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REVIEW

Cardiac Na^+/Ca^{2+} exchange stimulators among cardioprotective drugs

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Abstract
We previously reviewed our study of the pharmacological properties of cardiac Na^+/Ca^{2+} exchange (NCX1) inhibitors among cardioprotective drugs, such as amiodarone, bepridil, dronedarone, cibenzoline, azimilide, aprindine, and benzyl-oxyphenyl derivatives (Watanabe et al. in J Pharmacol Sci 102:7–16, 2006). Since then we have continued our studies further and found that some cardioprotective drugs are NCX1 stimulators. Cardiac Na^+/Ca^{2+} exchange current (I_{NCX1}) was stimulated by nicorandil (a hybrid ATP-sensitive K^+ channel opener), pinacidil (a non-selective ATP-sensitive K^+ channel opener), flecainide (an antiarrhythmic drug), and sodium nitroprusside (SNP) (an NO donor). Sildenafil (a phosphodiesterase-5 inhibitor) further increased the pinacidil-induced augmentation of I_{NCX1}. In paper, here I review the NCX stimulants that enhance NCX function among the cardioprotective agents we examined such as nicorandil, pinacidil, SNP, sildenafil and flecainide, in addition to atrial natriuretic (ANP) and dofetilide, which were reported by other investigators.

Keywords Cardiac Na^+/Ca^{2+} exchanger (NCX1) · Cardiac Na^+/Ca^{2+} exchange current (I_{NCX1}) · NCX1 stimulator · Patch-clamp method · Cardioprotective drug

Introduction

The plasma membrane Na^+/Ca^{2+} exchanger (NCX) is a bi-directional transporter that mediates the electrogenic exchange of $3Na^+$ for $1Ca^{2+}$. Among the three NCX subtypes, cardiac NCX (NCX1) is abundantly expressed in the heart, smooth muscle, and other tissues. NCX1 plays an important role in the regulation of intracellular Ca^{2+} homeostasis to maintain mechanical activity and normal electrical rhythm in the heart. In physiological conditions in the heart, NCX1 operates in either Ca^{2+} exit (generating an inward membrane current) mode or Ca^{2+} entry (outward membrane current) mode, depending on the membrane potential during the action potential (AP) and ion gradients across the plasma membrane. To maintain stable excitation–contraction coupling, the Ca^{2+} entry must be balanced by Ca^{2+} exit. The NCX1 and ATP-dependent Ca^{2+} pump are the two mechanisms that regulate Ca^{2+} exit via plasma membrane, and the dominant role of NCX1 has been well known. Especially during normal diastole of cardiomyocytes, NCX1 contributes to an approximately 20–30% reduction of $[Ca^{2+}]_i$ by expelling Ca^{2+} from the cytoplasm [1–3].

The canine NCX1 protein has a molecular mass of 110 kDa and consists of 970 amino acids. In 1999, two research groups suggested that mammalian NCX1 protein comprises nine trans-membrane segments (TMS) and a large hydrophobic loop between 5 and 6 TMS, with the NH_2- and COOH-terminals located on the external and internal sides, respectively [4, 5]. In 2013, two groups published ten TMS topology models of mammalian NCX1. The major difference between them is in the orientation of the three C-terminal TMS, but not in the large intracellular loop containing about 550 amino acids between TMS 5 and 6 [6, 7]. The large cytoplasmic domain is involved in the regulation of NCX1 by cytoplasmic factors including exchanger inhibitory peptide (XIP), Na^+, Ca^{2+}, and protein kinase C (PKC) [8, 9].

The pharmacological agents for NCX1 regulation are classified into two groups: stimulators and inhibitors. The pharmacology of NCX1 inhibitors has been reported by several researchers [9–11]. On the other hand, there are next to no reports on NCX1 stimulants among cardioprotective agents. This review is about the properties of NCX1 stimulators among cardioprotective drugs including nicorandil, pinacidil, flecainide, sodium nitroprusside (SNP), and

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sildenafil, which we have investigated to date, as well as atrial natriuretic peptide (ANP) and dofetilide.

NCX1 and NO/cGMP/PKG signaling pathway

In the cytoplasm, nitrate oxide synthase (NOS) produces NO, which activates a soluble guanylate cyclase (GC) and increases intracellular guanosine 3′,5′-cyclic monophosphate (cGMP) and thereby activates cGMP dependent protein kinase (PKG). Several studies have indicated that NCX1 function may be stimulated through the NO/cGMP/PKG signaling pathway in in vitro [12–14]. Horie et al. (1991) reported in single cardiac cells that nicorandil, which has nitrate-like activity and a KATP channel opening activity, decreases the resting level of intracellular Ca^{2+} ($[Ca^{2+}]_i$) via cGMP-mediated activation of plasma membrane transporters [15]. In addition, Baczkó et al. (2004) reported that, in rat cardiac myocytes, another KATP channel opener (pinacidil) prevents hypoxia/reoxygenation-induced Ca^{2+} overload [16]. This effect was due to hyperpolarization of the diastolic membrane potential, which may facilitate Ca^{2+} exit by NCX1. From these two reports, we suspected a relationship between KATP channel openers and NCX1. Therefore, we investigated the effects of KATP channel openers on NCX1 function and possible involvement of the NO/cGMP/PKG signaling pathway.

Nicorandil

Nicorandil (N-(2-hydroxyethyl)-nicotinamide nitrate) is widely used as an anti-angina drug with nitrate-like activity and KATP channel opening activity. This agent has cardioprotective effects by shortening action potential duration (APD) and hyperpolarizing membrane potential during cardiac ischemia/reperfusion injury. Nicorandil has multiple additional effects including anti-fibrotic activity, anti-apoptotic activity, and reactive oxygen species (ROS) prevention [17]. Regarding ion channels, nicorandil enhances Ca^{2+}-dependent K^+ current in rat smooth muscles [18], and cAMP-dependent Cl^- current in guinea-pig cardiomyocytes via increasing intracellular cGMP [19]. In addition, nicorandil acutely increases cGMP levels by activating soluble GC via NO-dependent or -independent pathways in smooth muscles and cardiac cells [20–23].

In patch-clamp and fluorescent Ca^{2+} indicator (Fura-2/AM) studies, we examined the acute effect of nicorandil on cardiac Na^+/Ca^{2+} exchange current (I_{NCX1}) in single guinea-pig ventricular cells. Nicorandil enhanced I_{NCX1} in a concentration-dependent manner, with EC_{50} values of 8.3 and 6.6 μM for the outward and inward I_{NCX}, respectively, and Hill coefficients of approximately 1 [24] (Fig. 1a–c). Since nicorandil has nitrate-like activity, we first focused on the NO/cGMP/PKG signaling pathway. We observed that 8-Br-cGMP at 100 μM significantly enhanced I_{NCX} compared to control in single guinea-pig ventricular cells [24]. The nicorandil-induced I_{NCX} was significantly inhibited by ODQ, a soluble GC inhibitor, at 10 μM [24] (Fig. 1e). Interestingly, the nicorandil-induced I_{NCX} increase was hardly prevented by L-NAME, an NO synthase (NOS) inhibitor, at 10 μM [24] (Fig. 1d). Liou et al. (2011) reported that nicorandil increased NO and eNOS phosphorylation in cardiac fibroblasts and these effects were time dependent [25]. It took more than 30 min for nicorandil to significantly increase both eNOS phosphorylation and NO generation [25]. Since the perfusion time (< 5 min) of nicorandil was shorter than 30 min in our experiment, the nicorandil-induced I_{NCX} increase must be NO independent in single guinea-pig ventricular cells. Similar results were obtained by Minamiyama et al. (2007), who reported that nicorandil elevated cGMP levels without NO generation in rat liver, aorta, and human coronary smooth muscle cells in vitro [23]. Although we did not examine the effect of PKG on I_{NCX}, our results suggest that nicorandil-induced I_{NCX1} increase is mediated by the PKG signaling pathway through an increase in intracellular cGMP.

