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Pharmacology of Brain Na^+/Ca^{2+} Exchanger: From Molecular Biology to Therapeutic Perspectives

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Abstract*——In the last two decades, there has been a growing interest in unraveling the role that the Na^+/Ca^{2+} exchanger (NCX) plays in the function and regulation of several cellular activities. Molecular biology, electrophysiology, genetically modified mice, and molecular pharmacology have helped to delve deeper and more successfully into the physiological and pathophysiological role of this exchanger. In fact, this nine-transmembrane protein, widely distributed in the brain and in the heart, works in a bidirectional way. Specifically, when it operates in the forward mode of operation, it couples the extrusion of one Ca^{2+} ion with the influx of three Na^+ ions. In contrast, when it operates in the reverse mode of operation, while three Na^+ ions are extruded, one Ca^{2+} enters into the cells. Different isoforms of NCX, named NCX1, NCX2, and NCX3, have been described in the brain, whereas only one, NCX1, has been found in the heart. The hypothesis that NCX can play a relevant role in several pathophysiological conditions, including hypoxia-anoxia, white matter degeneration after spinal cord injury, brain trauma and optical nerve injury, neuronal apoptosis, brain aging, and Alzheimer's disease, stems from the observation that NCX, in parallel with selective ion channels and ATP-dependent pumps, is efficient at maintaining intracellular Ca^{2+} and Na^+ homeostasis. In conclusion, although studies concerning the involvement of NCX in the pathological mechanisms underlying brain injury during neurodegenerative diseases started later than those related to heart disease, the availability of pharmacological agents able to selectively modulate each NCX subtype activity and antiporter mode of operation will provide a better understanding of its pathophysiological role and, consequently, more promising approaches to treat these neurological disorders.

I. Introduction

In the last two decades, there has been a growing interest in unraveling the role that the Na^+/Ca^{2+} exchanger (NCX[1]) plays in the function and regulation of several cellular activities. Biochemistry, molecular biology, electrophysiology, genetically modified mice, and molecular pharmacology have helped to delve deeper and more successfully into the physiological and pathophysiological role of this exchanger, as witnessed by more than 2000 papers written on this issue and published in the most internationally qualified journals (Philipson et al., 2002).

NCX was discovered and characterized in the late 1960s, when Baker et al. in the UK (Baker and Blaustein, 1968; Baker et al., 1969), Reuter and Seitz in Germany and Switzerland (Reuter and Seitz, 1968), and Martin and De Luca in the United States (Martin and De Luca, 1969) realized the presence of a countertransport mechanism that exchanged Na^+ and Ca^{2+} ions

[1]Abbreviations: NCX, Na^+/Ca^{2+} exchanger; TMS, transmembrane segment; PIP2, phosphatidylinositol 4,5 bisphosphate; PKC, protein kinase C; PKA, protein kinase A; PA, phosphoarginine; XIP, exchange inhibitory peptide; LTP, long-term potentiation; DA, dopamine; DTT, dithiothreitol; GSH, glutathione reduced; GSSG, glutathione oxidized; ROS, reactive oxygen species; SNP, sodium nitroprusside; NOS, nitric oxide synthase; VGCC, voltage-gated calcium channels; NMDA, *N*-methyl-D-aspartate; FRCRCFa, Phe-Arg-Cys-Arg-Cys-Phe; FMRFa, Phe-Met-Arg-Phe; pMCAO, permanent middle cerebral artery occlusion; DMB, 3′,4′-dimethylbenzamyl; CB-DMB, [*N*-(4-chlorobenzyl)]2,4-dimethylbenzamyl; KB-R7943, 2-[2-[4-(4-nitrobenzyloxy) phenyl] ethyl]isothiourea methanesulfonate; KB-R7898, 2-[2-[4-(3,4-dichlorobenzyloxy) phenyl] ethyl]isothiourea methanesulfonate; SEA0400, 2-[4-[(2,5-difluorophenyl)methoxyl-phenoxy]-5-ethoxyaniline; SM-15811, 4-phenyl-3-[(*N*-benzyl)-4-pyperidine]-3,4-dihydro-2(1*H*)-quinazolinone; A-β, amyloid-β.

across the plasma membrane of different excitable and nonexcitable cells. However, the most crucial advancement in NCX research was made in 1988 (Philipson et al., 1988) and 1990 (Nicoll et al., 1990), when Philipson and his colleagues purified and cloned the first isoform of this antiporter: NCX1. Remarkably, four years later, the same group of investigators cloned NCX2 (Li et al., 1994) and NCX3 (Nicoll et al., 1996b), two isoforms selectively expressed in the brain (Lee et al., 1994) and in the skeletal muscle (Nicoll et al., 1996b). Finally, in 1999, Philipson proposed a new topological model of the NCX1 exchanger (Nicoll et al., 1999) (Table 1).

The regulation of intracellular concentrations of Ca^{2+} and Na^+ ions in excitable cells is a relevant physiological phenomenon that maintains cellular homeostasis. In fact, although cytosolic Ca^{2+} ions play a key role in signaling at the cytosolic and nuclear levels (Choi, 1988), Na^+ ions play a major role in regulating cellular osmolarity, in inducing action potential (Lipton, 1999), and in transduction signaling (Yu et al., 1997). Indeed, the Na^+/Ca^{2+} exchanger, in parallel with selective ion channels and ATP-dependent pumps, maintains the physiological cytosolic concentrations of these ions (Blaustein and Lederer, 1999). In particular, the Na^+/Ca^{2+} exchanger can mediate Ca^{2+} and Na^+ fluxes across the synaptic plasma membrane in a bidirectional way (Blaustein and Lederer, 1999; Philipson and Nicoll, 2000) (Table 2). The stoichiometry of NCX is generally accepted to be three Na^+ ions/one Ca^{2+} ion; however, more recently, in addition to the major 3:1 transport mode, it has been demonstrated that ion flux ratio can vary from 1:1 to a maximum of 4:1, depending on the intracellular concentration of Na^+ and Ca^{2+} ions (Fujioka et al., 2000; Hang and Hilgemann, 2004). In resting excitable cells, when intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) rise and the cells require the return of $[Ca^{2+}]_i$ to resting levels (Carafoli, 1985), this exchange transport mechanism couples the uphill extrusion of Ca^{2+} to the influx of Na^+ ions into the cells down their electrochemical gradient. This mode of operation, defined as forward mode (Blaustein and Santiago, 1977), keeps the 10^4-fold difference in Ca^{2+} concentrations across the cell membrane. In contrast, in other physiological circumstances, when intracellular Na^+ concentrations ($[Na^+]_i$) rise or membrane depolarization occurs, thus reducing the transmembrane Na^+ electrochemical gradient, the Na^+/Ca^{2+} exchanger mediates the extrusion of $[Na^+]_i$ and the influx of Ca^{2+} ions. This mode of operation is defined as reverse way (Baker and McNaughton, 1976; DiPolo, 1979) (Fig. 1; Table 2).

