{2+} increase would activate the forward-mode of the exchanger, thereby promoting Ca^{2+} efflux. Conversely, Ach might recruit the Na^+-permeable TRPC channels, thus increasing Na^+ concentration beneath the PM and inducing Ca^{2+} entry through the reverse-mode of NCX. Alternatively, if TRPC channels are also engaged by ATP, they might not be in close proximity to NCX and therefore are not able to change the basal NCX mode (the forward mode). Therefore, the $InsP_3$-dependent Ca^{2+} discharge would stimulate the exchanger to clear Ca^{2+} out of the cytosol. Whatever the model, these findings demonstrate that NCX role in the regulation of Ca^{2+}-dependent processes in vascular endothelium may be governed by the incoming stimuli.

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