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Forefront of Na^+/Ca^{2+} Exchanger Studies: Molecular Pharmacology of Na^+/Ca^{2+} Exchange Inhibitors

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Abstract. The Na^+/Ca^{2+} exchanger (NCX) is an ion transporter that exchanges Na^+ and Ca^{2+} in either Ca^{2+} efflux or Ca^{2+} influx mode, depending on membrane potential and transmembrane ion gradients. In myocytes, neurons, and nephron cells, NCX is thought to play an important role in the regulation of intracellular Ca^{2+} concentration. Recently, the benzyloxyphenyl derivatives KB-R7943, SEA0400, and SN-6 have been developed as selective NCX inhibitors. Currently, SEA0400 is the most potent and selective inhibitor. These inhibitors possess different isoform-selectivities, although they have similar properties, such as Ca^{2+} influx mode-selectivity and I_1 inactivation-dependence. Recent site-directed mutagenesis has revealed that these inhibitors possess some molecular determinants (Phe-213, Val-227, Tyr-228, Gly-833, and Asn-839) for interaction with NCX1. These benzyloxyphenyl derivatives are expected to be useful tools to study the physiological roles of NCX. Moreover, such inhibitors may have therapeutic potential as a new remedy for ischemic disease, arrhythmias, heart failure, and hypertension.

Keywords: Na^+/Ca^{2+} exchanger (NCX), NCX inhibitor, inhibitory mechanism, ischemia/reperfusion injury

Introduction

The plasma membrane Na^+/Ca^{2+} exchanger (NCX) is a bi-directional transporter that catalyzes the exchange of Na^+ for Ca^{2+} (transport ratio = about 3:1) depending on the electrochemical gradients of the substrate ions (1 – 3). Under physiological conditions, the primary function of NCX is thought to be to pump Ca^{2+} to the outside of the cell using the Na^+ concentration gradient across the cell membrane (4). Under pathological conditions such as cardiac ischemia/reperfusion injury, the exchanger is thought to cause Ca^{2+} overload due to elevated levels of intracellular Na^+ (Na^+_i), leading to mechanical and electrical dysfunction of cardiomyocytes.

Mammalian NCX forms a multigene family comprising NCX1, NCX2, and NCX3. NCX1 is highly expressed in the heart, brain, and kidney and expressed at much lower levels in other tissues, whereas the expression of NCX2 and NCX3 is limited mainly to the brain and skeletal muscle (5). These three isoforms presumably have similar molecular topologies consisting of nine transmembrane segments and a large central cytoplasmic loop (6, 7) (see Fig. 4A). The former part, particularly the α-repeat regions, may participate in ion transport (8 – 10); the latter part, possessing the exchanger inhibitory peptide (XIP) region (11, 12) and regulatory Ca^{2+} binding sites (13, 14), is primarily involved in various regulatory properties. NCX1 has been shown to be secondarily regulated by the transport substrates Na^+ and Ca^{2+} (15). Intracellular Ca^{2+} at the submicromolar level activates NCX activity by promoting the recovery of the exchanger from the "I_2 inactivation state", whereas high Na^+_i restrains the exchange by facilitating the entry of the exchanger into the "I_1 inactivation state" (Na^+-dependent inactivation).

Recently, the benzyloxyphenyl derivatives KB-R7943 (16 – 18), SEA0400 (19), and SN-6 (20) have been developed as selective NCX inhibitors. Potent and selective NCX inhibitors would be very useful for clarifying the physiological and pathophysiological roles of NCX. This review will outline the characteristics and therapeutic potential of NCX inhibitors.

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Development of NCX inhibitors

NCX is thought to play an important role in the regulation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in myocytes, neurons, and nephron cells. Therefore, NCX inhibitors have long been targeted as new calcium regulators. As NCX transports Ca^{2+} bidirectionally, the pharmacological effects of NCX inhibitors depend on the particular transport modes active at a given time (Fig. 1). When NCX pumps Ca^{2+} from the cell (Ca^{2+} efflux mode), the NCX inhibitor is expected to increase $[Ca^{2+}]_i$. This will induce cardiotonic and hypertensive effects in the circulatory system. On the other hand, when NCX works as a pathway of Ca^{2+} entry (Ca^{2+} influx mode) under pathological conditions, such as ischemia/reperfusion injury and digitalism, the NCX inhibitor is expected to guard against Ca^{2+} overloading. Thus, it is possible that NCX inhibitors will be an epoch-making remedy for such conditions.