TABLE 2
Functional properties of the Na^+/Ca^{2+} exchanger

Transport mode	Bidirectional: Forward: Ca^{2+}; Efflux Reverse: Ca^{2+} Influx
Coupling ratio	3 Na^+/1 Ca^{2+} or 4 Na^+/1 Ca^{2+}
Reaction mechanism	Consecutive, Ca^{2+} first
Electrogenity	Na^+ translocation is electrogenic. Ca^{2+} movement is electroneutral.
Gating properties	Voltage dependency

Over the last five years, some exhaustive and basic reviews addressing the regulatory functions of the NCX have been published (Blaustein and Lederer, 1999; Egger and Niggli, 1999; Philipson and Nicoll, 2000; DiPolo and Beaugé, 2002), whereas reviews covering the pharmacological aspects of its modulation have been published only in 1997 and 1999 (Matsuda et al., 1997; Shigekawa and Iwamoto, 2001). Considering that a comprehensive review on the pharmacological modulation of brain NCX is still lacking, the present paper will particularly address the physiology and the molecular pharmacology of NCX in the brain and the possible therapeutic implications of its modulation.

II. Molecular Biology of Na^+/Ca^{2+} Exchanger

The Na^+/Ca^{2+} exchanger belongs to the superfamily of membrane proteins comprising the following members: 1) the NCX family, which exchanges three Na^+ ions for one Ca^{2+} ion or four Na^+ ions for one Ca^{2+} ion depending on $[Na^+]_i$ and $[Ca^{2+}]_i$ (Reeves and Hale, 1984; Fujioka et al., 2000; Hang and Hilgemann, 2004); 2) the Na^+/Ca^{2+} exchanger K^+-dependent family, which exchanges four Na^+ ions for one Ca^{2+} plus one K^+ ion (Schnetkamp et al., 1989; Lytton et al., 2002); 3) the bacterial family which probably promotes Ca^{2+}/H^+ exchange (Cunningham and Fink, 1996); 4) the nonbacterial Ca^{2+}/H^+ exchange family, which is also the Ca^{2+} exchanger of yeast vacuoles (Pozos et al., 1996); and 5) the Mg^{2+}/H^+ exchanger, an electrogenic exchanger of protons with Mg^{2+} and Zn^{2+} ions (Shaul et al., 1999). These membrane proteins are all peculiarly characterized by the presence of α-repeats, the regions involved in ion translocation.

Regarding the NCX family, three dominant genes coding for the three different NCX1 (Nicoll et al., 1990), NCX2 (Li et al., 1994), and NCX3 (Nicoll et al., 1996b)

TABLE 1
Historical steps in the characterization of the Na^+/Ca^{2+} exchanger

Year	
1968	Description of the exchanger activity in squid axon (Baker et al., 1968)
1988	Purification of the cardiac exchanger (Philipson and Nicoll, 1988)
1990	Cloning of NCX1 (Nicoll et al., 1990)
1994–1996	Cloning of brain specific isoforms NCX2 (Li et al., 1994) and NCX3 (Nicoll et al., 1996)
1997	NCX isoforms are differentially expressed in several tissues (Quednau et al., 1997)
1999	Proposal for a new topological model for NCX1 (Nicoll et al., 1999)

proteins have been identified in mammals. These three genes appear to be dispersed, since NCX1, NCX2, and NCX3 have been mapped in mouse chromosomes 17, 7, and 12, respectively (Nicoll et al., 1996b). At the post-transcriptional level, at least 12 NCX1 and 3 NCX3 proteins are generated through an alternative splicing of the primary nuclear transcripts (Kofuji et al., 1994). These variants arise from a region of the large intracellular f loop, are encoded by six small exons defined A to F, and are used in different combinations in a tissue-specific manner (Nakasaki et al., 1993; Lee et al., 1994). To maintain an open reading frame, all splice variants must include either exon A or B, which are mutually exclusive (Quednau et al., 1997). Excitable tissues, such as those of the brain and heart, are usually characterized by the presence of exon A, whereas kidney, stomach, and skeletal muscle tissues comprise NCX with exon B (Quednau et al., 1997).

NCX1 is composed of 938 amino acids, in the canine heart, having a theoretical molecular mass of 120 kDa and containing nine transmembrane segments (TMS). NCX1 amino terminus is located in the extracellular space, whereas the carboxyl terminus is located intracellularly (Fig. 2). The nine transmembrane segments

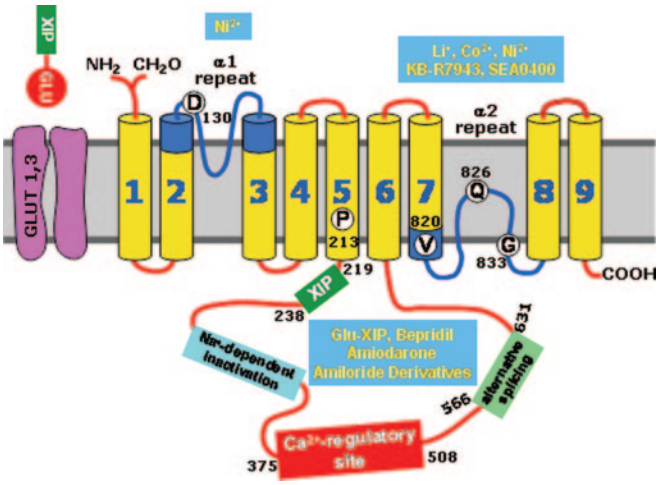

FIG. 2. Molecular pharmacology of NCX. Putative sites of the molecular action of drugs interfering with NCX activity.

