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Na^+ entry via TRPC6 causes Ca^{2+} entry via NCX reversal in ATP stimulated smooth muscle cells

Virginia S. Lemos [a,b], Damon Poburko [a,b], Chiu-Hsiang Liao [a,b], William C. Cole [c], Cornelis van Breemen [a,b,*]

[a] Department of Anesthesiology, Pharmacology and Therapeutics, University of British Columbia, Vancouver, Canada V6T 1Z1
[b] Child and Family Research Institute, Vancouver, Canada V5Z 4H4
[c] Smooth Muscle Research Group, Faculty of Medicine, University of Calgary, Alberta, Canada

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Abstract

Reversal of the Na^+/Ca^{2+}-exchanger (NCX) has been shown to mediate Ca^{2+} influx during activation of G-protein linked receptors. Functional coupling between the reverse-mode NCX and the canonical transient receptor potential channels (TRPCs) has been proposed to mediate Ca^{2+} influx in HEK-293 cells overexpressing TRPC3. In this communication we present evidence for similar functional coupling of NCX to endogenously expressed TRPC6 in rat aorta smooth muscle cells. Selective inhibition of reverse-mode NCX with KB-R7943 and of non-selective cation-channels with SKF-96365 abolished Ca^{2+} influx in response to agonist stimulation (ATP). Expression of a dominant negative TRPC6 mutant also reduced the Ca^{2+} influx in proportion to its transfection efficiency. Calyculin A, which is known to disrupt the junctions of the plasma membrane and sarco/endoplasmic reticulum, increased global Na^+ elevations and reduced stimulated Ca^{2+} influx. Together our data provide evidence that localized Na^+ elevations are generated by TRPC6 and drive reversal of NCX to mediate Ca^{2+} influx.

Keywords: Na^+/Ca^{2+}-exchanger; TRPC6 channels; Ca^{2+} influx; Plasma membrane; Sarcoplasmic reticulum; Microdomains; Na^+ entry; Smooth muscle cells; Corona green; Junctions

Reversal of the Na^+/Ca^{2+}-exchanger (NCX) is becoming recognized as a common physiological mechanism of Ca^{2+} influx in non-excitable cells. Recently we have shown that NCX reversal mediates a substantial proportion of the Ca^{2+} influx following adrenergic and purinergic stimulation [1,2]. Similarly, others have demonstrated agonist-induced Ca^{2+}-entry by reverse-mode NCX in several types of smooth muscle and non-excitable cells including adrenal chromaffin cells [3–5]. Reverse-mode NCX activity has also been shown to contribute to the increased vascular tone in salt-sensitive hypertensive rats [6].

Control of vascular smooth muscle (VSM) contraction depends on the regulation of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) to modulate the activity of the contractile proteins. Physiological elevation of $[Ca^{2+}]_i$ in response to stimulation of phospholipase C-coupled receptors typically consists of a transient phase due to Ca^{2+} release from the sarcoplasmic reticulum (SR) followed by a sustained elevation requiring Ca^{2+} influx across the plasmalemma (PM). In smooth muscle cells the two principal classes of Ca^{2+} channels are voltage-gated Ca^{2+} channels (VGCCs) and receptor-operated channels (ROCs). While L- and T-type VGCC have been extensively characterized, the molecular identity and mechanism(s) of activation of ROCs remain to be fully elucidated. The canonical transient receptor potential channels (TRPCs), some of which form non-selective cation channels (NSCC) rather than Ca^{2+}-selective channels, satisfy many of the functional criteria of ROCs [7].

* Corresponding author. Fax: +1 604 822 2281.
E-mail address: breemen@interchange.ubc.ca (C.van Breemen).

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Blaustein proposed that agonist-induced NCX reversal occurred following activation of receptor-operated NSCC due to localized elevation of the $[Na^+]_i$ at the PM–SR junctions where both NSCC and NCX are thought to be co-localized [8]. In support of this model, Groschner and coworkers demonstrated physical and functional linkages between TRPC3 and NCX in HEK-293 cells over-expressing TRPC3 [9]. TRPC6 is closely related to TRPC3 and is much more strongly expressed than TRPC3 in aortic smooth muscle cells [10], but evidence for functional coupling between NCX and TPRC6 has not yet been reported.

