福岡大医紀（*Med. Bull. Fukuoka Univ.*）：35(4), 211–218, 2008

Cardiovascular Na^+/Ca^{2+} Exchanger：Pathophysiologic Roles and Therapeutic Potentials

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Abstract：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 the ion gradients across the plasma membrane and membrane potential. In cardiac and vascular myocytes, NCX is thought to play an important role in the regulation of intracellular Ca^{2+} concentration. Recent studies using selective NCX inhibitors and gene targeting mice reveal etiological implications of NCX1 in cardiovascular diseases, such as cardiac ischemia/reperfusion injuries, heart failure and salt–dependent hypertension. This article presents the recent advance in our understanding of the pathological roles of NCX and the therapeutic potentials of NCX inhibitors.

Key words：**Na^+/Ca^{2+} exchanger, Benzyloxyphenyl NCX inhibitors, Cardiovascular disease, Ischemia/reperfusion injury, Heart failure, Salt–dependent hypertension**

Introduction

Calcium ions（Ca^{2+}）trigger physiological phenomena such as contraction, hormone secretion, gene expression and cell death in cardiac and vascular myocytes.[1)2)] These myocytes express various Ca^{2+} transport molecules, Ca^{2+} channels, Ca^{2+}–ATPases and Na^+/Ca^{2+} exchangers（NCXs）, which contribute to Ca^{2+} signaling in the plasma membrane and sarcoplasmic reticulum（SR）. In cardiomyocytes, altered Ca^{2+} handling is widely recognized as a contributing factor for both impaired contractile performance and electrical instability（i.e., arrhythmia）in human and experimental heart failure, and myocardial ischemia and reperfusion.[3)] It is also well documented that essential hypertension is somehow associated with an abnormality in cellular sodium ion（Na^+）and Ca^{2+} metabolism in vascular smooth muscle cells.[4)] Thus far, studies on altered intracellular Ca^{2+} handling in cardiovascular diseases have focused on abnormalities of L–type Ca^{2+} channels, Ca^{2+}–ATPases and ryanodine receptors.[3)4)]

On the other hand, there is little information available about the participation of NCX in cardiovascular diseases. Recently, the benzyloxyphenyl derivatives, such as KB–R7943 and SEA0400, have been developed as selective NCX inhibitors.[5)] Potent and selective NCX inhibitors would be very useful for clarifying the physiological and pathological roles of NCX. Furthermore, genetically engineered mice in which the NCX activity is either reduced or enhanced are also useful for elucidating the functions of NCX.[6)] Several studies using these experimental tools have provided compelling evidence that cardiovascular NCX may contribute to cardiac ischemia/reperfusion injury, heart failure and salt－sensitive hypertension. This review shall outline the therapeutic potentials of NCX inhibitors, along with the pathophysiologic roles of cardiovascular NCX.

Structure and Function of Cardiac NCX1

The NCX can transport Ca^{2+} either out of cells（forward mode）or into cells（reverse mode）in exchange for Na^+ moving in the opposite direction

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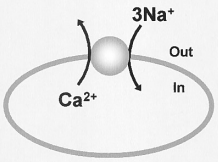
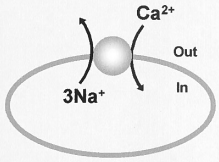
Table 1. Physiological and pharmacological implications of NCX inhibition

	Forward mode	Reverse mode
Transport mode		
Physiological function	Ca^{2+} extrusion after Ca^{2+} mobilization	Ca^{2+} entry under special conditions ($[Na^+]_i$ accumulation and depolarization)
Effect of NCX inhibition	Increase $[Ca^{2+}]_i$ Inhibit I_{ti}	Lower $[Ca^{2+}]_i$ Inhibit Ca^{2+} overload
Efficacy of NCX inhibition	Cardiotonic action Anti-arrhythmic action (against delayed after-depolarization) Hypertensive action	Anti-ischemic action (against ischemia/reperfusion injury) Digitalis detoxication Anti-hypertensive action (against salt-sensitive hypertension)

(Table 1). The coupling ratio is $3Na^+ : 1Ca^{2+}$, so the net transport of Ca^{2+} by NCX results in net charge transfer. NCX is therefore driven by the membrane potential (V_M) as well as the Na^+ and Ca^{2+} concentration gradients. 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. 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 (Table 1).