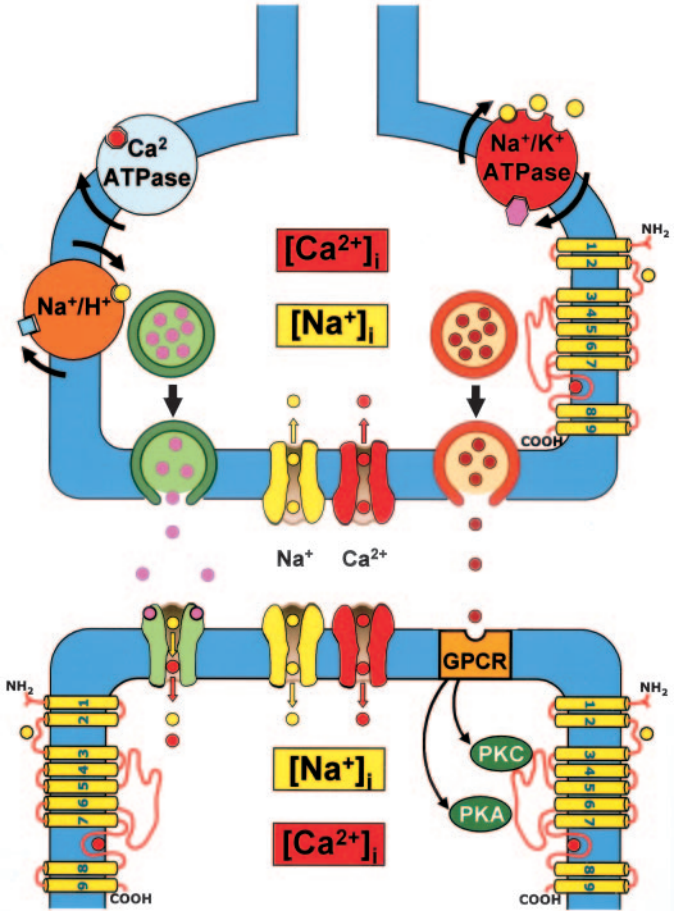

FIG. 1. Schematic diagram of NCX and other ionic transporter mechanisms involved in $[Na^+]_i$ and $[Ca^{2+}]_i$ homeostasis in a pre- and postsynaptic structure.

can be divided into an N-terminal hydrophobic domain, composed of the first five TMS (1–5), and into a C-terminal hydrophobic domain, composed of the last four TMS (6–9). These two hydrophobic domains are important for the binding and the transport of ions. The first (1–5) TMS are separated from the last four (6–9) TMS through a large hydrophilic intracellular loop of 550 amino acids, named the f loop (Nicoll et al., 1999). Although the f loop is not implicated in Na^+ and Ca^{2+} translocation, it is responsible for the regulation of NCX activity elicited by several cytoplasmic messengers and transductional mechanisms, such as Ca^{2+} and Na^+ ions, NO, phosphatidylinositol 4,5 bisphosphate (PIP2), protein kinase C (PKC), protein kinase A (PKA), phosphoarginine (PA), and ATP (Table 3). In the center of the f loop, a region of approximately 130 amino acids in length (371–508 amino acids) has been reported to exert a Ca^{2+} regulatory function. This region is characterized by a pair of three aspartyl residues and by a group of four cysteines (Nicoll et al., 1999; Qiu et al., 2001). At the N-terminal end of the f loop near the membrane lipid interface, an autoinhibitory domain, rich in both basic and hydrophobic residues and consisting of a 20-amino acid sequence (219–238), named exchange inhibitory peptide (XIP) (Matsuoka et al., 1997), has been identified. The f loop is also characterized by alternative splicing sites named β1-repeat and β2-repeat. These β-repeats are characterized by similar regions comprising 60 to 70 amino acids for which no functional role has yet been proposed (Hilgemann, 1990).

The NCX protein amino acid sequence found between TMS2 and TMS3 is called α-1 repeat, whereas the one found between TMS7 and TMS8 is named α-2 repeat. Both regions, α-1 and α-2 repeats, are located on the opposite site of the membrane and include two segments composed of 12 and 9 highly conserved residues separated by a nonconserved segment of 18 to 20 amino acids (Nicoll et al., 2002). Since the putative α-helices of the

TABLE 3
Regulatory mechanisms of Na^+/Ca^{2+} exchanger activity

$[Ca^{2+}]_i$	Activation of NCX with low affinity: 0.1–0.3 μM; high affinity: 20–50 nM (Di Polo, 1979)
$[Na^+]_i$	$[Na^+]_i$-dependent inactivation of outward Ca^{2+} currents (Hilgemann, 1990)
$[H^+]_i$	Inhibition of NCX activity (Doering and Lederer, 1993)
NO	Stimulates NCX activity via cGMP (Asano et al., 1995)
Redox status	Oxidizing and reducing agents stimulate activity (Reeves at al., 1986; Amoroso et al., 2000; Santacruz-Toloza et al., 2000)
PIP2	Elimination of the $[Na^+]_i$-dependent inactivation (Hilgemann and Ball, 1996)
ATP	Increased $[Na^+]_e$-dependent Ca^{2+} efflux (Blaustein and Santiago, 1977)
PKC	Increases the affinity of $Ca^{2+}{}_i$ and $Na^+{}_e$ for NCX (Caroni and Carafoli, 1983; Iwamoto et al., 1995, 1996a; Shulze et al., 2003)
PKA	Increased activity in exon A-carrying splicing isoforms of NCX (Caroni and Carafoli, 1983; Shulze et al., 2003)

α-repeats are amphipathic, the hydrophilic faces of these helices may form a portion of the ion translocation pathway (Nicoll et al., 1996a). Interestingly, the α-repeats form re-entrant loops of NCX1 that interact within the protein (Iwamoto et al., 2000; Qiu et al., 2001). Interestingly, the center of α2 repeat possesses a GIG sequence similar to the GYG sequence present in the P loop of K^+ channels (Philipson and Nicoll, 2000). With electrophoretic gels and under nonreducing conditions, NCX1 migrates as a 120- and a 70-kDa band. The 120-kDa band represents the native protein, and the 70-kDa protein is a proteolytic fragment, which includes a large part of the f loop and retains an Na^+/Ca^{2+} exchange activity (Saba et al., 1999; Van Eylen et al., 2001b).