Divalent and trivalent cations (Ni^{2+}, Cd^{2+}, and La^{3+}) and organic compounds such as 3',4'-dichlorobenzamyl and bepridil have long been known to act as NCX inhibitors (1, 2). However, these inhibitors were poorly specific to NCX and blocked other ion transporters and channels at low doses. In 1996, KB-R7943 (Fig. 2) was developed as a prototype for a selective NCX inhibitor (16, 17). This inhibitor was fairly specific to NCX because at up to 10 μM, it exerted little influence on some other ion transporters, such as the Na^+/H^+ exchanger, Na^+, K^+-ATPase, and Ca^{2+}-ATPases (16). It is now being widely used to study the physiological and pathological roles of the exchanger at the cellular and organ levels. Recently, however, KB-R7943 has been reported to possess nonspecific actions against ion channels, neuronal nicotinic acetylcholine receptors, the *N*-methyl-D-aspartate receptor, and the norepinephrine transporter (17, 19, 21, 22). In 2001, Matsuda et al. (19) reported on SEA0400 (Fig. 2), a newly developed, more potent, and selective NCX inhibitor. Surprisingly, SEA0400 was discovered from an original screening system independent of KB-R7943. It is completely coincidental that SEA0400 and KB-R7943 have a common benzyloxyphenyl structure, suggesting that this

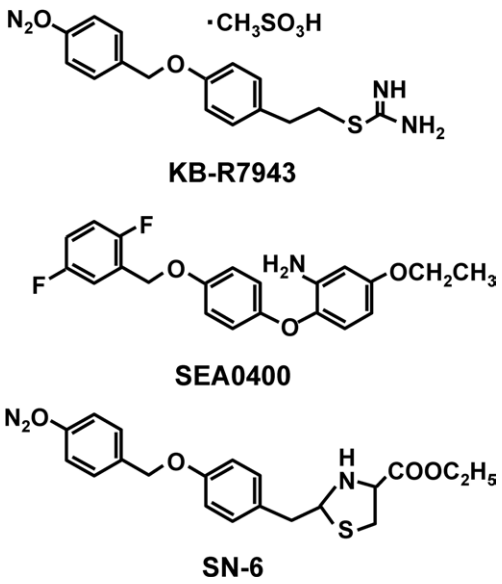


Fig. 2. Chemical structures of benzyloxyphenyl derivatives.

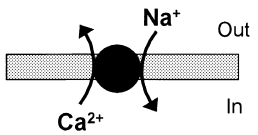
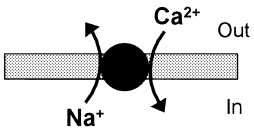
	Ca^{2+} efflux mode	Ca^{2+} influx mode
Transport mode		
Physiological function	Pumping out of Ca^{2+} after Ca^{2+} mobilization	Ca^{2+} influx under special conditions ($[Na^+]_i$ increases and depolarization)
Actions of NCX inhibitors	Increases in $[Ca^{2+}]_i$	Inhibition of Ca^{2+} overload Lowering of $[Ca^{2+}]_i$
Expected efficacy	Cardiotonic action Hypertensive action	Anti-ischemic action (against ischemia/reperfusion injury) Digitalis detoxication Anti-hypertensive action (against salt-sensitive hypertension)

Fig. 1. Physiological and pharmacological implications of Na^+/Ca^{2+} exchanger. Modified from Fig. 1 in Ref. 18 (Copyright© 2004) with kind permission of Kluwer Academic Publishers.

portion is important for inhibitory action against NCX. SEA0400 is highly specific for NCX because it hardly inhibits other receptors, channels, and transporters (19, 23). In 2002, SN-6 (Fig. 2) was found by screening newly synthesized benzyloxyphenyl derivatives for NCX1 inhibition (20, 24). This compound showed inhibitory potency for NCX1 similar to KB-R7943, but was more specific for NCX1 than KB-R7943.