Mammalian NCX forms a multigene family encompassing three exchangers : NCX1, NCX2, and NCX3.[5)6)] NCX1 is widely expressed in the heart, kidney, brain, arteries and other organs, whereas NCX2 and NCX3 expression is limited mainly to the brain and skeletal muscle. At the transcriptional level, at least 12 NCX1 splicing variants are expressed in a tissue specific manner.

In cardiac muscle, NCX primarily pumps Ca^{2+} from inside to outside the cell during repolarization and diastole, which balances Ca^{2+} entry via L-type Ca^{2+} channels during cardiac excitation. NCX also mediates Ca^{2+} influx during the action potential upstroke, and helps maintain elevated $[Ca^{2+}]_i$ during the action potential plateau and systole. Cardiac NCX1 is co-localized with Na^+, K^+-ATPase and $InsP_3$ receptor through the association with ankyrin-B in cardiac transverse tubules. This ankyrin-B-based protein complex is likely to contribute to "the regulatory system of the SR Ca^{2+} contents".[7)] In contrast, dihydropyridine receptor, ryanodine receptor and junctophilin-2 (JP-2) are co-localized in the distinct microdomain of transverse tubules. This JP-2-based protein complex functions as a "regulatory system for the Ca^{2+} release from SR".[8)] Interestingly, the loss of function mutation in either ankyrin-B or JP-2 causes arrhythmia and contractile dysfunction.[7)8)]

In vascular smooth muscle, the NCX, like Ca^{2+}-ATPases, is thought to contribute to Ca^{2+} extrusion from the cytosol in the relaxation process,[5)6)] but there is little information about vascular NCX in comparison to cardiac NCX. The immunocytochemical staining of vascular smooth muscle cells indicated that the NCX1 is localized in the plasma membrane microdomain which are adjacent to the junctional SR.[6)] This particular localization suggests that NCX1 may thus play a role in regulating the Ca^{2+} content of the SR stores, thereby modulating Ca^{2+} handling and vasoconstriction.

The NCX1 cDNA encoded a protein of 970 amino acids with a molecular mass of 120 kDa. Recent topological analyses have revealed NCX1 protein to consist of nine transmembrane segments (TMs) with a large cytoplasmic loop.[10)] The protein contains a pair of internal repeat sequences, the α-1 and α-2 repeats, that form oppositely oriented re-entrant loops. These α-repeat regions may participate in ion transport(Fig. 1). The NCX1 activity is regulated by at least two types of I_1 and I_2 inactivation. Intracellular Ca^{2+} at the submicromolar level activates NCX activity by promoting

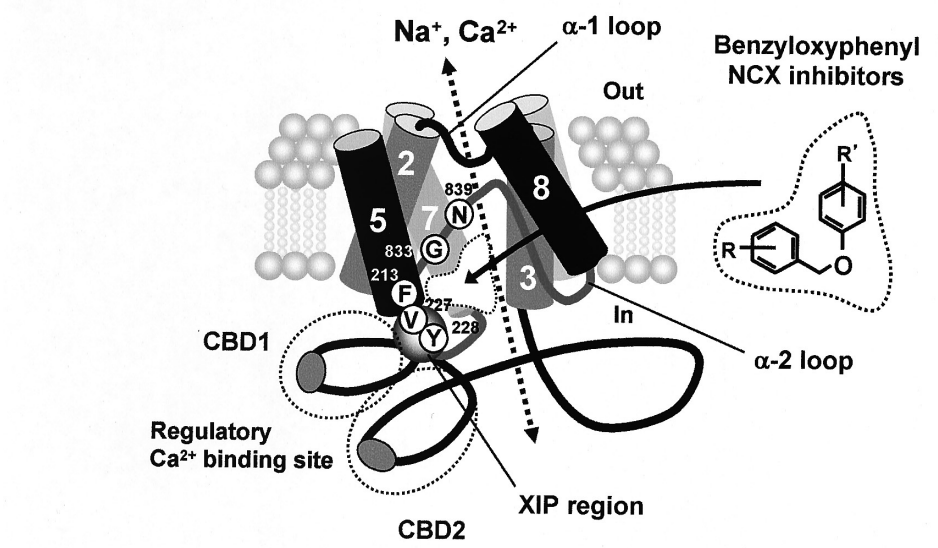


Fig. 1. Nine-transmembrane model of NCX1 and putative interaction domain of NCX inhibitors.
Benzyloxyphenyl NCX inhibitors may interact with a specific receptor site, leading to blocking ion transport pore(s). Transmembrane helices, partially illustrated, are indicated by cylinders. The amino acid residues of NCX1 whose mutation alters the sensitivities to benzyloxyphenyl derivatives are indicated. XIP: exchanger inhibitory peptide; CBD: Ca^{2+}-binding domain.