Interestingly, NCX2 and NCX3 have been found only in the brain and in the skeletal muscle. These two gene products consist of 921 and 927 amino acids and are characterized by molecular masses of 102 and 105 KDa, respectively. In addition, NCX2 displays a 65% sequence identity with NCX1, whereas NCX3 possesses a 73% sequence identity with NCX1 and 75% sequence identity with NCX2 (Nicoll et al., 1996b). All three NCX gene products share the same membrane topology.

III. Brain Distribution of Na^+/Ca^{2+} Exchanger Isoforms

The NCX1 gene is expressed in several tissues, including brain, heart, skeletal muscle, smooth muscle, kidney, eye, secretory, and blood cells, whereas transcripts encoded by the NCX2 and NCX3 genes have been found exclusively in neuronal and skeletal muscle tissues (Lee et al., 1994). In addition, NCX1 and NCX3 give rise to several splicing variants that appear to be selectively expressed in different regions and cellular populations of the brain (Quednau et al., 1997; Yu and Colvin, 1997).

A. Cerebral Cortex

NCX1 isoforms are intensively expressed in the pyramidal neurons of layers III and V within the molecular layer of the cerebral motor cortex. This area, which contains the terminal dendritic field of the pyramidal cells, displays a more intense NCX1 immunoreactivity than that of NCX2. In contrast, the somatosensory cortical area seems to express preferentially NCX2 transcripts. Such anatomical distribution reveals that the upper neurons of the motor system and the terminal neurons of the somatosensory system preferentially express distinct NCX isoforms (Canitano et al., 2002; Papa et al., 2003).

B. Hippocampus

Within the hippocampus, the transcripts of the three NCX isoforms display an intense labeling of most neuronal populations. In particular, high levels of the three NCX genes have been detected in the granular cell layers of the dentate gyrus and in the pyramidal cells of CA1, CA2, CA3, and CA4 subfields (Papa et al., 2003). The three NCX protein isoforms also display high levels of expression within the hippocampus. Thus, in the orient and radiatum layers of the CA1, NCX3 protein is more intense than NCX1 and NCX2. NCX1 protein expression is particularly intense in the granule cell layer and in the hilum of the dentate gyrus, which constitutes the terminal field of the perforant pathway, the major excitatory input to the hippocampus originating from the enthorinal cortex. In the CA3 area, the NCX1 and NCX3 genes of the mossy fibers projecting from the granule cells located in the dentate gyrus are more intense than those of NCX2. This peculiar distribution suggests that distinct Na^+/Ca^{2+} exchanger isoforms may play a crucial role in controlling the intracellular Na^+ and Ca^{2+} homeostasis of the major afferent, intrinsic, and efferent hippocampal projections. Such circuitries are crucial for the synaptic plasticity phenomena, such as those involved in long-term potentiation (LTP) and long-term depression (Madison et al., 1991). Furthermore, NCX plays a major role in hippocampal ischemia. In fact, it has been demonstrated that under anoxic/glucopenic conditions, the inhibition of the Na^+/Ca^{2+} exchanger influences the hippocampal electrophysiological responses (Schroder et al., 1999) and the excitatory neurotransmitter release (Amoroso et al., 1993; Trudeau et al., 1999).

C. Mesencephalon and Basal Ganglia

NCX isoforms are also expressed in crucial areas of the extrapyramidal control of motor coordination. In fact, NCX1 mRNA can be detected in the substantia

nigra pars compacta, in which dopaminergic cell bodies are localized; the NCX1 protein isoform is present in the striatum, in which the terminal projection fields of dopaminergic nigrostriatal neurons are found (Canitano et al., 2002; Papa et al., 2003).

Interestingly, both the transcript and the protein, encoded by the three NCX genes, are abundantly expressed in the nucleus accumbens (Canitano et al., 2002; Papa et al., 2003), a brain region involved in the motivational control of motor coordination (Canitano et al., 2002; Papa et al., 2003).

D. Cerebellum

The analysis of the expression of Na^+/Ca^{2+} exchanger transcripts and proteins in the cerebellum reveals their presence in the afferent projections and in the intrinsic neurons of this crucial brain region (Canitano et al., 2002; Papa et al., 2003). In particular, NCX1 protein is expressed in the excitatory mossy fibers that originate in the extracerebellar structures and that branch off to the granule cell layer, a site where the glomerular structure is formed by the mossy fiber terminals, the granule cell dendrites, and the Golgi cell axons (Canitano et al., 2002; Papa et al., 2003). Consistent with this regional brain distribution, functional studies, performed on cerebellar granule cells, suggest that $[Ca^{2+}]_i$ increase, induced by glutamate, may occur through the Na^+/Ca^{2+} exchanger operating in the reverse mode (Kiedrowski et al., 1994).

E. Median Eminence

In the median eminence, a region devoid of neuronal perykaria and provided with nerve endings originating from neurons located in several hypothalamic regions, positive immunostaining for NCX1, NCX2, and NCX3 isoforms has been demonstrated (Papa et al., 2003). Since median eminence plays a relevant role in controlling dopamine (DA) and anterior pituitary hormone release (Annunziato et al., 1979), the presence of NCX1, NCX2, and NCX3 in this area suggests that NCX may exert an important function in the modulation of DA release and anterior pituitary hormone secretion.

The distribution of the three NCX isoforms in the mammalian brain may, therefore, give useful insights into unraveling the physiological and, possibly, the pathophysiological role played by the different NCX isoforms in the regulation of neuronal function.

IV. Regulation of Na^+/Ca^{2+} Exchanger Activity

Several factors are involved in the regulation of Na^+/Ca^{2+} exchanger activity: the two transported ions, Na^+ and Ca^{2+}; the intracellular pH; metabolic related compounds, ATP, PA, PIP2, PKA, and PKC; redox agents, hydroxyl radicals, H_2O_2, dithiothreitol (DTT), $O_2^{-}\cdot$, Fe^{3+}, Fe^{2+}, Cu^{2+}, $OH^{\cdot}$, glutathione reduced (GSH), and glutathione oxidized (GSSG); and the gaseous mediator, NO (Table 3).