Inhibitory properties of benzyloxyphenyl derivatives

Figure 3 shows the dose-response curves for the effects of three benzyloxyphenyl derivatives on Na^+_i-dependent $^{45}Ca^{2+}$ uptake in fibroblasts expressing NCX1, NCX2, and NCX3 (20, 25, 26). Their inhibitory potencies for NCX1 are SEA0400 (IC_{50} = 0.056 μM) ≫ SN-6 (2.9 μM) = KB-R7943 (4.3 μM). These inhibitors have different isoform selectivities: KB-R7943 is 3-fold more effective on NCX3 than on NCX1 and NCX2 (25, 27), whereas SEA0400 predominantly blocks NCX1, only mildly blocks NCX2, and exerts almost no influence upon NCX3 (26). SN-6 is 3- to 5-fold more inhibitory to NCX1 than to NCX2 and NCX3 (20). Accordingly, these benzyloxyphenyl derivatives should be properly used depending on the target organs, which express specific NCX isoforms.

Interestingly, all benzyloxyphenyl derivatives inhibit the Ca^{2+} influx mode by NCX1 much more effectively than the Ca^{2+} efflux mode (16, 17, 24 – 26, 28), although its mechanism is unknown at present. Recently, we have found that the inhibitory effects of benzyloxyphenyl derivatives are related to the kinetics of I_1 inactivation (20, 26). In short, XIP region mutants K229Q (or Y224W/Y226W/Y228W/231W) and F223E, which display either completely eliminated or accelerated I_1 inactivation, respectively, exhibit markedly reduced sensitivity or hypersensitivity to inhibition by benzyloxyphenyl derivatives, also respectively. The K229Q mutant apparently works as a constitutively active exchanger, as do Y224W/Y226W/Y228W/231W. These mutants hardly enter the I_1 inactive state. The marked reduction of drug sensitivities in these mutants suggests that benzyloxyphenyl derivatives may specifically target the I_1 inactive state. This possibility received support from the evidence that benzyloxyphenyl derivatives have hypersensitivities to F223E, which are able to very quickly enter an I_1 inactive state. Therefore, the interaction of benzyloxyphenyl derivatives with the exchanger seems to stabilize the I_1 inactive state or accelerate the rate of entry into the I_1 inactive state.

Interaction domains of benzyloxyphenyl derivatives

As described above, KB-R7943, SEA0400, and SN-6 have different isoform selectivities. NCX1, NCX2, and NCX3 share approximately 70% of their overall amino acid sequences. Therefore, the interaction domain of each benzyloxyphenyl derivative seems to differ among isoforms. To identify the critical region(s) involved in isoform selectivity, we constructed a series of chimeric exchangers by changing portions of a homologous sequence of NCX1 with that of NCX3 and searched for the region where the drug affinity changed. In KB-R7943, the α-2 repeat region (Fig. 4A) was determined to be almost exclusively responsible for the differential drug responses between NCX1 and NCX3 (25). On the other hand, in SEA0400 and SN-6, the first intracellular loop and the fifth transmembrane (and part of the XIP region) were mostly responsible for the difference in the drug responses (20, 26). Further site-directed mutagenesis within these regions revealed some critical residues involved in drug sensitivity. Gly-833 and Asn-839 in NCX1 are common molecular determinants required for inhibition by all benzyloxyphenyl derivatives (20, 25, 26). In contrast, Phe-213 and Val-227/Tyr-228 are specific determinants corre-

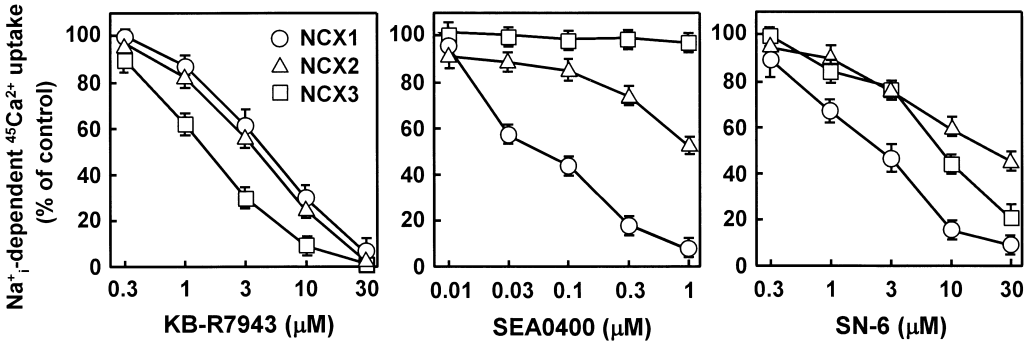


Fig. 3. Dose-response curves for the effects of benzyloxyphenyl derivatives on Na^+_i-dependent $^{45}Ca^{2+}$ uptake (i.e., Ca^{2+} uptake mode) in fibroblasts expressing NCX1, NCX2, and NCX3. The initial rates of $^{45}Ca^{2+}$ uptake into cells were measured in the presence or absence of indicated concentrations of NCX inhibitors. Modified from Refs. 20, 25, and 26 with permission.