Furthermore, to clarify the site of action of nicorandil on NCX1, we used the fibroblast cell line, CCL39 stably expressing a canine heart NCX1 isoform and its mutant. In this NCX1 mutant, amino acids (Δ)247–671 are deleted, which is a large portion of the long intracellular loop between TMS 5 and 6, and which includes the XIP region, Ca^{2+} binding domain, phosphorylation sites, and various modulating sites [8, 9, 26]. We examined the effects of nicorandil on I_{NCX} in cells expressing wild-type NCX1, its mutants, and in isolated guinea-pig cardiac ventricular myocytes [24] (Fig. 2a, c). The enhancement ratios of I_{NCX1} by nicorandil were similar between the wild-type NCX1 expressing cells and the guinea-pig cardiac ventricular myocytes [24] (Fig. 2c). On the other hand, nicorandil did not increase I_{NCX1} in the mutant expressing cells [24] (Fig. 2b, c). These results indicated that the large intracellular loop between TMS 5 and 6 may be responsible for the site of action of nicorandil on NCX1.

Pinacidil

Pinacidil, which was initially developed as an antihypertensive drug, is a non-selective KATP channel opener without nitrate-like activity. The non-selective KATP channel openers have properties that open both plasma membrane KATP (pmKATP) and mitochondria KATP (mitoKATP) channels.

In the patch-clamp study and Fura-2/AM study, we examined the effect of pinacidil on I_{NCX1} in single guinea-pig cardiac ventricular myocytes. Pinacidil enhanced I_{NCX1} in a concentration-dependent manner with EC_{50} values of 23.5

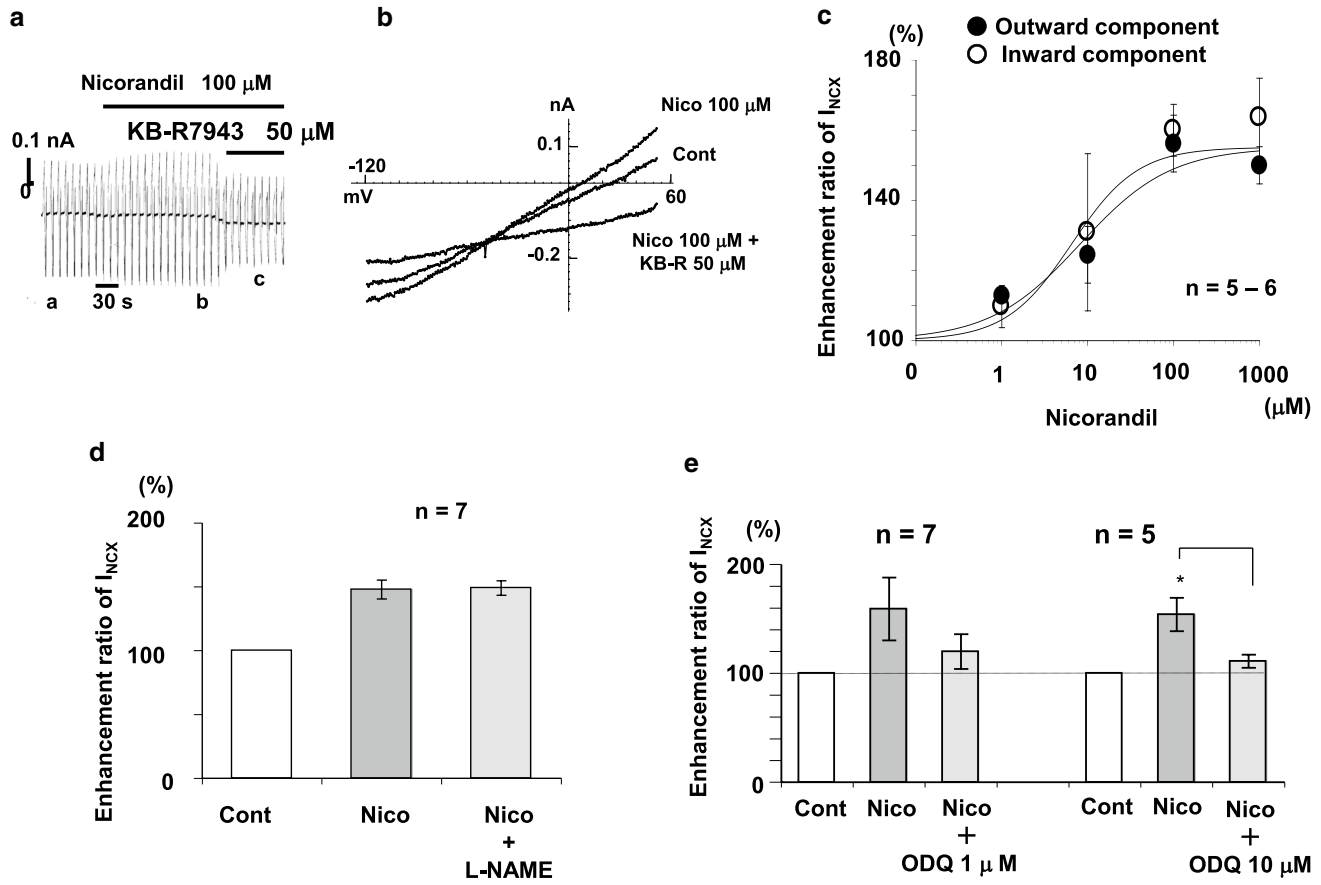

Fig. 1 Effect of nicorandil on I_{NCX1} (modified from [24] with permission). **a** Chart recording on I_{NCX1}. The ramp pulse was initially depolarized from a holding potential of −60 to +60 mV, then hyperpolarized to −120 mV, and then depolarized back to −60 mV at a rate of 680 mV/s. A ramp pulse was given every 10 s. I_{NCX} was inhibited completely by KB-R7943, a potent inhibitor of I_{NCX}, at 50 μM. **b** I–V curves on I_{NCX1}. **c** Concentration–response curves of the pinacidil on I_{NCX}. **d** and **e** Summarized data of L-NAME and ODQ on I_{NCX1}. *Cont* control, *Nico* nicorandil, *KB-R* KB-R7943

and 23.0 μM for the outward and inward I_{NCX1}, respectively, and Hill coefficients of approximately 1 [27] (Fig. 3a).