A. Intracellular Ca^{2+} Concentrations

The site level at which $[Ca^{2+}]_i$ regulates NCX activity is different from the one required for Ca^{2+} transport (Levitsky et al., 1994). In fact, submicromolar concentrations (0.1–0.3 μM) of intracellular Ca^{2+} are needed to activate the antiporter (DiPolo, 1979; Hilgemann et al., 1992). Indeed, the removal of intracellular Ca^{2+} ions completely blocks NCX activity (Philipson and Nicoll, 2000). This regulatory function of low micromolar Ca^{2+} is more evident when the Na^+/Ca^{2+} exchanger is working in the reverse mode. However, it is not completely clear how low μM Ca^{2+} can also regulate NCX when it operates in the forward mode (Matsuoka et al., 1995). The location of such regulatory site has been identified in the 134-amino acid-length region, situated in the center of the intracellular f loop. This region is characterized by a pair of three aspartyl residues and by a group of four cysteines (Matsuoka et al., 1995).

B. Intracellular Na^+ Concentrations

In addition to the submicromolar intracellular Ca^{2+} regulatory site, an increase in $[Na^+]_i$ can also regulate the Na^+/Ca^{2+} exchanger (Hilgemann, 1990). In particular, when intracellular Na^+ increases, it binds to the transport site of the exchanger molecule, and after an initial fast outward Na^+/Ca^{2+} current, an inactivation process occurs (Hilgemann et al., 1992). This inactivation process, very similar to the phenomenon occurring in voltage-dependent ionic channels, is named Na^+-dependent inactivation. The region of the intracellular f loop, in which this regulatory site is located, has been identified in a 20-amino acid portion of the N-terminal part of the loop termed XIP (Matsuoka et al., 1997). Studies in vitro have characterized a negatively charged region of the intracellular f loop (445–455 amino acids) of the NCX protein that is able to cross link with synthetic XIP, thus suggesting that this amino acid sequence constitutes the binding site of XIP (Hale et al., 1997). On the other hand, since deletion mutagenesis of amino acids 562 to 685 results in an exchange activity that is no longer regulated by XIP (Matsuoka et al., 1993), it is likely that XIP interacts with residues 445 to 455 and with another region of the f loop located between residues 562 and 685. Indeed, this region is believed to be a Na^+ regulatory site (Li et al., 1991). Regarding the mechanism by which XIP inhibits NCX activity, it has been proposed that when the XIP-binding site is ligand occupied, a conformational change is induced in the C-terminal portion of the f loop, thus resulting in the inhibition of the ion transport (Li et al., 1991). XIP is provided with relevant pharmacological implications. In fact, those exogenous peptides, having the same amino acid sequence as XIP, act as potent inhibitors of NCX activity (Li et al., 1991; Pignataro et

al., 2004b). Interestingly, Ca^{2+} ions, at low micromolar concentrations, binding its regulatory site, decrease the extent of this Na^+-dependent inactivation. In fact, mutations in the Ca^{2+} regulatory binding site alter the activation and inactivation kinetics of exchange currents by modulating Na^+-dependent inactivation (Matsuoka et al., 1995).

C. Intracellular H^+ Concentrations

H^+ strongly inhibits NCX activity under steady-state conditions (Table 3) (Doering and Lederer, 1993). Changes in intracellular pH values, as little as 0.4, can induce a 90% inhibition of NCX activity. Since this H^+ ion modulatory action is α-chymotrypsin sensitive, the action site of the proton can be attributed to the antiporter's hydrophilic intracellular loop (Espinosa-Tanguma et al., 1993). Intriguingly, such inhibitory action depends on the presence of intracellular Na^+ ions (Doering and Lederer, 1994). Hence, the action exerted by H^+ ions is pathophysiologically relevant with regards to brain and heart ischemia. In fact, when intracellular H^+ and Na^+ ion homeostasis is deregulated, the anoxic conditions resulting in these cells may selectively interfere with the activity of the different NCX gene products.

D. ATP, Protein Kinase A, Protein Kinase C, and Phosphatidylinositol 4,5 Bisphosphate

Acting as a phosphoryl donor molecule, ATP may increase the activity of the exchanger in a number of ways (Blaustein and Santiago, 1977). Firstly, ATP directly participates in the NCX molecule phosphorylation process by PKA and PKC (Caroni and Carafoli, 1983). Secondly, it increases PIP2 production (Hilgemann and Ball, 1996). Finally, by activating G-protein-coupled receptors, via endogenous and exogenous ligands, ATP can stimulate the activity of the Na^+/Ca^{2+} exchanger through the pathway involving PKC or PKA activation (DiPolo and Beaugé, 1998). The mechanism underlying the phosphorylating effect on the exchanger seems to be related to an increase in its affinity for both internal Ca^{2+} and external Na^+ and to a decrease in its inhibition by internal Na^+. Each of the NCX isoforms has distinctive putative phosphorylation sites, although their roles have not yet been elucidated (Linck et al., 1998). ATP cellular depletion inhibits NCX1 and NCX2 but does not affect NCX3 activity. The exchange activity of NCX1 and NCX3 is modestly increased by those agents that activate PKA and PKC (Linck et al., 1998). More recently, the mechanism by which PKA and PKC activate NCX has been clarified. In fact, it has been demonstrated that the regulation of PKA-induced phosphorylation is due to the existence of an NCX1 macromolecular complex that contains the kinase PKA holoenzyme. This holoenzyme consists of two PKA catalytic subunits and two identical PKA regulatory subunits (Schulze et al., 2003). Together with PKA, other critical regulatory enzymes are also associated with NCX1, including PKC and serine-threonine protein phosphatases, PP1 and PP2A (Schulze et al., 2003). Particularly a pathway involving PKC has been shown to stimulate NCX1 (Iwamoto et al., 1995, 1996a). In a more recent paper, it has been demonstrated that PKC-dependent regulation of NCX isoforms also involves NCX3 but not NCX2 (Iwamoto et al., 1998a). In the same paper, three phosphorylation sites in the NCX1 protein, Ser-249, Ser-250, and Ser-357, have been identified. Among these, Ser-250 is the amino acid that is predominantly phosphorylated (Iwamoto et al., 1998a).

The other mechanism by which ATP can activate NCX occurs through PIP2 production. This mechanism of activation is related to the relevant PIP2 influence on Na^+-dependent inactivation of NCX. In fact, PIP2 directly interacts with the XIP region of the exchanger, thus eliminating its inactivation and stimulating NCX function. Indeed, exchangers with mutated XIP regions no longer respond to PIP2 or to PIP2 antibodies (Hilgemann et al., 1992; Hilgemann and Ball, 1996; He et al., 2000).

