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Cross-talk between plasma membrane Na⁺/Ca²⁺ exchanger-1 and TRPC/Orai containing channels: key players in arterial hypertension

MV Pulina¹, A Zulian¹, SG Baryshnikov¹, CI Linde¹, I Karashima¹, JM Hamlyn¹, P Ferrari², MP Blaustein¹, and VA Golovina¹

¹Dept. of Physiol. Univ. of Maryland Med Sch., Baltimore, MD 21201, USA

²Praxis-sigma tau Research Institute, Settimo Milanese, Milan, Italy

Abstract

Arterial smooth muscle (ASM) Na⁺/Ca²⁺ exchanger type 1 (NCX1) and TRPC/Orai-containing receptor/store-operated cation channels (ROC/SOC) are clustered with α2 Na⁺ pumps in plasma membrane microdomains adjacent to the underlying junctional sarcoplasmic reticulum. This arrangement enables these transport proteins to function as integrated units to help regulate local Na⁺ metabolism, Ca²⁺ signaling and arterial tone. They thus influence vascular resistance and blood pressure (BP). For instance, up-regulation of NCX1 and TRPC6 has been implicated in the pathogenesis of high BP in several models of essential hypertension: ouabain-induced hypertensive rats, Milan hypertensive strain rats, and Dahl salt-sensitive hypertensive rats, which all have elevated plasma ouabain levels. Enhanced expression and function of arterial smooth muscle NCX1 and TRPC/Orai1-containing channels in experimental and clinical hypertension implies that these proteins are potential targets for pharmacological intervention.

Keywords

Arterial smooth muscle cells; Ca²⁺ signaling; Store-operated channels; Receptor-operated channels; TRPC6; ouabain-induced hypertensive rats; Milan hypertensive strain rats; Dahl salt-sensitive hypertensive rats

1. Regulation of Ca²⁺ signaling in vascular smooth muscle: important role of Na⁺/Ca²⁺ exchanger and TRPC/Orai-containing channels

The contractility of vascular smooth muscle is reliant upon increases in the intracellular Ca²⁺ concentration (Lagaud et al., 1999; Zou et al., 2000; McDaniel et al., 2001). Intracellular [Ca²⁺] is regulated by various Ca²⁺ entry, exit and storage mechanisms in a variety of cell types including vascular myocytes (Sanders, 2001). The role of voltage-gated Ca²⁺ channels (VGCCs) in Ca²⁺ entry in vascular smooth muscle cells (SMCs) has long been recognized (Nelson et al., 1990). Accumulating evidence indicates, however, that non-selective cation channels, such as store-operated, receptor-operated and stretch-activated channels (SOCs, ROCs and SACs, respectively) also play essential role in regulation of vascular tone by mediating the entry of Ca²⁺ and Na⁺ (Welsh et al., 2002; Bryden et al., 2008). Importantly, Ca²⁺ can also either exit or enter vascular SMCs through the Na⁺/Ca²⁺ exchanger type 1 (NCX1) which is driven by the Na⁺ electrochemical gradient across the

plasma membrane (PM) under the control of the Na⁺ pump (Blaustein and Lederer, 1999) and the non-selective cation channels. The Na⁺ entry through these channels and consequent depolarization should promote Ca²⁺ entry not only via L-type Ca²⁺ channels but also via NCX1 and, thereby, contribute to myogenic tone, which is a key in the setting of vascular resistance in hypertension (Davis and Hill, 1999). Indeed, in contrast to the heart, where NCX1 primarily mediates Ca²⁺ extrusion (Dibb et al. 2007), in SMCs, which are partially depolarized in arteries with tone, NCX1 primarily mediates Ca²⁺ entry (Iwamoto et al. 2004; Kashihara et al. 2009). The implication is that multiple mechanisms, including not only VGCCs but also ROCs, SOC, and NCX1 contribute to the maintenance of Ca²⁺ entry, elevated free cytosolic Ca²⁺ concentration ([Ca²⁺]_{cyt}), and arterial tone (Welsh et al. 2002; Iwamoto et al. 2004; Kashihara et al. 2009); the latter influences total peripheral vascular resistance (TPR) and blood pressure (BP) (Blaustein and Hamlyn 2010).