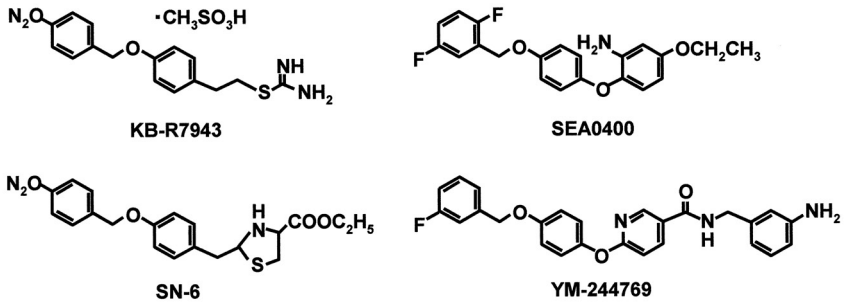


Fig. 2. Chemical structures of benzyloxyphenyl NCX inhibitors.

the recovery of the exchanger from the "I_2 inactivation state", whereas high Na^+_i retains the exchange by facilitating the entry of the exchanger into the "I_1 inactivation state" (Na^+-dependent inactivation). The large loop between TM5 and TM6 possesses the exchanger inhibitory peptide (XIP) region (Fig. 1). Phosphatidylinositol 4, 5-bisphosphate (PIP_2) removes the I_1 inactivation by direct interaction with the XIP domain,[11] while ATP depletion promotes I_1 inactivation. The Ca^{2+} regulatory Ca^{2+} binding domains, CBD_1 and CBD2 (see Fig. 1), which consist of acidic clusters, are also located in the intracellular loop. Recently, the crystal structures for CBD1 and CBD2 have been characterized, proposing the model of the NCX regulatory mechanism with Ca^{2+}-deoendent conformational changes.[12]

Properties of NCX inhibitors

Since NCX is an important transporter that regulates the intracellular Ca^{2+} concentration, NCX inhibitors have long been the favorite target of pharmaceutical enterprises that have sought to develop new calcium regulators.[5] In 1996, KB-R7943(Fig. 2), a prototype selective NCX inhibitor, was developed.[13] KB-R7943 suppresses the Ca^{2+} uptake through NCX in cultured cardiomyocytes

and vascular smooth muscle cells (IC_{50}=1.2－2.4 μM) and the outward exchange current (I_{NCX}) in cardiomyocytes (IC_{50}=0.32 μM). Subsequently, a new type of selective NCX inhibitor SEA0400 was developed.[14)15)] The inhibitory action of SEA0400 is about 80 times stronger than that of KB–R7943. Recently, SN–6, which is more specific than KB–R7943, and YM–244769, a potent NCX inhibitor with lower cell toxicity, were developed from KB–R7943 and SEA0400 derivatives, respectively.[16)17)]

These benzyloxyphenyl NCX inhibitors have different isoform selectivities.[5)] KB–R7943 and YM－244769 are more inhibitory to NCX3 than to NCX1 or NCX2. On the other hand, SEA0400 and SN–6 predominantly blocks NCX1. Recent site－directed mutagenesis have revealed the important amino acids (Phe–213, Val–227, Tyr–228, Gly–833 and Asn–839) responsible for inhibition by benzyloxyphenyl derivatives.[5)] Therefore, as shown in Fig. 1, these inhibitors probably interact with a specific receptor site, thus leading to blocking of the ion transport pathway (pores) formed within the membrane regions.