E. Phosphoarginine

PA, present in millimolar concentrations in the cytosol, activates NCX function in the forward mode of operation by an intracellular Mg^{2+}- and Ca^{2+}-dependent way (DiPolo et al., 1998, 2004). Such mechanism of activation is different from the one characterizing ATP. In fact, ATP and PA regulation is associated with different structures inside and outside the exchanger protein. Particularly, PA should interact with a new zone of NCX named the PA region, which is related to intracellular transport sites for Na^+ and Ca^{2+} (DiPolo et al., 2004).

F. Redox Agents

In the last 15 years, several groups of investigators using different cellular models, such as cell-expressing cloned splicing variants of the brain, heart isoforms, cardiac sarcolemma vesicles, cells transiently transfected with NCX1 isoform, and giant excised patches, have found that the Na^+/Ca^{2+} exchanger is sensitive to different combinations of redox agents (Reeves et al., 1986; Amoroso et al., 2000; Santacruz-Toloza et al., 2000). In particular, the stimulation of the exchange activity requires the combination of a reducing agent (DTT, GSH, or Fe^{2+}) with an oxidizing agent (H_2O_2 and GSSG) (Santacruz-Toloza et al., 2000). The effects of both agents are mediated by metal ions (e.g., Fe^{2+}). The antiporter's sensitivity to changes in the redox status can assume particular relevance during oxidative stress. In fact, in this condition, the modulation of reactive oxygen species (ROS) could affect the transport of Na^+ and Ca^{2+} ions through the plasma membrane.

G. Gaseous Mediator: NO

The ubiquitous gaseous mediator NO seems to be involved in the modulation of NCX activity. In fact, Asano

et al. (1995) provided evidence that NO, released by NO donors, is able to stimulate NCX in the reverse mode of operation in neuronal preparations and astrocytes through a cGMP-dependent mechanism. In contrast, in C6 glioma cells, the stimulatory action on NCX reverse mode of operation, elicited by the NO donor sodium nitroprusside (SNP), is not elicited by NO release but by the presence of iron in SNP molecule (Amoroso et al., 2000).

In addition, a direct relationship between the constitutive form of nitric oxide synthase (NOS), the enzyme involved in NO synthesis, and NCX has recently been demonstrated. Indeed, heat stress by inducing NOS phosphorylation causes NOS complexation with NCX, thus decreasing its activity (Kiang et al., 2003).

V. Pharmacological Modulation of Na^+/Ca^{2+} Exchanger Activity

Since the time researchers discovered that cardiac glycosides increased cytosolic sodium by binding to the extracytoplasmatic face of the Na^+/K^+ ATPase pump α-subunit (Eisner and Smith, 1991), a great deal of interest has been devoted to the pharmacological modulation of NCX. The reasons for this enormous interest lay in the hope of finding clinically effective drugs for those pathophysiological conditions in which a stimulation or an inhibition of the NCX might have achieved beneficial effects. Unfortunately, several drawbacks hampered the achievement of this goal: a lack of scientific knowledge on the specific role played by NCX activity in several physiological and pathophysiological conditions, such as neurological and cardiac diseases; a limited understanding of the role played by the different NCX gene products and splicing variants in the same cells; a lack of knowledge about the molecular biology and the regulatory sites of the NCX molecule; the poor availability of NCX selective activators and inhibitors incapable of interfering with other cellular ion transporting mechanisms, i.e., Na^+ epithelial channels; K^+ channels; plasma membrane store-operated Ca^{2+} channels; VGCC-, NMDA-, and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate receptor-operated channels; Na^+/H^+ exchanger; Na^+/K^+ ATPase; and plasma membrane Ca^{2+} ATPase; and a lack of pharmacological probes able to selectively stimulate or inhibit the activity of each NCX gene product and their multiple splicing variants.

Although the specific scientific literature of the past two decades has been characterized by an overwhelming number of works on NCX, only few of them have dealt with its pharmacological modulation. Accordingly, field scientists attending the last international conference held in October 2001 in Banff, Alberta, Canada, adamantly advocated the need to develop new drugs that could specifically modulate NCX activity and, hence, help to evaluate the antiporter's pathophysiological and therapeutic role.

After the discovery of NCX activity in 1969, studies have reported that some compounds can interfere with this antiporter (Kaczorowski et al., 1989). In the last 35 years, in fact, several inorganic and organic compounds have been reported to activate or block the NCX activity (Fig. 2). However, the selectivity of this action has often been questioned (Matsuda et al., 2001; Reuter et al., 2002; Pignataro et al., 2004b). For this reason, the following section will review the major classes of pharmacological compounds that influence NCX activity and will critically examine the specificity of their action.

VI. Inhibitors

A. Inorganic Cations

1. Divalent Cations. Many divalent cations have been reported to block the Na^+/Ca^{2+} exchanger (Iwamoto and Shigekawa, 1998b) (Fig. 2). This inhibitory effect can be due either to a direct action on the exchanger molecule or to the replacement of Ca^{2+} ions as a substrate for the antiporter.

Although Cd^{2+} (Hobai et al., 1997), Co^{2+} (Hilgemann, 1989), Zn^{2+} (Colvin et al., 2000), and Ni^{2+} (Kimura et al., 1987) are all NCX inhibitors, none of these cations can be considered as specific blockers. Some cations, like Ni^{2+}, Mn^{2+}, and Cd^{2+}, may also function as substrates for the exchanger (Iwamoto and Shigekawa, 1998b). Among these divalent cations, Ni^{2+} is the element most commonly used for blocking NCX activity during electrophysiological measurements (Fujioka et al., 1998; Main et al., 1997). The use of Ni^{2+} as a NCX inhibitor is limited because its blocking concentrations are in the order of 2 to 5 mM, levels at which Ni^{2+} is also able to inhibit other membrane currents (Iwamoto and Shigekawa, 1998b). Interestingly, the IC_{50} value of Ni^{2+} for NCX activity is increased 2- to 3-fold by membrane depolarization, suggesting that the affinity of Ni^{2+} for the inhibitory site is affected by membrane potential (Iwamoto and Shigekawa, 1998b). Regarding the mode selectivity, Ni^{2+} inhibits NCX in the reverse mode of operation (Iwamoto and Shigekawa, 1998b). The affinity of Ni^{2+} for NCX proteins differs in the three NCX gene products. In fact, NCX3 is 10-fold less sensitive to Ni^{2+} or Co^{2+} inhibition than NCX1 and NCX2 (Iwamoto and Shigekawa, 1998b). The inhibitory mechanism exerted by Ni^{2+} is apparently exerted by competing against extracellular Ca^{2+} for the external transport site (Iwamoto and Shigekawa, 1998b). In particular, using cysteine-substituted mutants it was possible to identify three amino acids residues influencing Ni^{2+} sensitivity of NCX1, Asp-130, at the level α1-repeat, Asp-825, and Glu-837 at the level of α2-repeats (Shigekawa et al., 2002) (Fig. 2). All these results underline the fact that the degree of sensitivity of the different NCX gene prod-

ucts to pharmacological agents may vary depending upon the type of NCX gene product involved.