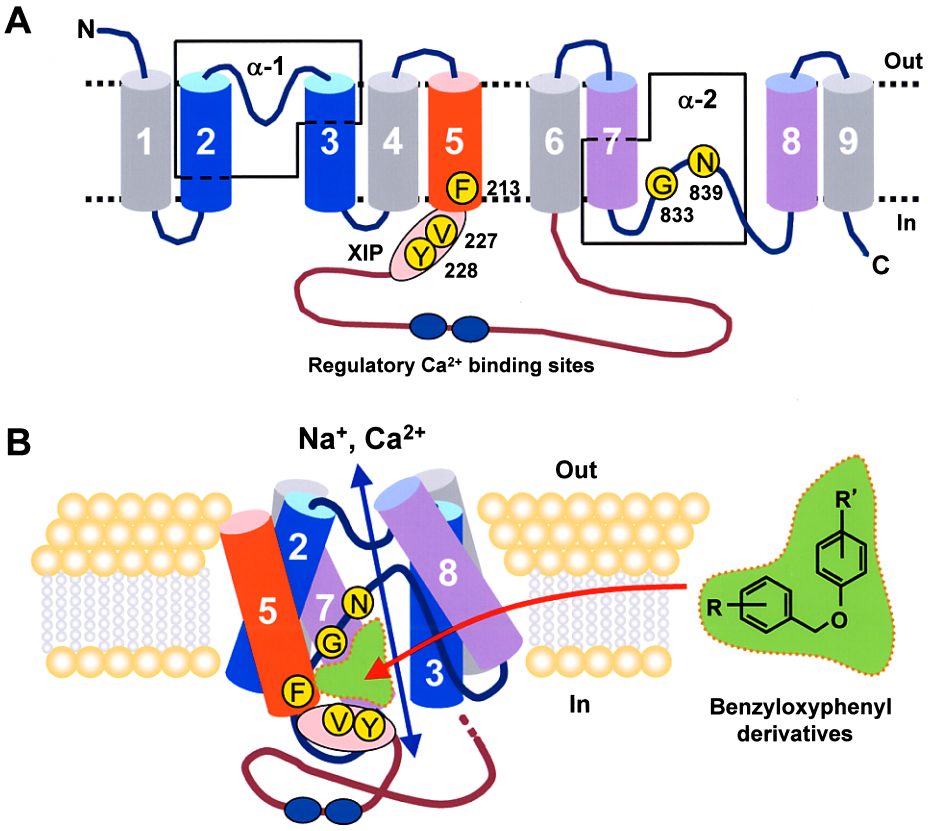


Fig. 4. Putative interaction domains for benzyloxyphenyl derivatives. A: a nine-transmembrane model of NCX1 taken from recent topology analyses (6, 7, 10). The amino acid residues of NCX1 whose mutation alters the sensitivities to benzyloxyphenyl derivatives are indicated. B: illustration representing the imagined structural domains responsible for benzyloxyphenyl derivatives. The indicated helix packing of transmembrane segments 2, 3, 7, and 8 is suggested by cross-linking data (29). Modified from Ref. 20 with permission.

sponding to inhibition by SEA0400 and SN-6, respectively. Figure 4 illustrates models for the topology and helix packing of NCX1 (6, 7, 10, 29), in which molecular determinants for inhibition by benzyloxyphenyl derivatives are indicated. It is inferred that the ion transport pathway of NCX consists of the membrane loops of the α-1 and α-2 regions that face each other and transmembrane helixes at both sides of two membrane loops. As shown in Fig. 4B, Phe-213, Val-227, Tyr-228, Gly-833, and Asn-839 may participate in the formation of the interaction domains with benzyloxyphenyl derivatives. It is conjectured that benzyloxyphenyl derivatives inhibit ion transport by blocking pores formed within the membrane regions.