We have previously shown that the rat aortic smooth muscle cells used in our current experiments express TRPC1, TRPC4, and TRPC6 [11]. While TRPC1 and TRPC4 are thought to be activated by SR Ca^{2+} depletion (reviewed in [12]), TRPC6 appears to be activated by diacylglycerol (DAG) and to form a relatively non-selective cation channel with a Na^+:Ca^{2+} permeability ratio ~1:5 [13,14,10]. TRPC6 has emerged as an essential component of α_1-adrenoceptor activated Ca^{2+}-entry in vascular smooth muscle cells [10,14,15], which in some cases has been shown to be secondary to Na^+ entry. Consistent with this concept, we have recently shown that purinergic stimulation of cultured RASMC leads to a tonic elevation of $[Na^+]_i$ [2]. In this study we present evidence, which shows that agonist induced NCX reversal is due to Na^+-entry through TRPC6.

Materials and methods

Cell culture and transfection. Rat aortic smooth muscle cells (RASMC) were cultured as previously described [16]. Cells were stored in 90% DMEM 10% DMSO in liquid nitrogen, used between passages 8–13, and cultured at 37 °C in 95% O_2/5% CO_2. Cells were seeded on 12 mm culture-coated glass coverslips (VWR Scientific) treated with Matrigel (30× dilution, BD Sciences). Empty pcDNA3 vector or dominant-negative TRPC6 (dnTRPC6; C-myc epitope tagged) were transiently transfected into cells 24 h after plating using 2 μl Transfectin® (Bio-Rad) and 1 μg DNA per coverslip. Transfection efficiency was calculated at 31.6 ± 2.2% by immuno-fluorescently labeling $TRPC6^{dn}$ with primary antibody against C-myc (1:200; Clone 9E10, Sigma–Aldrich) that was visualized with Alexa-488 goat anti-mouse secondary antibody (1:200; Cat number A11029; Invitrogen). Nuclei were counterstained with Hoechst 33342 (0.1 μg/ml). Cells were used 24–48 h after transfection.

Measurement of cytosolic $[Ca^{2+}]$ and $[Na^+]$. Immediately prior to experimentation, DMEM was replaced with Hepes buffered physiological saline solution (PSS) with the following composition (in mM): 5 NaCl, 145 KCl, 1.2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 5 Hepes, adjusted to pH 7.4 with NaOH. Cells were loaded with Fluo-4 AM or CoroNa Green (Molecular Probes; 5 μM each, 0.1% DMSO, 1.25 μM pluronic F127, and 200 μM probenecid) for 45 min at room temperature followed by a 45 min washout and de-esterification period before coverslips were mounted in a perfusion chamber on the microscope stage. Experiments were performed in PSS at room temperature. As indicated, cells were pre-incubated with KB-R7943 (10 μM) or SKF-96365 (50 μM) for 10 min or with Calyculin A (100 nM) for 20 min prior to stimulation with ATP. Fluo-4 and CoroNa Green were excited at 488 nm (Argon/Krypton laser) and fluorescent images (525–530 nm) were acquired with a 40× water-dipping objective (NA 0.8) on an Olympus BX50WI microscope equipped with a Nipkow spinning-disc confocal head. Images were acquired at 0.5 Hz with a 500 ms exposure. Image analysis was performed off-line using Ultraview 4.0 Software (Perkin-Elmer) to select regions on interest from which fluorescence traces were extracted and further analyzed with Excel (Microsoft) and Prism (GraphPad). Fluorescence traces were subject to a linear correction for bleaching and dye leakage and normalized to initial fluorescence values.