KATP channels are regulated by the NO/cGMP/PKG signaling pathway in rat and rabbit hearts [28, 29]. On the other hand, two groups suggested a possible link between KATP channels and NO generation in the rabbit mesenteric artery and rat heart [30, 31]. We examined the relationship between the I_{NCX1} increasing effect of pinacidil and the NO/cGMP/PKG signaling pathway. In our study, L-NAME, ODQ, and KT-5823, a PKG inhibitor, completely blocked the pinacidil-induced I_{NCX1} increase [27] (Fig. 3b). Glibenclamide, a non-selective KATP channel blocker, completely blocked the pinacidil-induced I_{NCX1}, but 5-HD, a selective mitoKATP channel inhibitor, did not [27] (Fig. 4a). These results suggest that the pinacidil-induced I_{NCX1} increase may be due to pmKATP channel opening, but not due to mitoKATP channel opening.

The next question that arises is how pinacidil generates NO. We tested NO production by pinacidil using a fluorometric assay kit in single cardiomyocytes. Pinacidil increased NO about twice as much as the control and pinacidil-induced NO was significantly inhibited by glibenclamide and L-NAME [27] (Fig. 4b). These results suggest that pinacidil may generate NO directly, or indirectly by pmKATP channel opening, and increase I_{NCX1} by phosphorylation via PKG as a result of activation of the NO/cGMP/PKG signaling pathway.

Reactive oxygen species (ROS) enhance NCX1 function in cardiac ventricular myocytes [32–34]. Krenz et al. (2002) have reported that KATP channel opening contributed to generation of ROS in vascular smooth muscles [35]. There may be a positive feedback relationship for ROS release by interaction between pmKATP channel and mitoKATP channel opening in cardiomyocytes. However, in our study, 30 μM pinacidil-induced I_{NCX1} was not inhibited by 1 mM N-2-(mercaptopropionyl) glycine (MPG), an ROS scavenger, or by 5-HD [27] (Figs. 3c, 4a right). There are two reports that affirm these results. Pinacidil generated

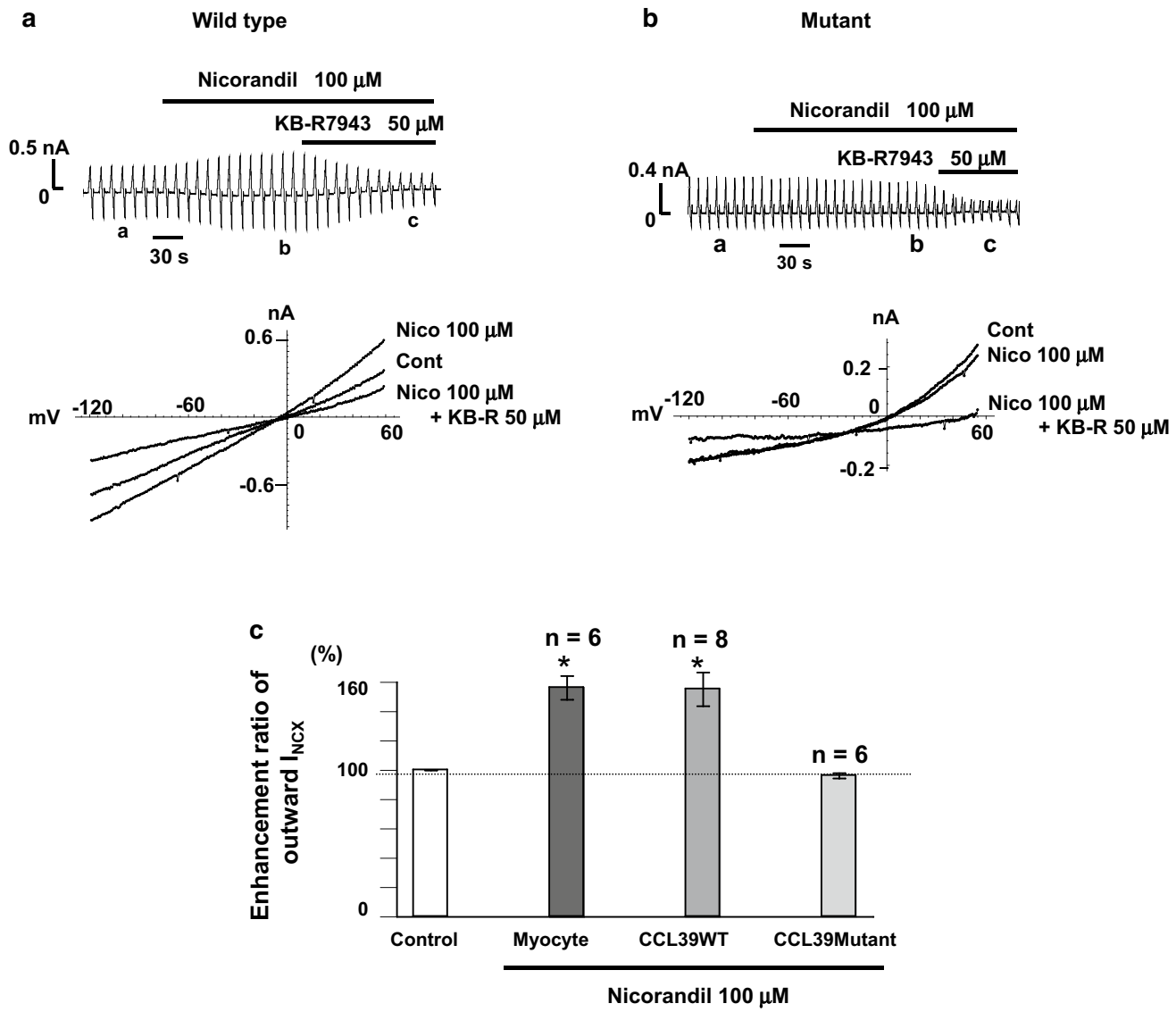

Fig. 2 Effect of nicorandil on I_{NCX} in wild type and mutant NCX1 expressing CCL39 cells (modified from [24] with permission). **a** and **b** Chart recordings of nicorandil on I_{NCX1} in NCX1 wild type and mutant Δ247–671, and I–V curves. *Cont* control, *Nico* nicorandil, *KB-R*, KB-R7943. **c** Summarized data. Control: Myocyte or CCL39WT or CCL39Mutant without nicorandil

ROS in a concentration-dependent manner in the rabbit heart, but it took more than 30 min for pinacidil to significantly increase ROS [36]. Holmuhamedov et al. (1998) indicated that in isolated cardiac mitochondria pinacidil acutely decreased mitochondrial membrane potential by mitoKATP channel opening in a concentration-dependent manner at a concentration of 100 μM or higher [37]. These reports suggest that 30 μM pinacidil application for 2–3 min may not generate ROS by mitoKATP channel opening. Therefore, pinacidil-induced I_{NCX1} is not caused by ROS and/or mitoKATP channel opening.

There are multiple isoforms of phosphodiesterases (PDEs) in cardiomyocytes that can hydrolyze cAMP and/or cGMP. Sildenafil, a PDE5 inhibitor, has specific properties such as inhibiting hydrolyzation of cGMP and increasing intracellular cGMP accumulation [38]. In our patch-clamp study, sildenafil at 10 μM further increased 10 μM pinacidil-induced I_{NCX1} [27] (Fig. 4c). In the case of a low or high concentration of pinacidil, the signaling pathway that enhances NCX1 function may be different.