Numerous reports indicate that mammalian SOC and ROC are homo- and heterotetramers formed by members of “classical” or “canonical” family of seven proteins (TRPC1–TRPC7) that are homologous to the *Drosophila* transient receptor potential (TRP) channel (Beech et al. 2004). The diversity of heteromeric assemblies of TRPC isoforms may partly contribute to functional heterogeneity in the vasculature. Two recently-discovered families of transmembrane proteins, Orai1 [also known as Ca²⁺ release activated Ca²⁺ (CRAC) channel modulator] and STIM1 (stromal interacting molecule 1), may also contribute to SOC-mediated Ca²⁺ entry (Zhang et al. 2005b; Yeromin et al. 2006; Cahalan 2009). Orai1 may form the Ca²⁺ selectivity filter of the CRAC channel (Yeromin et al. 2006), which may be another type of SOC. The role of Orai1 in store-operated Ca²⁺ entry (SOCE) was confirmed in arterial SMCs (Baryshnikov et al. 2009; Potier et al. 2009; Ng et al. 2010). STIM1, the putative Ca²⁺ sensor in the SR, regulates SOC and CRAC channels (Zhang et al. 2005b; Cahalan 2009). Evidence indicates that depletion of SR Ca²⁺ stores triggers STIM1 to translocate into defined SR-PM “junctional” areas in which coupling to Orai proteins can occur (Wu et al. 2006). Stim1 associates not only with Orai1 but also with TRPC proteins (Li et al. 2008; Liao et al. 2008; Yuan et al. 2009), suggesting that in some tissues SOC and CRAC channels are regulated by similar molecular components.

2. Functional interaction of NCX1 with TRPC/Orai proteins within the plasma membrane-junctional SR regions

The structural integrity of PM-junctional SR units and the spatial relationship between SOC/ROC and the SR Ca²⁺ stores may play an important role in regulating Ca²⁺ influx and vascular tone. Indeed, in many cell types, the peripheral SR lies within 8–20 nm of the PM, with which it appears to form “junctions” (Somlyo and Franzini-Armstrong 1985). Van Breemen and colleagues (1995) postulated that in smooth muscle Ca²⁺ entering the cells is directly accumulated by the peripheral SR (“Superficial buffer barrier”) before it reaches the myofilaments. Thus, diffusion of ions from the PM-SR junctions to “bulk” cytosol must be markedly restricted. This may explain why Ca²⁺ influx fails to induce contraction in some smooth muscle when SR Ca²⁺ stores are depleted by SERCA inhibitors (Flemming et al., 2002). We previously demonstrated that SOCE occurs at PM-SR junctions (Golovina 1995; Lee et al. 2006).

Ca²⁺ homeostasis in vascular SMCs is influenced not only by direct Ca²⁺ entry through TRPC channels, but also by Na⁺ entry through these non-selective cation channels (Arnon et al. 2000; Eder et al. 2005; Poburko et al. 2007; Fellner and Arendshorst 2008). The Ca²⁺/Na⁺ permeability ratio varies between different members of the TRPC family from 1 to 9 (Nilius et al. 2007). The entering Na⁺ apparently accumulating in a restricted space between the PM and adjacent SR then also promotes Ca²⁺ entry through nearby NCX1 (Arnon et al. 2000; Eder et al. 2005; Poburko et al. 2007). We did not observe, however, significant

changes in SOCE in human aortic SMCs when experiments were performed at low (5 mM) $[Na^+]_o$ (Baryshnikov et al. 2009). This may be explained by relative differences in the contribution of NCX1 to SOCE in different cell types. Silencer RNA data demonstrated a function interaction between NCX1 and TRPC6 in rat mesenteric arterial SMCs; knockdown of NCX1 markedly (by ~25%) reduced TRPC6 expression (Pulina et al. 2010). NCX1 is also functionally associated with Orai1 in human aortic SMCs (Baryshnikov et al. 2009). Knockdown of Orai1 gene down-regulated expression of NCX1 and PM Ca^{2+} pump and significantly attenuated not only SOCE but also Ca^{2+} extrusion from primary cultured human aortic SMCs (Baryshnikov et al. 2009). These results imply a mutual interaction between the regulation of Ca^{2+} homeostasis by NCX1 and regulation of Ca^{2+} (and Na^+) entry through SOCs and ROCs.