Chemicals and solutions. KB-R7943 (Tocris), SKF-96365 (Sigma), and Calyculin A (Calbiochem) were prepared as stock solutions in DMSO and diluted to the desired concentration in physiological saline solution (PSS) immediately before use. ATP (Sigma) was prepared as stock solution in water (pH adjusted to 7.4 with NaOH) and further diluted in PSS before use. The dominant negative TRPC6 construct from *Homo sapiens* (NCBI Accession No.: BC093660) was a generous gift from Dr. William Cole at the University of Edmonton, Canada.

Results and discussion

Ca^{2+}-influx through receptor-operated channels and reverse-mode NCX

In these RASMC ATP activates metabotropic P2Y G-protein coupled receptors [2,16]. Stimulation of RASMC with ATP (1 mM) induced a biphasic increase in $[Ca^{2+}]_i$ consisting of a large transient increase followed by a plateau phase typical of activation of PLC coupled receptors (Fig. 1A). KB-R7943 (10 μM), a concentration that selectively inhibits reverse-mode NCX [1,2], abolished the tonic phase of the $[Ca^{2+}]_i$ increase (Figs. 1A and B) indicating that a major component of Ca^{2+} entry stimulated by ATP is mediated by rev-NCX. SKF-96365 (50 μM; Figs. 1C and D), known to inhibit TRPC6 and TRPC1, mimicked the effects of KB-R7943, abolishing the plateau phase of the $[Ca^{2+}]_i$ elevation. SKF-96365 also slightly decreased the amplitude of the $[Ca^{2+}]_i$ transient, which is consistent with its inhibition of basal Ca^{2+} entry that would be expected to partially decrease resting SR Ca^{2+} content [11]. These findings are highly significant (Figs. 1B and D) and consistent with functional coupling between NCX and NSCC in RASMC.

Dominant negative suppression of TRPC6 channel function decreases Ca^{2+} influx

Current evidence indicates that some of the plasmalemmal ion channels that are activated by SR depletion and/or receptor activation are NSCC and members of the transient receptor potential channel (TRP) family [7]. Expression of a dominant negative TRPC6 in RASMCs to suppress the function of the native TRPC6 [17] induced a decrease in the tonic phase of the $[Ca^{2+}]_i$ response (Fig. 2A). This effect was not observed in cells transfected with the empty vector (Fig. 2B). In 33.3% (24 out of 72 cells) of the cells imaged on coverslips that were transfected with the dnTRPC6 the Ca^{2+} plateau was completely abolished (Fig. 2C), which is consistent with the measured transfection efficiency of 31.6 ± 2.2%. The close correlation between influx inhibition and transfection efficiency suggests that dnTRPC6 likely caused complete inhibition of the Ca^{2+} plateau in those cells in which it was expressed (Figs. 2C and D). In HEK293 cells, over-expression of