Intriguingly, benzyloxyphenyl inhibitors block the reverse mode of NCX1 much more effectively than the forward mode under unidirectional ionic conditions. Recent mutational and electrophysiological analyses provide an explanation for the reverse mode–selectivity of benzyloxyphenyl derivatives. Surprisingly, the inhibitory potency of NCX inhibitors is directly coupled to the rate of I_1 inactivation (i.e., Na^+_i－dependent inactivation). Under unidirectional ionic conditions, the reverse mode is induced when $[Na^+]_i$ is high, whereas the forward mode is generated when $[Na^+]_i$ is reduced. NCX1 molecules thus tend to undergo I_1 inactivation under the experimental conditions employed for studying the reverse mode, thereby suggesting an apparent, but not substantial, reverse mode selectivity.

Ischemia/Reperfusion Injury

Recent reports have suggested that NCX inhibitors efficiently prevent myocardial ischemia/reperfusion injury. KB－R7943 and SEA0400 strongly suppress Ca^{2+} overload, hypercontracture, and cell damage induced by the Ca^{2+} paradox (the sudden and massive damage following the transient depletion and repletion of Ca^{2+}) and hypoxia (anoxia)/reoxygenation in cardiomyocytes. KB–R7943 and SEA0400 also significantly prevent myocardial ischemia/reperfusion injury (contractile dysfunction) in the isolated perfused hearts treated with the inhibitors either before or after ischemia.[5)] Recently, genetic manipulations in cells and mice have provided compelling evidence that NCX1 inhibition protects against myocardial reperfusion injury. Heterozygous NCX1－knockout mice in which the NCX1 function is reduced by half are resistant to myocardial reperfusion injury.[18)] Conversely, NCX1–overexpressing hearts from transgenic mice are hypersensitive to reperfusion injury.[5)] These findings support the evidence that NCX1 mediates the Ca^{2+} entry and overload primarily during reperfusion as a result of Na^+ gain (perhaps due to Na^+ pump inhibition and/or acidification－induced Na^+/H^+ exchange) (Fig. 3). Taken together, the above evidence suggests that the inhibition of Ca^{2+} overload by NCX inhibitor may have a therapeutic potential for preventing reperfusion injury, myocardial stunning, and arrhythmias.

In renal ischemia/reperfusion, Ca^{2+} overload is thought to be an important factor for renal damage. In fact, KB－R7943 and SEA0400 prevent the renal injury induced by ischemia/reperfusion.[19)20)] We also observed that the ischemia/reperfusion–induced renal dysfunction and histological damage were markedly moderate in NCX1－knockout mice.[19)] Moreover, NCX inhibitors ameliorated the hypoxia/reoxygenation－induced injury in LLC–PK_1 cells, derived from proximal tubules.[15)16)] These findings suggest that Ca^{2+} overload via NCX1 plays an important role in renal reperfusion injury, and NCX inhibitors could therefore be a beneficial remedy for renal reperfusion injury.

Heart Failure

Ventricular arrhythmias are a critical cause of sudden death in patients suffering from heart failure. Several reports suggest that cardiac NCX1 is up–regulated in animal models of heart

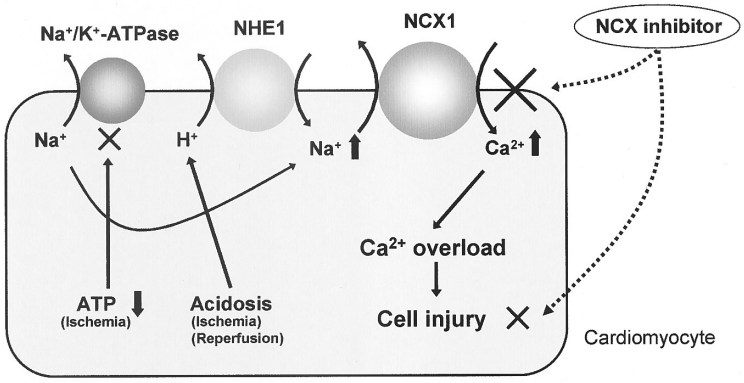


Fig. 3. Protective mechanism of NCX inhibitors in cardiac ischemia/reperfusion injury.
Myocardial ischemia (hypoxia) leads to various changes in cell metabolism and energy production, resulting in a decrease in pH_i (acidosis) and ATP production. During reperfusion, the Na^+/H^+ exchanger type 1 (NHE1) accelerates H^+ extrusion from myocytes in exchange for Na^+. In addition, the depleted ATP inhibits Na^+/K^+-ATPase activity, finally leading to a large increase in $[Na^+]_i$. Subsequently, accumulated Na^+ changes the reversal potential of NCX1 and then favors Ca^{2+} overloading by the reverse mode during reperfusion. NCX inhibitors can block this Ca^{2+} overload and prevent the following cell injury.