The functional interaction of NCX1 with TRPCs and with Orai1 raises the possibility that they co-localize in small membrane clusters. In fact, immunocytochemical observations indicate that NCX1, TRPCs and Orai1 are all confined to the PM microdomains that overlie the closely apposed junctional SR (Juhaszova et al. 1996; Golovina 2005; Baryshnikov et al. 2009; Zulian et al. 2010) where STIM1 accumulates after store depletion (Wu et al. 2006). These PM microdomains also contain the high ouabain-affinity $\alpha 2 Na^+$ pumps (Juhaszova et al. 1997; Lee et al. 2006; Song et al. 2006) which are involved in control of agonist-mediated vasoconstriction (Shelly et al. 2004), myogenic tone and BP (Zhang et al. 2005a). High power images of a portion of hASMC show that the NCX1 labeling pattern (Fig. 1a, *inset*) is remarkably similar to the pattern observed with antibodies directed against the Orai1 (Fig. 1b, *inset*). Indeed, when Fig. 1ci (NCX1, green) is overlaid on Fig. 1cii (Orai1, red), extensive overlap of the labels is observed (Fig. 1ciii), as indicated by the large amount of yellow in the image. Notably, reactivity was not detected in the PM in the absence of the primary anti-NCX1 or anti-Orai1 antibodies (not shown). Also, both NCX1 and TRPC6 labels are distributed in a distinct reticular pattern that parallels the organization of the underlying ER-Tracker-stained SR (Zulian et al., 2010). Moreover, co-immunoprecipitation experiments provide evidence for association of NCX1 and TRPC3 in protein complexes (Rosker et al. 2004). These findings indicate that PM microdomains that include TRPC/Orai1-containing channels, NCX1 and $\alpha 2 Na^+$ pumps function as integrated units that help to regulate Ca^{2+} signaling and vascular tone, and are, thus, critical for the determination of vascular resistance in hypertension.

3. Dysregulation of NCX1 and TRPC/Orai-containing channels is implicated in the pathogenesis of high blood pressure

Arterial smooth muscle NCX1 and TRPC channel proteins apparently play a key role in salt-dependent hypertension (Iwamoto et al. 2004; Bae et al. 2007). A common feature of many animal models of salt-dependent hypertension is an elevated plasma level of endogenous ouabain (Hamlyn et al. 1991; Krep et al. 1995; Kaide et al. 1999), an adrenocortical hormone (Boulanger et al. 1993; Shah et al. 1998). Plasma endogenous ouabain also is significantly elevated in ~45% of patients with essential hypertension (Rossi et al. 1995). Moreover, in rodents, the prolonged administration of low doses of ouabain induces sustained BP elevation, termed 'ouabain-induced hypertension' (Manunta et al. 1994; Pulina et al. 2010). Binding of ouabain to its receptors, the high ouabain affinity $\alpha 2 Na^+$ pumps, modulates Ca^{2+} signaling pathways in arterial SMCs and endothelial cells leading to increase in arterial tone, peripheral vascular resistance, and, thereby, elevated BP (Blaustein et al. 2009). In contrast to $\alpha 2 Na^+$ pumps, rodent $\alpha 1 Na^+$ pumps have a very low affinity for ouabain (O'Brien et al. 1994) and may be absent from the PM-SR junctions (Lee et al., 2006). Furthermore, neither ouabain nor adrenocorticotrophic hormone (ACTH) can induce hypertension when the $\alpha 2$ ouabain-binding site is mutated to a ouabain-resistant form

(Dostanic-Larson et al. 2005; Lorenz et al. 2008). It is noteworthy, in rodents, $\alpha 1$ Na⁺ pumps have a very low affinity for ouabain (O'Brien et al. 1994). The key molecular links that lead from ouabain's interaction with Na⁺ pumps to the sustained elevation of BP are, however, not completely understood.

Our recent studies demonstrate that freshly dissociated mesenteric artery myocytes from ouabain hypertensive rats exhibit Ca²⁺ dysregulation: elevated resting [Ca²⁺]_{cyt} and augmented phenylephrine (1 μ M)-induced Ca²⁺ signals (Pulina et al. 2010). The altered Ca²⁺ homeostasis is associated with up-regulated expression of key PM transporters: $\alpha 2$ Na⁺ pumps, NCX1 and TRPC1 and TRPC6 (Pulina et al. 2010). Inhibition of the $\alpha 2$ Na⁺ pumps would be expected to increase [Na⁺]_{cyt} in the local spaces between the PM and junctional SR, and reduce the Na⁺ electrochemical gradient, thereby driving Ca²⁺ into the cells via adjacent NCX1. Up-regulation of NCX1 (by 5–6-fold) in arterial SMCs from ouabain hypertensive rats should tend to accelerate, further, Ca²⁺ entry and promote net Ca²⁺ gain (Pulina et al. 2010). Pharmacological and genetic engineering studies reveal that NCX1 contributes to the ouabain-induced increases in arterial SMC Ca²⁺ signaling, myogenic tone and BP (Iwamoto et al. 2004; Zhang et al. 2010). The vasoconstrictor action of ouabain (Zhang et al. 2005a), as well as ACTH, deoxycorticosterone acetate (DOCA)-salt, and Dahl-salt hypertension are blocked by NCX inhibitors or by reduced NCX1 expression (Iwamoto et al. 2004; Zhang et al., 2010).