KATP channel openers and NO production

How is KATP channel opening involved in the production of NO? KATP channels in vascular smooth muscle cells regulate the membrane potential. Opening of smooth muscle KATP channels by KATP channel openers causes membrane hyperpolarization. The opening of KATP channels in the

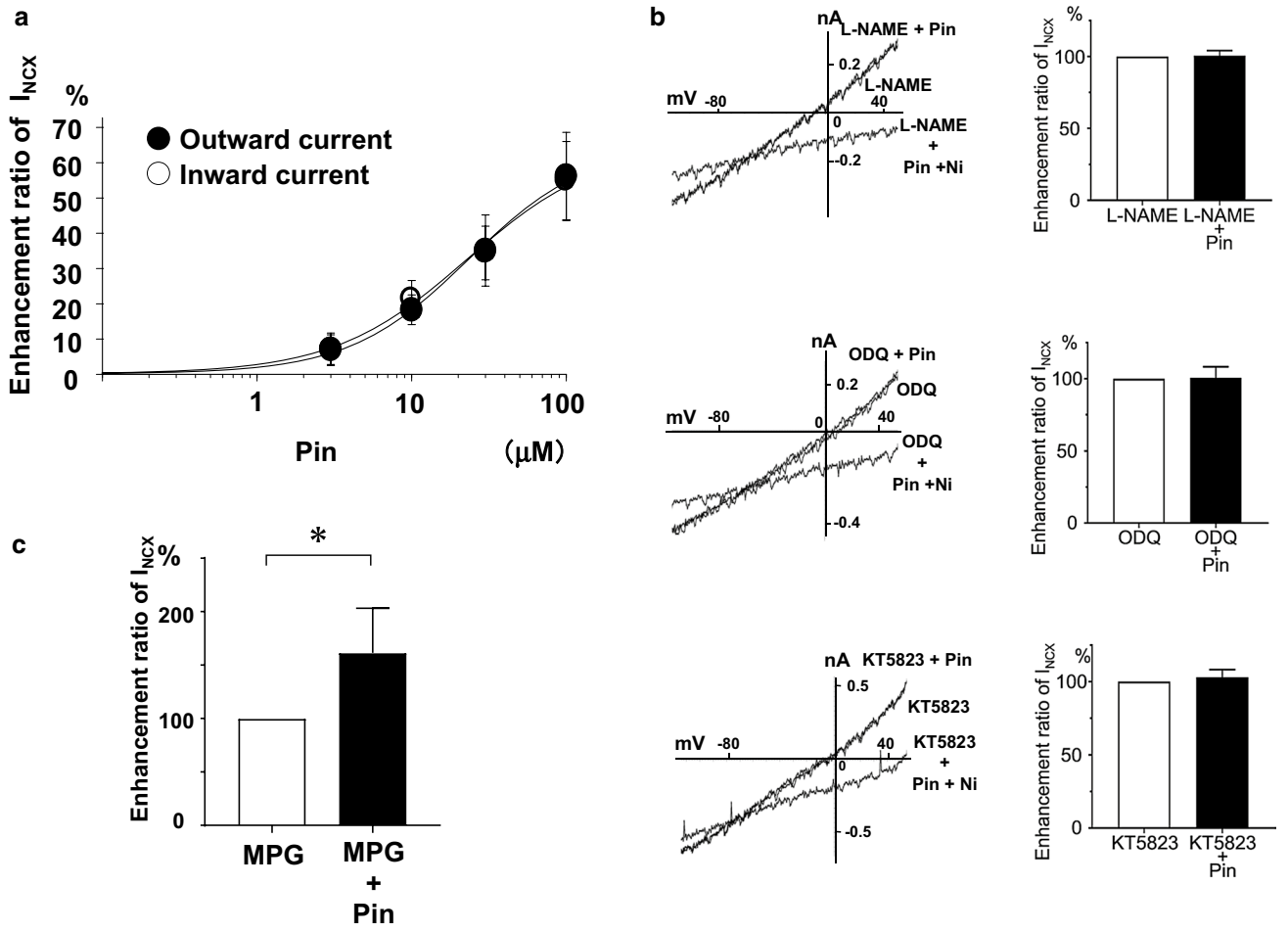

Fig. 3 Effect of pinacidil on I_{NCX1} (modified from [27] with permission). **a** Concentration–response curves of the pinacidil (*Pin*) on I_{NCX1}. **b** I–V curves and summary data of L-NAME (top), ODQ (middle) and KT5823 (bottom) on *Pin*-induced I_{NCX1}. **c** Summary data of MPG on *Pin*-induced I_{NCX1}

endothelium may elevate intracellular Ca^{2+} concentration, which stimulates the secretion of vasoactive factors via NOS activation [39]. NO release was dependent on the activation of endothelial KATP channels in the pig endocardial artery [40]. Recently, it was reported in endothelial colony-forming cells that the KATP channel openers nicorandil and iptakalim led to Ca^{2+} influx and activation of CaMKII, with increased phosphorylation levels of CaMKII, eNOS, and Akt, while these phosphorylations were abolished by glibenclamide [41]. These results suggest that intracellular Ca^{2+} increase may contribute to the opening of KATP channels, but this concept is still controversial. Katakam et al. (2015) indicated for the first time that diazoxide, a mitoKATP channel opener, depolarized mitochondria and increased $[Ca^{2+}]_i$ in cultured neurons [42]. Diazoxide thus increased nNOS phosphorylation and increased NO production. Our study suggests that nicorandil, a hybrid KATP channel opener, increases I_{NCX1} though the cGMP/PKG signaling pathway. Since the pinacidil-induced NO increase was inhibited by glibenclamide and L-NAME in a fluorometric assay, pinacidil may directly or indirectly generate NO [27] (Fig. 4b). From these results, we proposed that pinacidil, which does not possess nitrate-like activity, increases I_{NCX1} though the NO/cGMP/PKG signaling pathway.

However, how does KATP channel opening induce the pinacidil activation of NOS? The opening of KATP channels in the endothelium may elevate intracellular Ca^{2+} concentration, which stimulates NOS activation [39]. NCX contributes to Ca^{2+} homeostasis in endothelial cells. In vascular endothelial cells, consistent with a pivotal role of NCX in Ca^{2+}-dependent activation of eNOS, NCX protein was detected in caveolin-rich membrane fractions containing both eNOS and caveolin-1. This suggests that a functional interaction between endothelial NCX and eNOS may take place in caveolae [43]. Therefore, KATP channels, NOS, and NCX may be colocalized in caveolae of the plasma membrane. It is known that NCX1 increased or up-regulated in animal and clinical studies in heart failure (HF).