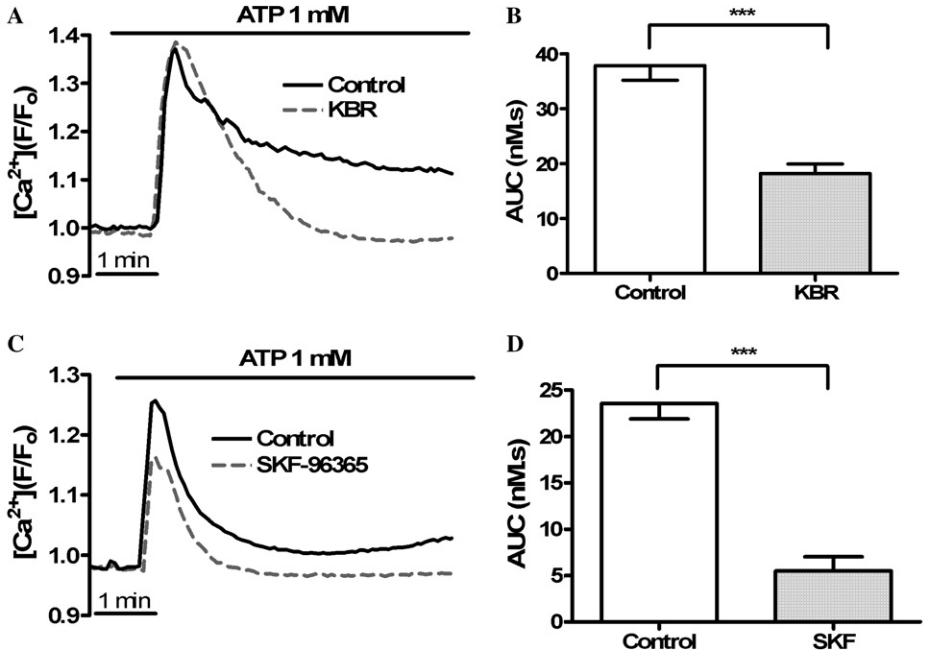

Fig. 1. Reverse-mode Na^+/Ca^{2+}-exchange mediates Ca^{2+} influx through non-selective cation channels in smooth muscle cells. (A) Average trace of ATP-induced Ca^{2+} response in the presence (grey trace, $n = 38$) and in the absence (black trace, $n = 35$) of KB-R7943 (KBR, 10 μM). (B) KBR decreased the area under the curve (AUC) of the plateau phase of Ca^{2+} response. (C,D) SKF-96365 (50 μM) also inhibits Ca^{2+} influx in smooth muscle cells. Average trace (C) and area under the curve (D) for control (black) and treated cells (grey). Area under the curve was calculated with Prism (GraphPad), from 45 s after the start of the response until 400 s. ($^{***}p < 0.0001$, two-tailed unpaired t test, n = number of cells imaged.)