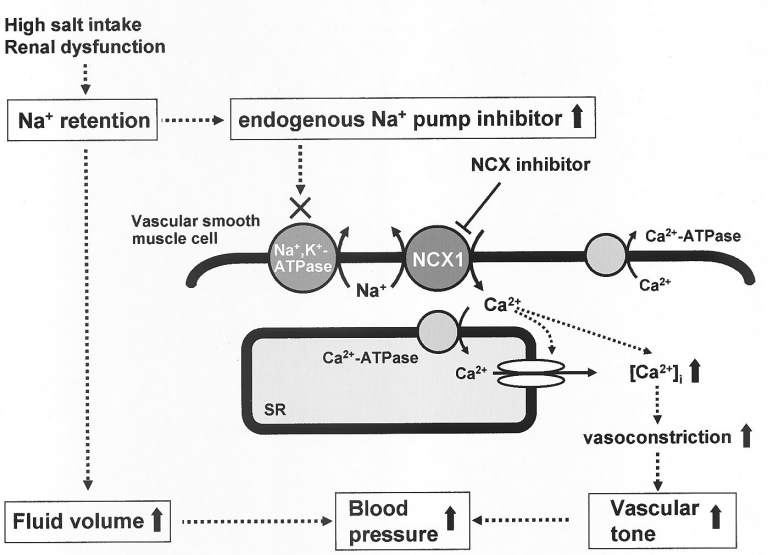


Fig. 4. Antihypertensive mechanism of NCX inhibitor on salt-sensitive hypertension.
High salt intake (or Na^+ retention) causes the levels of endogenous cardiotonic steroids (CTS), that inhibit Na^+/K^+-ATPase ($\alpha 2$ subtype), to rise in the plasma (although Na^+ retention also increases plasma volume, resulting in elevating blood pressure). This results in the increase in subplasma membrane $[Na^+]$ of arterial smooth muscle. The restricted $[Na^+]$ accumulation elevates $[Ca^{2+}]_i$ by vascular NCX1 isoform-mediated Ca^{2+} entry. This enhances arterial tone and causes hypertension. NCX inhibitors can block this Ca^{2+} entry and exert an antihypertensive effect in salt-sensitive hypertension. SR, sarcoplasmic reticulum.

failure and the human heart with end-stage failure.[6] Stimulation of the forward mode of NCX1 would reduce $[Ca^{2+}]_i$ and unload SR Ca^{2+}, leading to contractile dysfunction ; this mode also initiates an arrhythmogenic I_{ti}, which is related to delayed after depolarizations (DADs) and triggered activity. Stimulation of the reverse mode of NCX1 would increase SR Ca^{2+} (over)loading and contractility with the risk of impairing diastolic function. Under these pathological conditions, the inhibition of NCX1 may thus be cardioprotective by normalizing both $[Ca^{2+}]_i$ and SR Ca^{2+} loading and by blocking I_{ti}. In reality, however, alterations of the NCX1 expression and activity are complicated in heart failure models and even in the failing human heart,[6] and are likely attributable to several factors, including etiological causes, compensatory regulations, diastolic or systolic performance, hemodymanics, neuronal or humoral regulation, and the failing stage.

A recent study in canine tachycardiac pacing-induced failing hearts suggests that the partial inhibition of NCX1 by XIP peptides improves the contractility in the failing heart cell.[21] Quite interestingly, NCX1 inhibition normalizes contractile Ca^{2+} cycling by indirectly activating the Ca^{2+}-dependent SR Ca^{2+} uptake. The reverse has also been reported. The overexpression of NCX1 in rabbit myocytes by adenovirus vectors decreases SR Ca^{2+} loading and cell shortening,[22] which are likely those observed in the human failing heart. Furthermore, the homozygous overexpression of NCX1 in transgenic mice leads to defective excitation-contraction coupling leading to cardiac hypertrophy,[23] although heterozygous overexpressors do not display a heart failing phenotype.[24][25] These results support the idea that NCX1 inhibition has the potential to be an effective therapeutic treatment for improving contractile Ca^{2+} cycling in heart failure. However, it is not currently known whether benzyloxyphenyl NCX inhibitors ameliorate contractile dysfunction in heart failure models.