The arterial myocytes from ouabain hypertensive rats also exhibit increased Ca²⁺ entry through SOCs and ROCs. This effect is a consequence of up-regulated expression of TRPC1 and TRPC6 proteins (~3-fold and >6-fold, respectively) (Pulina et al. 2010). Importantly, the actions of *in vivo* ouabain on arterial Ca²⁺ signaling can be mimicked *in vitro* in primary cultured arterial SMCs. Like chronic *in vivo* ouabain infusion, prolonged (72 hr) treatment of cultured arterial SMCs with nanomolar ouabain up-regulates expression of NCX1 and TRPC6 and increases Ca²⁺ entry through SOCs and ROCs (Pulina et al. 2010). Thus, the aforementioned effects of *in vivo* ouabain administration appear to be triggered by ouabain itself and not by the increase in BP or by other circulating factors.

It is noteworthy that Milan hypertensive, (DOCA)-salt and Dahl salt-sensitive (Dahl//SS) hypertensive rat models have elevated plasma endogenous ouabain levels (Hamlyn et al. 1991; Leenen et al. 1994; Ferrandi et al. 1997). Therefore, it is not surprising that high blood pressure in these models is also associated with up-regulation of NCX1 and TRPC6 (Table 1) which might be triggered by the high plasma endogenous ouabain. Figure 2a shows that a high salt diet for 12 days markedly increases blood pressure in Dahl/SS rats. NCX1 and TRPC6 are greatly up-regulated in mesenteric arteries from these rats compared to Dahl/SS rats on normal salt diet (Fig. 2b,c). Expression of NCX1 is also increased (~13-fold) in mesenteric artery SMCs from Milan hypertensive strain (MHS) rats (Fig. 3a,b) (Zulian et al. 2010). MHS rats are a genetic model of hypertension with an adducin gene polymorphism linked to enhanced renal tubular Na⁺ re-absorption (Ferrandi et al., 1996, 1999). Figure 3, c and d, shows that removal of extracellular Na⁺ [conditions that favor Na⁺ extrusion and Ca²⁺ entry via NCX1 (Blaustein and Lederer, 1999)] induced a rapid increase in [Ca²⁺]_{cyt}. The increase in [Ca²⁺]_{cyt} in arterial SMCs in response to removal of extracellular Na⁺ ("Na⁺-free"), a measure of Na⁺/Ca²⁺ exchange activity, was significantly greater in arterial SMCs from MHS than from Milan normotensive strain (MNS) rats. Nevertheless, this ~30% increment (Fig. 3b) was *far* smaller than the ~13-fold increase in NCX1 expression (Fig. 3a) (Zulian et al., 2010). This difference may be explained, in part, by buffering of NCX1-mediated Ca²⁺ entry in the PM-junctional SR regions by the SR and mitochondria that limits its diffusion into the cytosol. Indeed, the estimated transient increase in the sub-PM Ca²⁺ concentration upon substitution of extracellular Na⁺ by NMDG in rat ASMCs is >13-fold greater than the observed increase in [Ca²⁺]_{cyt} (Poburko et al., 2006). Furthermore,

SERCA2 expression is ~2.5-fold higher in arterial myocytes from MHS than from MNS rats (Zulian et al., 2010).

Augmented expression of NCX1 and TRPC proteins has also been observed in arterial SMCs from spontaneously hypertensive rats (SHR) (Taniguchi et al. 2004; Liu et al. 2009; Chen et al. 2010), and in cultured pulmonary artery myocytes from humans with primary pulmonary arterial hypertension (PAH) (Yu et al. 2004; Zhang et al. 2007a,b) (Table 1). Furthermore, Giachini and colleagues (2009) demonstrated that expression of Orai1 and STIM1, as well as SOC-dependent contraction of endothelium-denuded aortic rings, are significantly greater in stroke-prone SHR compared with WKY rats. Up-regulation of TRPC6 may underlie the abnormally enhanced proliferation of pulmonary artery SMCs from PAH patients (Yu et al. 2004). A recent study identified a single-nucleotide polymorphism in the TRPC6 gene promoter that is associated with idiopathic PAH and that apparently influences TRPC6 activity in pulmonary artery SMCs (Yu et al. 2009). Regardless of the mechanism(s) involved in up-regulation of these transport systems (NCX1, TRPC/Orai1-containing channels), they may play an important role in the development and/or maintenance of many forms of hypertension (Table 1).