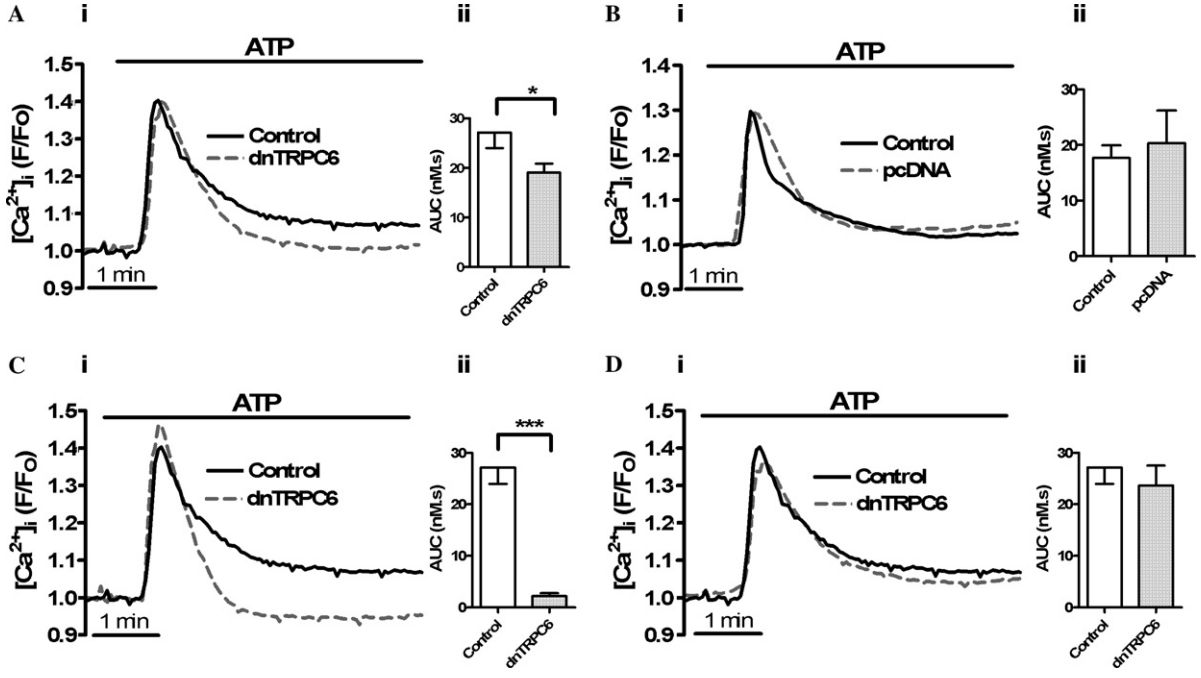

Fig. 2. Ca^{2+} influx through reverse-mode NCX following ATP stimulation is decreased when TRPC6 channel function is suppressed by dominant-negative expression of TRPC6 protein. (i) Average traces and (ii) area under the curve (AUC) for the ATP-induced Ca^{2+} response. (A) Averaged response to ATP (1 mM; $n = 72$) of all cells imaged from coverslips transfected with dnTRPC6 compared with control cells ($n = 102$). (B) In cells transfected with an empty vector the decrease in Ca^{2+} influx was not seen ($n = 25$ and 24 for control and transfected cells, respectively). Responses from cells transfected with dnTRPC6 were separated into those cells with a plateau less than (C) and greater than (D) resting fluorescence. (C) Ca^{2+} influx was abolished in 33.3% ($n = 24$) of cells corresponding to the transfection efficiency of the dominant negative plasmid. (D) In the other 66.7% ($n = 48$) of cells, there was no significant difference in Ca^{2+} plateau compared to control traces. Note, control traces in (C,D) is same as (A). ($^{*}p < 0.05$, $^{***}p < 0.0001$; two-tailed unpaired t test; n = number of cells imaged.)

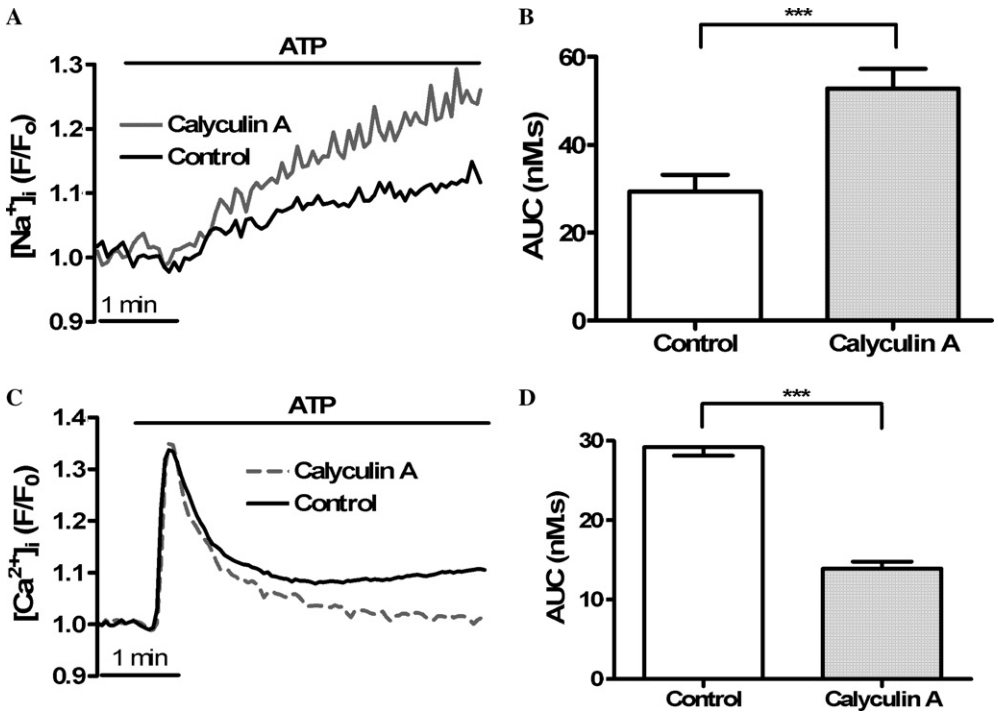

Fig. 3. Disruption of the PM–SR junctions with Calyculin A prevents NCX reversal that facilitates the diffusion of Na^+ into the bulk cytosol and inhibits Ca^{2+} influx. (A,B) Calyculin A increases ATP-mediated global $[Na^+]_i$ ($n = 14$) as compared to control untreated cells ($n = 18$). Average trace (A) and area under the curve (B) for control (black; n=) and treated cells (grey, n=). (C,D) Calyculin A inhibits ATP-mediated Ca^{2+} influx. Average trace (C) and area under the curve (D) for control (black; $n = 95$) and treated cells (grey, $n = 57$). (***$p < 0.0001$, two-tailed unpaired t test; n = number of cells imaged.)