Pharmacological actions of benzyloxyphenyl derivatives

The pharmacological effects of benzyloxyphenyl derivatives have been well studied in the heart. KB-R7943 (up to 10 μM) has no significant effect on Ca^{2+} transient, contractility, or spontaneous beating of normal cardiomyocytes of rats and guinea pigs (16, 30, 31). KB-R7943 also has no apparent effect on the left ventricular developed pressure (LVDP), left ventricular dP/dt_{max}, left ventricular end-diastolic pressure (LVEDP), and the heart rate of isolated rat perfused hearts (32). SEA0400 (0.001–1 μM) and SN-6 (1–10 μM) also exhibit similar properties in perfused hearts (33, 34). These findings are consistent with the results that benzyloxyphenyl derivatives have almost no effect on the Ca^{2+} efflux mode of NCX. Probably, the Ca^{2+} influx mode of NCX is not so important in the excitation-contraction coupling in these small animals.

The classic medicine for cardiac insufficiency, cardiotonic steroid (digitalis), manifests cardiotonic action by inhibiting the Na^+ pump. However, the agent at toxic doses induces arrhythmia. In model experiments, when high concentration ouabain or strophanthidin is added to isolated atrial preparations or isolated cardiomyocytes, arrhythmia is observed as contractive tension increases. KB-R7943 decreases markedly the incidence of

arrhythmia (30, 31). However, unexpectedly, KB-R7943 has hardly any effect on the cardiotonic action; that is, the cardiotonic action seems to be a phenomenon arising through accumulation of intracellular Na^+, suppression of the pumping out of Ca^{2+} by NCX and increases in $[Ca^{2+}]_i$. This mechanism is important for understanding the action of cardiotonic steroids.

The onset of ischemia/reperfusion injury is associated with intracellular Ca^{2+} overload and generation of oxygen free radicals due to reperfusion. NCX has long been thought to be the principal pathway of this Ca^{2+} overload. In fact, KB-R7943 and SEA0400 strongly suppress Ca^{2+} overload (and hypercontraction) of cardiomyocytes due to Ca^{2+} paradox and anoxia/reoxygenation (16, 35). KB-R7943, SEA0400, and SN-6 also significantly suppress myocardial ischemia/reperfusion injury (cardiac dysfunction) in isolated perfused hearts (32 – 34). The abilities of SEA0400 to guard against these ischemic injuries are reported to be more effective than those of KB-R7943 (33, 36). The effectiveness of post-ischemic treatment with KB-R7943 is thought to corroborate that Ca^{2+} overload through NCX occurs principally during reperfusion (32). SN-6 is reported to exhibit effective protective effects on the ischemia /reperfusion-injured heart by the dual actions of NCX inhibition and scavenging oxygen radicals (34). In addition, KB-R7943 and SEA0400 decrease the incidence of ventricular fibrillation in acute myocardial infarction models of anesthetized rats (32, 33). KB-R7943 and SEA0400 are also reported to be effective in ischemia /reperfusion injuries of the kidney (37, 38) and brain (19, 39) and in brain edema generated by radiofrequency lesion (40). On the other hand, some reports show negative effects by benzyloxyphenyl derivatives on ventricular arrythmias (41, 42), although the reasons are unknown.

Potential of NCX inhibitors as new remedy

As described above, benzyloxyphenyl derivatives potently suppress ischemia/reperfusion injuries of the heart, kidney, and brain while hardly affecting normal cardiac functions. These characteristics are ideal for a remedy for ischemia/reperfusion injury. Interestingly, we found that SN-6 predominantly works as a blocker of Ca^{2+} overload via NCX under hypoxic/ischemic conditions because this inhibitor preferentially acts on the exchanger under ATP-depleted conditions. Such a property of SN-6, which might be derived from its interaction with the endogenous XIP region, seems to be advantageous to developing it clinically as a new anti-ischemic drug for several organs. However, it has not been determined if NCX inhibitors alone are effective in various cardiac insufficiency models. It is anticipated that much more therapeutic information on SEA0400 or SN-6 will be accumulated. In recent years, the possibility has been pointed out that endogenous digitalis-like substances are associated with the etiology and pathophysiology of essential hypertension. It is highly possible that Ca^{2+} influx through NCX plays a role in the hypertensive mechanism (43). It is expected that NCX inhibitors are effective in essential hypertension.

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