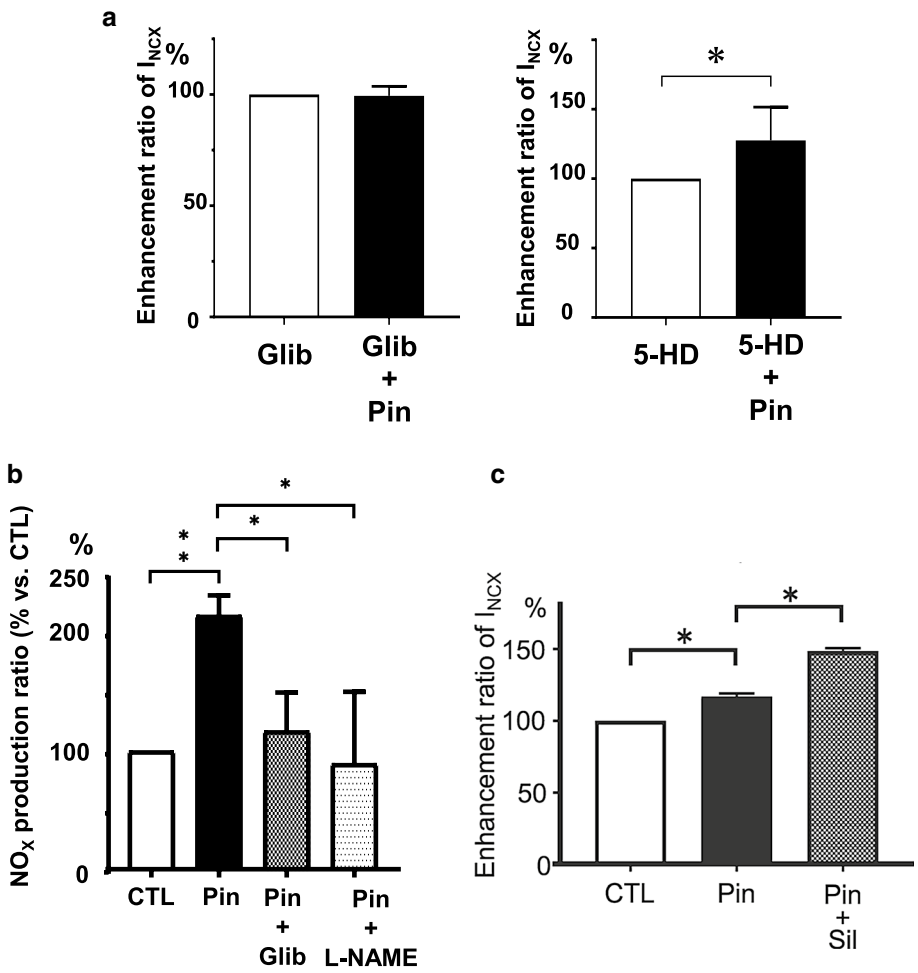

Fig. 4 **a** Summary data of glibenclamide, 5-HD and MPG on pinacidil-induced I_{NCX1} increase (modified from [27] with permission). **b** Summary data of pinacidil on NO in isolated cardiac ventricular myocytes. The effect of glibenclamide and L-NAME on pinacidil-induced NO (modified from [27] with permission). **c** Summary data of sildenafil on pinacidil-induced I_{NCX} increase (modified from [27] with permission). *CTL* control, *Glib* glibenclamide, *Pin* pinacidil, *Sil* sildenafil

Cardiomyocytes as well as endothelial cells and smooth muscle cells contain caveolin-1, -2, and -3 [44]. The muscle-specific isoform, caveolin-3, increased in HF [45]. NCX1 co-precipitated with caveolin-3 [44]. Myocardial NO signaling may be elevated in HF. The increase in caveolin-3 and sarcolemmal caveolae is associated with augmented nitric oxide signaling in canine pacing-induced HF [45]. Endothelial NOS (eNOS) and neuronal NOS (nNOS) are constitutively expressed in cardiomyocytes and endothelial cells, and inducible NOS (iNOS) is also expressed in normal cardiomyocytes [46, 47]. Various ion channels in cardiomyocytes are colocalized with different types of NOS, as reviewed by Gonzales et al. (2009) [48]. There may be a close relationship between KATP channel opening and NOS activation in cardiomyocytes. If there are microdomains such as caveolae where KATP channels, NOS, GC, and PKG are colocalized just below the cardiac cell membrane [43, 48], NOS may be activated by the opening of KATP channels and induce NO production. We found two reports that support this hypothesis. One notes that KATP channel opening by levosimendan may activate nNOS and thereby generate NO in the hippocampus and temporal cortex [49]. Another report indicates that vasodilatation caused by KATP channel opening by minoxidil was inhibited by L-NAME in rat renal vascular smooth muscles [50]. Assuming that the KATP channel opening may mechanically or redox chemically activate NOS, which may be colocalized with the KATP channel, the activation of NOS generates NO and activates the cGMP/PKG signaling pathway in cardiomyocytes. In endothelial colony-forming cells, the KATP channel openers nicorandil and iptakalim led to Ca^{2+} influx, and activated CaMKII with increased phosphorylation levels of CaMKII, eNOS, and Akt, while their phosphorylation was abolished by glibenclamide [41]. KATP channel opening decreases $[Ca^{2+}]_i$ in cardiomyocytes. Therefore, the mechanism of NOS activation in cardiomyocytes by KATP channel opening may be different from that of endothelial cells. However, we have not been able to find any report to date on the molecular link between KATP channel opening and NOS activation. On the contrary, we found reports that KATP channel openers such as pinacidil, diazoxide, cromakalim, and minoxidil did not increase cGMP in rat intact aorta smooth muscle [51, 52]. Whether all KATP channel openers activate NOS and increase I_{NCX1} needs to be investigated.

SNP and ANP

Vasodilatating NO donors such as sodium nitroprusside (SNP) and alpha-human atrial natriuretic peptide (α-hANP) are widely used for the treatment of congestive heart failure. SNP is an atrial and venous dilator that decreases cardiac preload and afterload. ANP activates GC and increases cGMP as a second messenger. ANP has various effects, including as an anti-inflammatory, and inhibitory effects on the rennin–angiotensin system and sympathetic tone [53].

SNP and 8-Br-cGMP, a membrane-permeable analog of cGMP, stimulated NCX1 activity by stimulating soluble GC via an NO-dependent or NO-independent pathway in vascular smooth muscle cells, C6 glioma cells, and astrocytes [12, 14, 54]. We examined the effects of SNP and 8-Br-cGMP on I_{NCX1} in guinea-pig cardiomyocytes by the patch-clamp method. SNP at 1 mM and 8-Br-cGMP at 100 μM stimulated I_{NCX1} [24, 27] (Fig. 5a). Nashida et al. (2011) reported that SNP decreases intracellular Ca^{2+} concentration by activation of the Ca^{2+} exit mode of NCX1 through the NO/cGMP/PKG signaling pathway [55]. Furukawa et al. (1991) reported that α-human ANP at 100 nM increases the Ca^{2+} efflux via NCX1 (ionomycin-induced $^{45}Ca^{2+}$ efflux) in rat aorta vascular smooth muscle cells and suggested that the NCX1 function increase by ANP may be dependent on the cGMP/PKG signaling pathway [12].