TRPC3 provided important proof of principle for the functional interaction of TRCPs with NCX, in that the carbachol-mediated Na^+ current carried by TRPC3 stimulated Ca^{2+} entry via the NCX [9]. Our current findings provide further evidence for this mechanism and reveal the physiological interaction of endogenously expressed TRPC6 with the NCX.

Disruption of the sarcoplasmic reticulum-plasmalemmal junctions with Calyculin A inhibits stimulated Ca^{2+} influx and increases global Na^+ entry

Localized Ca^{2+}-transport between the extracellular space and the SR has been demonstrated in several types of vascular smooth muscle and is highly dependent on junctional membrane complexes between the PM and SR [18–22]. The arrangement of ion transport proteins within domains where the superficial SR apposes the PM (PM–SR junctions) is thought to provide localized regulation of $[Na^+]$ in the subplasmalemmal cytosol and thus NCX-activity, which regulates SR Ca^{2+} levels [23,24]. For example, refilling of the SR Ca^{2+} store in certain types of VSM hinges on the functional and spatial coupling of NSCC and NCX in the PM–SR junctions [1], as disruption of these junctions with calyculin A prevents refilling of SR with Ca^{2+} [25]. Stimulation of RASMC with ATP increased global $[Na^+]_i$ to the lower limit of the $[Na^+]$ required to reverse the NCX [2]. Based on the assumption that local elevations of $[Na^+]_i$ near the point of Na^+-entry would exceed the average $[Na^+]_i$, ATP-mediated elevations of $[Na^+]_i$ could easily support the reversal of the NCX demonstrated in Fig. 1A.

In our current study we found that calyculin A (100 nM) potentiated the ATP-induced increase in global $[Na^+]_i$ (Figs. 3A and B) and inhibited the $[Ca^{2+}]_i$ elevation (Figs. 3C and D). We have previously shown that this concentration of calyculin A disrupts PM–SR junctions and prevents revNCX-mediated refilling of the SR in intact venous smooth muscle [25]. This was explained by the separation of the cell and SR membranes and loss of the cytoplasmic microdomains within the PM–SR junctions. The present results of a loss of the $[Ca^{2+}]_i$ plateau in the face of an augmented $[Na^+]_i$ elevation upon disruption of the PM–SR junctions by calyculin A can be explained along similar lines. First, disruption of the PM–SR junctions may have removed a physical barrier to the diffusion of Na^+ influx into the bulk cytosol. This would increase the portion of Na^+ influx detected by CoroNa green while preventing the localized elevation of $[Na^+]_i$ in the diffusionally restricted spaces required for NCX reversal. Prevention of reversal of the NCX would have two effects: one, inhibition of stimulated Ca^{2+} entry and two, inhibition of Na^+ extrusion, both entirely consistent with the findings illustrated in Fig. 3.

In summary, this study illustrates that the primary function of TRPC6 is to generate localized Na^+ elevations,

which indirectly mediate Ca^{2+} influx via their functional linkage to the NCX. Since the ratio of extracellular Na^+ to Ca^{2+} is ~100:1, opening of NSCCs would cause considerable Na^+-influx accompanied by depolarization. Such depolarization would not only reduce the driving force for Ca^{2+} entry through voltage-gated Ca^{2+} channels, but would also reduce the $[Na^+]_i$ required for reversal of the NCX. In conclusion our data show that purinergically stimulated Ca^{2+} influx in RASMC is a consequence of Na^+ entry through functional TRPC6-containing channels, and that these channels rely upon the close associations of the PM and SR to generate the local elevations of $[Na^+]_i$ required for NCX reversal. This communication also shows the need for future high resolution imaging of localized cytoplasmic Na^+ gradients and co-localization of NCX and TRPCs.

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