2. Trivalent Cations. Among the different lanthanides, La^{3+} was one of the first inorganic cations to be tested as an effective NCX inhibitor in several excitable cells (Kwan and Putney, 1990; Amoroso et al., 1993, 1997). However, the K_i of La^{3+} for NCX inhibition was in the high micromolar range (500 μM), whereas its affinity for other Ca^{2+}-transporting systems, such as VGCC and the plasma membrane Ca^{2+} ATPase, was much greater (Shimizu et al., 1997). Such discrepancy suggests that lanthanide concentrations capable of blocking NCX are also effective in inhibiting the other Ca^{2+}-transporting mechanism.

Other lanthanides as Nd^{3+}, Tm^{3+}, and Y^{3+} can also inhibit the Na^+/Ca^{2+} exchanger (Trosper and Philipson, 1983), but their potency is lower than that of La^{3+} (Trosper and Philipson, 1983). Interestingly, Gd^{3+}, another lanthanide, has been shown to block VGCC without interfering with NCX activity (Canzoniero et al., 1993).

B. Organic Derivatives

1. Peptides.

a. Endogenous Constrained Cyclic Peptides: Phe-Arg-Cys-Arg-Cys-Phe. Among one class of cyclic exapeptides found to inhibit cardiac NCX activity, Phe-Arg-Cys-Arg-Cys-Phe (FRCRCFa) is apparently the most potent, IC_{50} value = 10 ± 3 μM (Khananshvili et al., 1996). This peptide is able to block both the forward and the reverse modes of Na^+-Ca^{2+} exchange of all three NCX subtypes (Khananshvili et al., 1996). It has been proposed that the positively charged and conformationally constrained Arg-Cys-Arg-Cys structure might be a pharmacophore. Therefore, FRCRCFa may interact with two negatively charged domains. The first negative domain (amino acids 56–96) represents a major part of the short intracellular loop connecting TMS1 and TMS2; the second sequence (amino acids 723–733) is located on the NCX cytosolic side at the level of the f loop, where the action site of XIP effect is presumably located (Khananshvili et al., 1996). FRCRCFa rapidly (<20 ms) interacts with the inhibitory site of the exchanger molecule and prevents a specific conformational transition of ion/protein interaction without altering the interaction of the ions with the exchanger's transport sites (Khananshvili et al., 1995).

b. The Molluscan Tetrapeptide Phe-Met-Arg-Phe and Its Related Peptides. Phe-Met-Arg-Phe (FMRFa) is a molluscan peptide having potent biological effects on excitable cells, such as cardiomyocytes and neurons. In addition, an endogenous peptide with an FMRFa sequence has been detected in the brain and in other organs of several vertebrates (Khananshvili et al., 1993; DiPolo et al., 1994; Van Eylen et al., 1994). FMRFa, along with other related peptides, blocks NCX activity (IC_{50} values = 1 μM to 1 mM). Interestingly, the action of these molluscan peptides is reversible and is exerted both in the forward and in the reverse mode of NCX operation. As it stands, no evidence is available on the antiporter subtype selectivity of these molluscan peptides. The NCX inhibition exerted by these peptides is eliminated by trypsin preincubation, thus suggesting that its action occurs at the intracellular f loop level of the exchanger, viz., a trypsin-sensitive site (Khananshvili et al., 1993). However, since FMFRa, along with its related peptides, does not present clear homologies with the amino acid sequence of the XIP peptide, it is possible to speculate that these two peptides bind NCX molecule to two distinct sites. FMRFa, in effect, itself has a low inhibitory potency (IC_{50} 750 μM) that completely resides in its carboxyl-terminal RFa portion (Khananshvili et al., 1993). Its inhibitory potency can increase by 300 to 500 times if the NH_2-terminal Phe is substituted by either Val or His (IC_{50} value = 1–2 μM) (Khananshvili et al., 1993). This suggests the importance of potentiating the inhibitory activity of the NH_2 terminal portion. Moreover, since the inhibitory action of FMRFa and opioid derivatives on NCX activity is mutually exclusive, these two classes of compounds may act through the same molecular site (Khananshvili et al., 1993).

c. Callipeltin A. Callipeltin, a cyclic depsipeptide obtained from the New Caledonian Lithistida Sponge *Callipelta* sp., is a macrocyclic lacton comprising the following amino acids: Ala, Leu, and Thr (two residues) in the L configuration; Arg in the D configuration; two *N*-methyl amino acids, *N*-MeAla and *N*-MeGln; and a methoxy Tyr, 3,4-dimethyl-L-glutamine and a 4-amino-7-guanidino-2,3 dihydroxypentanoic acid, formally derived from L-Arg. Similarly to FRCRFa, the presence of two positively charged Arg residues confers to this New Caledonian sponge product a highly inhibitory property. The peptide's peculiar lipophilicity and resistance to proteolytic degradation can further explain its high inhibitory potency (IC_{50} value = 0.85 μM) on NCX activity in the Na^+_i-dependent Ca^{2+} uptake and its ability to induce a full NCX inhibition (Trevisi et al., 2000). Evidence on its ability to inhibit the exchanger forward mode of operation and on its NCX subtype selectivity is still lacking.