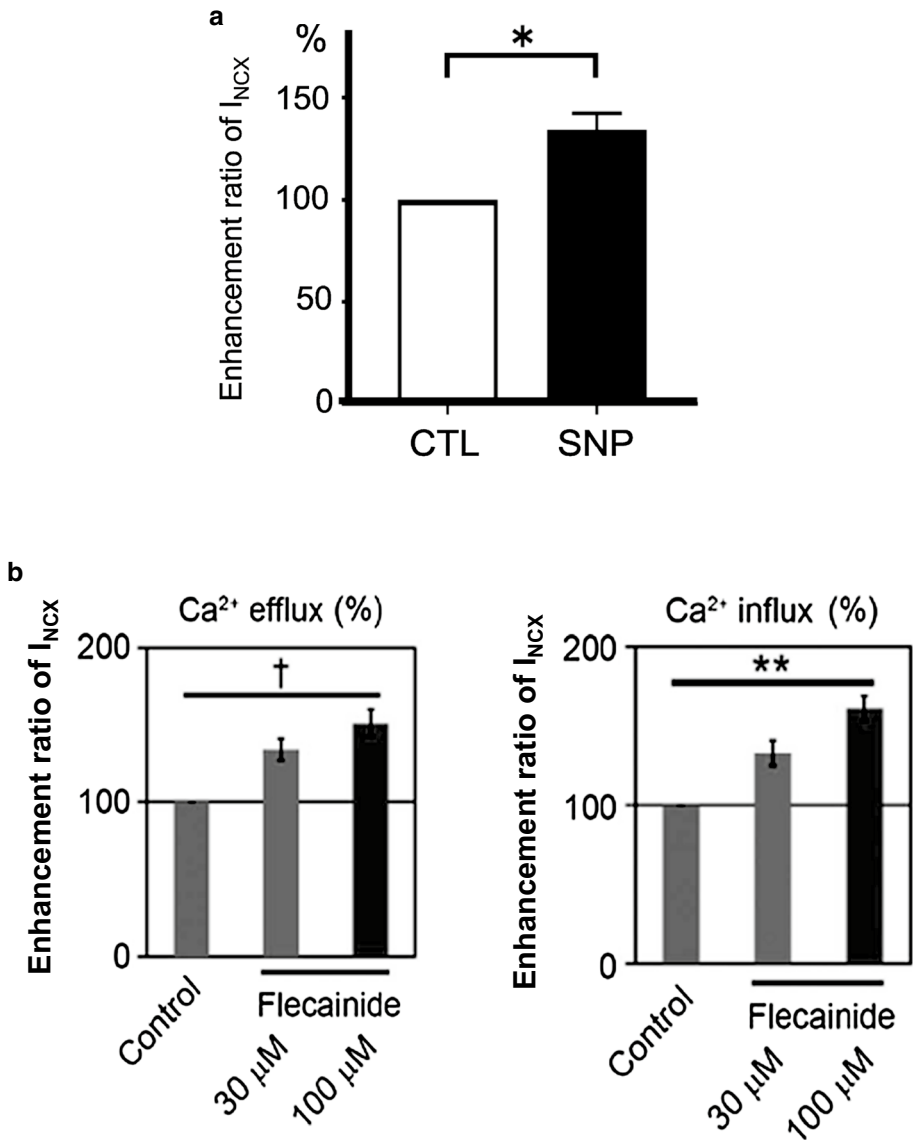

Fig. 5 Effect of flecainide and SNP on I_{NCX1}. **a** Summary data of SNP at 1 mM on I_{NCX1} (modified from [27] with permission). **b** Summary data of flecainide on I_{NCX1} (modified from [59] with permission)

Antiarrhythmic drugs that activate NCX1 function

Flecainide

Flecainide is a class I_c antiarrhythmic agent in Vaughan Williams classification and is used primarily in the treatment of supraventricular arrhythmias [56]. The acute effects of flecainide are inhibition of peak Na^+ channels (peak I_{Na}), late Na^+ channels (late I_{Na}), L-type Ca^{2+} channels ($I_{Ca\text{-}L}$), two voltage-gated K^+ channels, i.e., delayed rectifier K^+ channels at the rapid component (I_{Kr}) and the transient outward K^+ channels (I_{to}), and the human Ether-à-go-go-Related Gene (*hERG*) potassium channel [57].

Flecainide at 5 μM decreased the amplitude of DADs in dog Purkinje fibers [58]. We examined the effect of flecainide on I_{NCX1} in single guinea-pig cardiac ventricular cells. Flecainide at 30–100 μM stimulated I_{NCX1} by 30–60% in a concentration-dependent manner by the patch-clamp method [59] (Fig. 5b). Sikkel et al. (2013) reported that flecainide at 5 μM significantly stimulated NCX1-mediated Ca^{2+} efflux in isolated rat cardiomyocytes and suggested that this effect contributed to reducing $[Na^+]_i$ [60].

Cardioprotective drugs that inhibit I_{Na}, I_{Ca}, and I_K such as amiodarone, bepridil, aprindine, and dronedarone inhibited I_{NCX1} in a concentration-dependent manner in isolated guinea-pig cardiomyocytes, as reviewed previously [10]. In addition, ranolazine and carvedilol, which inhibited I_{NCX1}, also suppressed I_{Na}, I_{Ca}, and I_K [61, 62]. However, strangely, only flecainide, which suppressed I_{Na}, I_{Ca}, and I_K, activated I_{NCX1} in our study. Further studies are required to elucidate the molecular mechanisms of flecainide that activated the NCX1 function.

Dofetilide

Dofetilide, a Class III antiarrhythmic drug in Vaughan Williams classification, prolongs APD by inhibiting delayed outward rectifying K^+ current and has a positive inotropic effect in guinea-pig cardiomyocytes [63]. Dofetilide increased the amplitude of DADs induced by cardiac glycoside acetyl-strophanthidin in isolated cardiac Purkinje fibers using microelectrode techniques [64]. Dofetilide dose-dependently increased I_{NCX1} with EC_{50} values of 0.149 μM and 0.249 μM for the inward and outward components, respectively, in rat cardiac ventricular myocytes [65]. However, there has been no report on the molecular mechanisms of activation of I_{NCX1} by dofetilide.

NCX1 stimulators that protect or inhibit delayed afterdepolarizations (DADs)

An augmented NCX1 function may play an important role in cardiac arrhythmogenesis. The cardiac arrhythmia is induced by concomitant triggers such as extrasystole, intracellular Ca^{2+} overload, and spontaneous Ca^{2+} release. The activated Ca^{2+} efflux mode of NCX1 may cause DADs, and ventricular arrhythmias [66, 67]. Therefore, NCX inhibitors may have antiarrhythmic actions by inhibiting intracellular Ca^{2+} overload in cardiomyocytes, or by directly inhibiting the inward I_{NCX1} [68, 69]. In our study, 30 μM carvedilol, which suppressed I_{NCX1}, also inhibited ouabain-induced DADs with 0.1 Hz pulse stimuli in isolated guinea-pig ventricular myocytes [62] (Fig. 6a). DADs are almost entirely due to the inward I_{NCX1}, not Ca^{2+}-activated Cl^- current or Ca^{2+}-activated non-selective cation current [70]. Though nicorandil, pinacidil, and flecainide protected against or attenuated both spontaneous and triggered activities such as ouabain- or acetylstrophantidin-induced DADs in in vitro and in vivo studies [71–76], these three drugs enhanced I_{NCX1} in our patch-clamp experiment using guinea-pig cardiac ventricular myocytes. Furthermore, in this study nicorandil also protected against ouabain-induced DADs in single guinea-pig cardiac ventricular myocytes [24] (Fig. 7b, c). Why do nicorandil, pinacidil, and flecainide, which increase I_{NCX1}, prevent or suppress DADs?