Salt-dependent Hypertension

Hypertension is a leading risk factor for death due to stroke, myocardial infarction, and end-stage renal failure. The critical importance of an excess salt intake in the pathogenesis of hypertension is widely recognized.[26] However, the mechanism by which excess salt intake elevates blood pressure is still not well known, although cardiotonic steroids (CTS), such as endogenous ouabain and other steroids, have been proposed as candidate intermediaries.[27] Quite recently, it has been shown that SEA0400 lowers the arterial blood pressure in salt- or ouabain-dependent hypertensive rat models ; but, interestingly, not in normotensive rats or other types of hypertensive rats.[28] This antihypertensive profile is pretty different from those of Ca^{2+} channel blockers, which lower the blood pressure in almost all hypertensive models. SEA0400 may exhibit peripheral vasodilation by preventing CTS-induced vasoconstriction in salt-dependent hypertensive animals.[28] Figure 4 shows the proposed pathway responsible for salt-dependent hypertension. In humans and animals, a high salt intake (or Na^+ retention) causes the endogenous CTS levels to rise in the plasma.[29] This results in an increase in the subplasma membrane $[Na^+]$ of arterial smooth muscle by inhibiting Na^+/K^+-ATPase ($\alpha 2$ subtype).[30] The restricted $[Na^+]$ accumulation elevates $[Ca^{2+}]_i$ by vascular NCX1 isoform (i.e., NCX1.3)-mediated Ca^{2+} entry. This enhances the arterial tone and causes hypertension. SEA0400 blocks this Ca^{2+} entry, and exerts an antihypertensive effect in salt-sensitive hypertension — an idea supported by experiments with genetically engineered mice. Heterozygous NCX1 knockout mice are resistant to developing salt-dependent hypertension, whereas transgenic mice with a vascular specific expression of NCX1 readily develop hypertension after high salt intake.[24] Intriguingly, SEA0400 suppresses the blood pressure in salt-dependent hypertensive mice expressing wild-type NCX1, but not in SEA0400-insensitive NCX1 mutants, thus suggesting that SEA0400 exerts its antihypertensive effect by blocking NCX1 overexpressed in arterial smooth muscle. Vascular NCX1 thus seems to be a new therapeutic or diagnostic target for salt-sensitive hypertension. NCX inhibitors might therefore lower salt-dependent hypertension without a tightly controlled low-salt diet.

Conclusion

Recent studies using physiological or pharmacological techniques and genetically engineered mice have suggested the functional and etiological implications of NCX1 in cardiac and vascular smooth muscle cells. NCX1 is multi-regulated by intracellular Na^+ and Ca^{2+}, PIP_2, and protein kinases, and controls $[Ca^{2+}]_i$ and SR Ca^{2+} content mainly by Ca^{2+} extrusion in myocytes during the contraction-relaxation cycle. An alteration of NCX1 activity produces abnormalities in myocyte Ca^{2+} regulation, thus resulting in mechanical dysfunction and electrical instability associated with myocardial ischemia/reperfusion injury, heart failure, arrhythmia, and hypertension. During myocardial reperfusion, NCX1 underlies the Ca^{2+} overload, thus resulting in cell injury. In heart failure, up-regulated NCX1 produces contractile dysfunction by reducing $[Ca^{2+}]_i$ and SR Ca^{2+} content. Ischemia and digitalis toxicity induce SR Ca^{2+} overload, thus leading to arrhythmogenic I_{ti} by NCX1, which is related to DADs and triggered activity. In addition, salt-sensitive hypertension is triggered by Ca^{2+} entry via NCX1 in arterial smooth muscle. Benzyloxyphenyl NCX inhibitors may block these forms of abnormal Ca^{2+} transport by NCX1 and may then improve related morbid conditions. Several experiments suggest that NCX inhibitors have a therapeutic potential for myocardial ischemia/reperfusion injury, arrhythmia and salt-dependent hypertension, but their effects on heart failure and reperfusion-induced arrhythmia are not clear. Treatment with NCX inhibitors is thus expected to be an innovative therapeutic approach for several cardiovascular diseases.

Acknowledgement

This review was supported by a Grant-in-Aid for Young Scientists (B) (20790216).

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(Received on July 10, 2008,
Accepted on September 5, 2008)