A unique organizational arrangement of $\alpha 2$ Na⁺ pumps, NCX1 and TRPC/Orai1 proteins at PM-SR junctions enables these transport systems to function cooperatively to help regulate Ca²⁺ signaling. As a consequence, these proteins work together to modulate arterial myogenic tone. Therefore, they likely make a key contribution to the elevated vascular resistance, a hallmark of sustained hypertension (Cowley, 1992). These appear to be some of the critical molecular mechanisms involved in the long-term, “whole body autoregulation” of vascular resistance. Enhanced expression and function of arterial smooth muscle NCX1 and TRPC/Orai1-containing channels in experimental and clinical hypertension implies that these proteins are potential targets for pharmacological intervention.

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Figure 1. Immunofluorescent localization of NCX1 and Orai1 in hASMC

a and **b**: images of cell double labeled with anti-NCX1 antibody (**a**) and anti-Orai1 antibody (**b**). *Insets* in **a** and **b** (enlargements of the *boxed areas*) indicate that NCX1 and Orai1 labels show similar distributions. *Panels ci* and *cii* are pseudocolor images (green – anti-NCX1; red – anti-Orai1) of enlarged boxes from **a** and **b**, respectively. *Panel ciii*: colocalization of NCX1 (*ci*) and Orai1 staining (*cii*); the yellow, orange and yellow/green areas in the overlay indicate regions of overlap between the two epitopes. Reprinted with permission from (Baryshnikov et al. 2009).

Figure 2. Up-regulated NCX1 and TRPC6 in de-endothelialized mesenteric arteries from hypertensive Dahl salt-sensitive (DS) rats on high salt (HS) diet

a. Development of hypertension in DS rats on HS diet. Normotensive male DS rats were split into 2 groups; one was fed normal salt (NS) and the other, HS (6%) diet. A HS diet for 12 days markedly increases BP in DS rats (Student's t-test, * = $P < 0.05$). **b** and **c**: Western blot analysis of NCX1 (**b**) and TRPC6 (**c**) expression in mesenteric artery from DS rats on NS and HS diets. Representative Western blots are shown (30 μ g/lane); comparable results were obtained in 4 immunoblots.

Figure 3.

Augmented NCX1 expression and enhanced Ca^{2+} entry via the reverse mode of NCX1 in freshly dissociated mesenteric artery myocytes from Milan hypertensive strain (MHS) rats. **a** and **b**. Western blot analysis of NCX1 protein expression in smooth muscle cell membranes from mesenteric arteries of MNS and MHS rats. **a**. Representative immunoblot (30 μ g/lane). **b**. Summary data are normalized to the amount of β -actin and are expressed as mean $\pm$ S.E.M. from 9 immunoblots (total of 22 rats). ***P < 0.001 vs. MNS arterial SMCs. **c**, **d**. Activation of the reverse mode of NCX1 in arterial ASMCs from MNS (black) and MHS (grey) rats. **a**. Representative time course records showing changes in $[Ca^{2+}]_{cyt}$ in single arterial SMCs; time of treatment with Na^{+} -free solution is indicated. Nifedipine (10 μ M) was added 10 min before the records shown, and was maintained throughout the experiment. **b**. Summarized data show the NCX1-mediated Ca^{2+} entry in 33 MNS and 23 MHS mesenteric artery SMCs. *P < 0.05 vs. MNS arterial myocytes. Reprinted with permission from (Zulian et al. 2010).

Table 1

NCX1 and SOC/ROC proteins are up-regulated in many types of hypertension (HTN)

	Hypertension	Artery Smooth Muscle							References
		NCX1	SOCs				ROCs		
			TRPC1	TRPC5	Orai1	STIM1	TRPC3	TRPC6	
1	Ouabain* (vs. vehicle)	↑	↔	N/R	N/R	↔	↑	Pulina et al. (2010)	
2	Milan HTN* (vs. Milan NT)	↑	↔	N/R	N/R	↔	↑	Zulial et al. (2010)	
3	Dahl/SS high vs low salt*#	↑	N/R	N/R	N/R	N/R	↑	Fig. 2 (current manuscript)	
4	DOCA-salt*#	N/R	↔	N/R	N/R	↔	↑	Bae et al. (2007)	
5	SHR (vs. WKY)	↑	↑	N/R	N/R	↑	↔	Taniguchi et al. (2004) Liu et al. (2009) Chen et al. (2010)	
6	Stroke-prone SHR (vs. WKY)	N/R	N/R	↑	↑	N/R	N/R	Guachini et al. (2009)	
7	Human Primary Pulmonary HTN	↑	↔	N/R	N/R	↑	↑	Yu et al. (2004) Zhang et al. (2007a) Zhang et al. (2007b)	

* Hypertension associated with elevated plasma ouabain levels

Salt-sensitive hypertension models

N/R - No reports