Both nicorandil and pinacidil have a KATP channel opening effect. Pharmacological properties in common to these two drugs are shortening APD and hyperpolarizing membrane potential by ATP-sensitive K^+ (KATP) channel opening. In our study, nicorandil inhibited I_{Ca} and shortened APD via KATP channel opening [24] (Fig. 7a). The I_{NCX1} increase by nicorandil and pinacidil may be as a result of phosphorylation by PKG via the cGMP/PKG signaling pathway. While the Na^+/K^+ pump in the plasma membrane is also activated by cGMP, the cGMP-mediated increase in NCX1 function decreases $[Ca^{2+}]_i$ in cardiac cells [13]. Furthermore, both the functional densities of NCX1 and the Na^+/K^+ pump were 3- to 3.5-fold more in the transverse tubule plasma membrane than that in the external plasma membrane in rat cardiac ventricular myocytes [77]. In rat vascular smooth muscle cells, the Na^+/K^+ pump may affect the gap junction conductivity by changing $[Ca^{2+}]_i$ of the microdomain via modulation of NCX1 activity [78]. The functional interaction between NCX1 and the Na^+/K^+ pump may be pivotal for the contraction of cardiac muscle. Especially in the microdomain of the plasma membrane in the heart, NCX1 and the Na^+/K^+ pump may closely interact to regulate $[Na^+]_i$ and $[Ca^{2+}]_i$. Both nicorandil and pinacidil may decrease resting $[Ca^{2+}]_i$

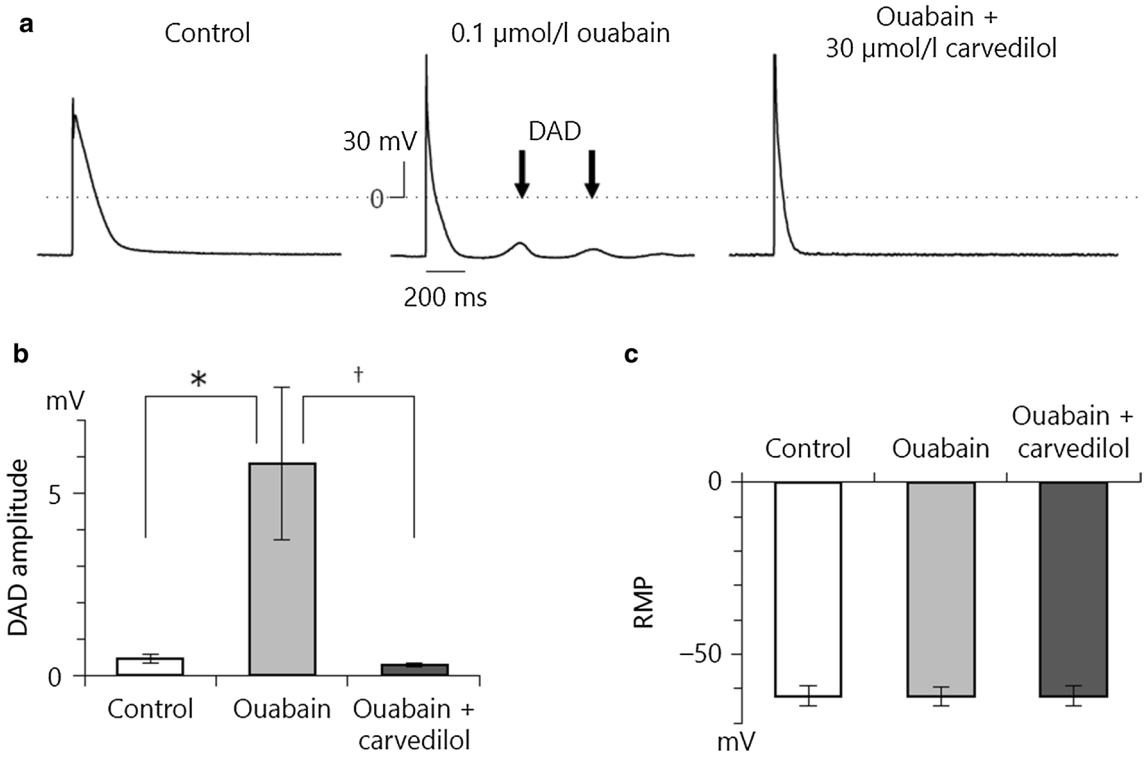

Fig. 6 Effect of carvedilol on DADs ([62] with permission). **a** (Left) Control condition. (Middle) DADs were induced by ouabain and electrical stimulation. (Right) The inhibitory effect of carvedilol on DADs. **b** Summarized data of carvedilol on DAD amplitude. **c** Summarized data of carvedilol on resting membrane potential (RMP)

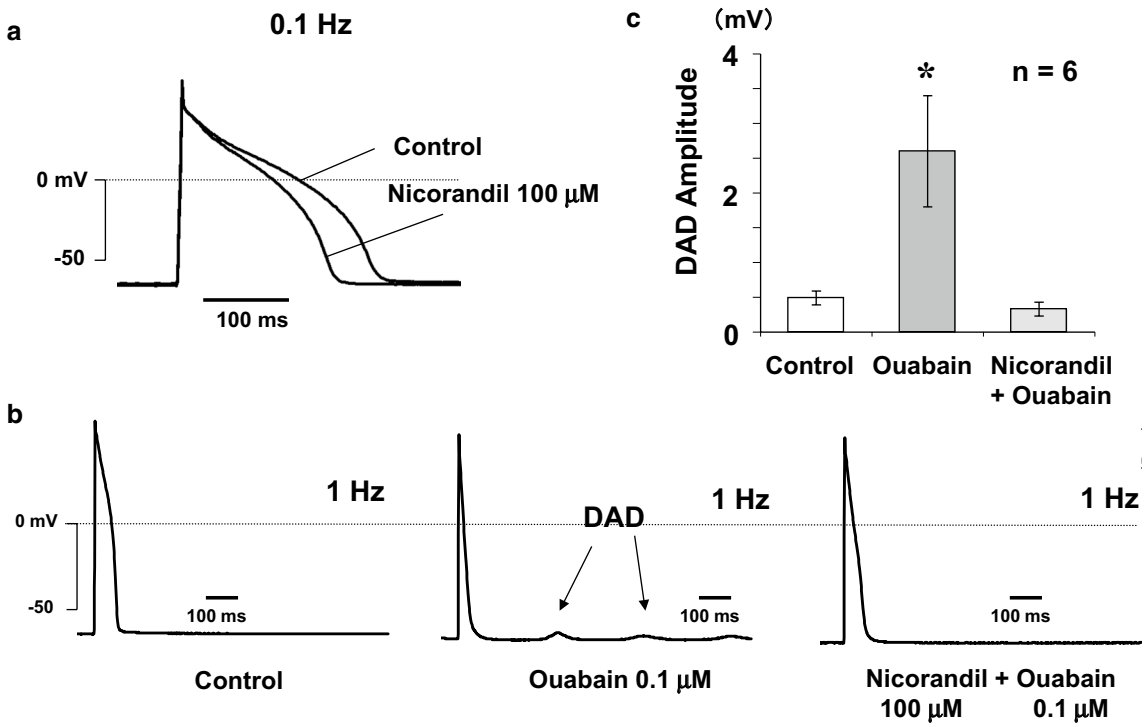

Fig. 7 Effect of nicorandil on action potential and DADs ([24] with permission). **a** The effect of nicorandil on action potential in a ventricular myocyte. **b** (Left) Control condition. (Middle) DADs were induced by ouabain. (Right) The inhibitory effect of nicorandil on DADs. **c** Summarized data of nicorandil on DADs

Table 1 Properties of NCX1 stimulants

NCX1 stimulants	Drug class	EC_{50} value, potential	Preparation
Nicorandil	KATP channel opener Nitrate generator	EC_{50} 15.0 μM (outward), 8.7 μM (inward) Approximately 60% enhancement (100 μM, outward and inward)	Guinea-pig ventricular myocytes [24]
Pinacidil	Non-selective KATP channel opener	EC_{50} 23.5 μM (outward), 23.0 μM (inward) Approximately 55% enhancement (100 μM, outward and inward)	Guinea-pig ventricular myocytes [27]
SNP	NO donor	34.3 ± 8.1% enhancement (1 mM, outward)	Guinea-pig ventricular myocytes [27]
α-hANP	Peptide hormone	46 ± 10% enhancement (100 nM, inward)	Rat Aorta vascular smooth muscle [12]
Sildenafil	PDE5 inhibitor	Pinacidil-induced I NCX increase (outward, 10 μM pinacidil 16 ± 8.1%, + 10 μM sildenafil 48.5 ± 2.2% enhancement)	Guinea-pig ventricular myocytes [27]
Flecainide	Class Ic antiarrhythmic drug	Approximately 60% enhancement (100 μM, outward and inward)	Guinea-pig ventricular myocytes [59]
Dofetilide	Class III antiarrhythmic drug	EC 50 0.249 μM (outward), 0.149 μM (inward) Approximately 120% enhancement (1 μM, outward)	Rat Ventricular myocytes [65]

EC_{50} Half-maximum concentration for enhancement of the drug, []: Reference No.

via the activation of both NCX1 and the Na^+/K^+ pump by the cGMP/PKG signaling cascade in cardiomyocytes. The cardioprotective effects of nicorandil and pinacidil against DADs may be mainly due to shortening APD in addition to the enhancement of Ca^{2+} efflux by NCX1.

$[Na^+]_i$ is a key modulator of intracellular Ca^{2+} cycling in the heart. An enhancement of late I_{Na} increases the intracellular Na^+ concentration and thereby increases Ca^{2+} influx via the outward mode of NCX1 during the plateau phase of the action potential (AP). Late I_{Na}-mediated $[Na^+]_i$ loading may increase diastolic $[Ca^{2+}]_i$, Ca^{2+} extrusion by the inward mode of NCX1, and DADs formation [79, 80]. The inhibitory effect of flecainide on cardiac $Na_v1.5$ channels increased the triggering threshold by inhibiting both peak I_{Na} and late I_{Na}. Therefore, flecainide indirectly reduced $[Ca^{2+}]_i$ by the Ca^{2+} efflux mode of NCX1 as well as the incidence of DADs [81]. On the other hand, in one report, flecainide at 6 μM failed to abolish isoproterenol-induced DADs but suppressed isoproterenol-induced triggered activity in mice [82]. Further studies are required to clarify whether or not flecainide inhibits DADs.

Up-regulation of NCX1 gene expression and NCX1 inhibitor

Xu et al. (2009) reported that chronic administration of KB-R7943, an NCX inhibitor, up-regulated NCX1 gene expression in both isolated cardiomyocytes and intact mouse heart [83]. In response to chronic NCX1 inhibition, p-38 forms NCX1-p38 complex [83]. Furthermore, NCX1-p38 complex results in NCX1 up-regulation via activation of p-38 signaling pathway [83]. During hypertrophy and heart failure, up-regulation of NCX1 can be considered as a compensatory adaptation to improve contractile function. However, this compensation invites an increased risk of arrhythmia, such as DADs.

Summary

The KATP channel openers nicorandil and pinacidil, and ANP and SNP, as well as the Na^+ channel blocker flecainide and the K^+ channel blocker dofetilide, increased NCX1 function (Table 1). The effects of nicorandil and ANP on NCX1 may be mediated by a PKG signaling pathway through an increase in intracellular cGMP (Fig. 8). The effect of pinacidil on NCX1 is mediated by a PKG signaling pathway and pmKATP channel opening (Fig. 8). Little is known about the coexistence and functional cooperation mechanism among NCX1, NOSs and KATP channels in caveolae on the membrane in cardiomyocytes. The effect of SNP on increasing NCX1 may be dependent on the NO/cGMP/PKG signaling pathway (Fig. 8). On the other hand, the molecular mechanisms of flecainide and dofetilide, which activated the NCX1 function, have not been reported. The up-regulation of NCX1 during hypertrophy and heart failure can be considered a compensatory adaptation to improve contractile function. However, this compensation increases risk of arrhythmia. Therefore, further studies are also required to elucidate the role of NCX1 gene expression for myocardial protection.

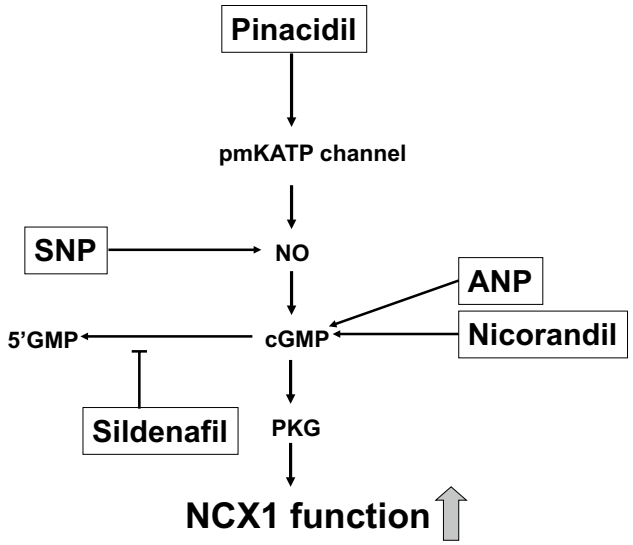

Fig. 8 Scheme of a possible signaling pathway for NCX1 activation by nicorandil, pinacidil, ANP, SNP, and sildenafil. Nicorandil and ANP increase cGMP, SNP generates NO, sildenafil accumulates cGMP, and four agents subsequently activate PKG. Pinacidil opens the pmKATP channel, which generates NO and activates guanylate cyclase, increases cGMP, and subsequently activates PKG. PKG directly or indirectly phosphorylates and stimulates NCX1

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Compliance with ethical standards

Conflict of interest The author of this manuscript has no conflict of interest to declare.

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