d. Endogenous Exchange Inhibitory Peptides. Since it was demonstrated that a 20-amino acid sequence (219–238) of the intracellular f loop of the exchanger molecule, XIP, played an autoinhibitory function through an Na^+-dependent inactivating mechanism (Nicoll et al., 1990; Matsuoka et al., 1997) and that synthetic peptides with the same amino acid sequence could exert an inhibitory action on NCX function (Li et al., 1991; DiPolo and Beaugé, 1994), a great effort has been made to synthesize and to characterize the molecular pharmacology of different XIP analogs. XIP, an amphipathic molecule, potently inhibits Na^+/Ca^{2+} exchange activity in both modes of operation with a K_i of 0.1 to 1.0 μM in a noncompetitive manner (Fig. 2) (Li et al., 1991). NCX1, NCX2, and NCX3 have homologous

XIP regions. The three corresponding inhibitory peptides, XIP1, XIP2, and XIP3, have some residue variations, despite having well conserved sequences. For instance, XIP1 is provided with two basic residues at positions 17 and 19, whereas at these same positions, XIP2 has two neutral residues, a proline and a serine (He et al., 1997). Interestingly, whereas XIP1 is a good inhibitor of NCX1 activity, XIP2 and XIP3 exhibit only a weak inhibitory property on NCX1 (Linck et al., 1998). As previously mentioned, XIP probably interacts with the residues 445 to 455 of the f loop, previously described as a Ca^{2+} regulatory site, and with the residues 562 to 685, believed to be an Na^+ regulatory site (Fig. 2). In regard to the mechanism by which XIP inhibits NCX activity, some authors have proposed that when the XIP-binding site is ligand occupied, a conformational change is induced in the C-terminal portion of the f loop, thus resulting in the inhibition of the ion transport (Li et al., 1991). Moreover, XIP can also open an inward current of unknown origin when applied to the extracellular medium.

The entire extent of XIP is important for maximal potency, although the major inhibitory components are found between the amino acid residues 5 and 16 (Fig. 3) (He et al., 1997). In this sequence, basic and aromatic residues are crucially important for the inhibitory function of XIP. However, since substitutions of Arg-12 and Arg-14 with Ala or Gln, respectively, dramatically decrease XIP potency, both residues also play a key role in possible charge-charge interactions (He et al., 1997). Finally, it should be underlined that although some residues vary in their position, the XIP region is well conserved in all the known NCX gene products (He et al., 1997).

Since XIP hardly penetrates the cell membrane because of its prevalent hydrophilia, an XIP bearing a molecule of glucose attached to the Tyr-6 residue has recently been synthesized (Fig. 3). By virtue of this strategy, the peptide more easily penetrates into the cell, since the glucose molecule, actively transported into the cell through the glucose transporters (glucose transporters 1 and 3), carries the attached peptide (Namane et al., 1992). Interestingly, this Tyr-6-glycosilated form of XIP (Fig. 2), intracerebroventricularly infused in male rats bearing permanent middle cerebral artery occlusion (pMCAO), caused a dramatic increase in infarct volume (Pignataro et al., 2004b). These results suggest that NCX plays a pivotal role in the mechanisms that lead to neuronal death under ischemic conditions. Therefore, a pharmacological modulation of its activity may represent one of the possible therapeutic strategies for stroke treatment.

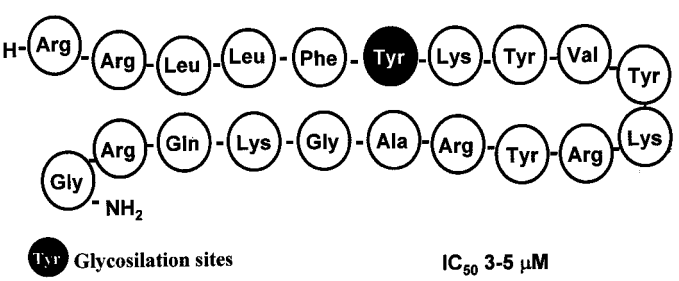

FIG. 3. Structure of the 6-tyrosine glycosilated form of the exchange inhibitory peptide XIP.

C. Heterocycles

1. Amiloride Derivatives. The synthesis of amiloride and amiloride analogs as K^+-sparing diuretics capable of inhibiting kidney epithelial Na^+-channels was first described by Cragoe et al. (1967). Subsequently, these compounds were shown to inhibit other ion transport processes such as NCX, Na^+/H^+ exchanger, and VGCC (Murata et al., 1995). Since then, in an attempt to evaluate NCX activity, amiloride has been used as a probe to block NCX function (Sharikabad et al., 1997). However, two major drawbacks have limited its use. Firstly, millimolar concentrations are required for its NCX inhibitory activity; secondly, it lacks specificity, for it can also inhibit both the epithelial Na^+ channel at micromolar concentrations and the Na^+/H^+ exchanger in the millimolar range. More recently, to overcome these hindrances, two classes of amiloride analogs have been developed (Fig. 4). The amiloride analogs of the first class, such as 5-[*N*-methyl-*N*-(guanidinocarbonylmethyl)] amiloride, bear substituent on the 5-amino nitrogen atom of the pyrazine ring (Taglialatela et al., 1988b). They lack inhibitory properties on the epithelial Na^+ channel and the plasma membrane NCX, even though they display great effectiveness in inhibiting the Na^+/H^+ exchange in the 1 to 10 μM range (Taglialatela et al., 1988b, 1990a; Amoroso et al., 1990). The compounds of the second class, having no inhibitory effect on the Na^+/H^+ exchanger, bear substituents on the terminal guanidino nitrogen atom and behave as specific inhibitors (K_i = 1–10 μM) of the epithelial Na^+ channels and NCX. Among these compounds, dimethylbenzamylamiloride (DMB), 3′,4′-dichlorobenzamyl, and α-phenylbenzamyl have been shown to be selective inhibitors of the Na^+/Ca^{2+} exchanger in excitable cells, such as neurons, in which the kidney epithelial Na^+ channels are not expressed (Taglialatela et al., 1988b, 1990a) (Figs. 2 and 4). In contrast, [*N*-(4-chlorobenzyl)]2,4-dimethylbenzamyl (CB-DMB) (Figs. 4 and 5) appears to be the most specific inhibitor of NCX activity (K_i = 7.3 μM), for it has no inhibitory properties against the Na^+/H^+ antiporter (K_i > 500 μM) and the epithelial Na^+ channels (K_i > 400 μM) (Sharikabad et al., 1997).

The amiloride derivatives are able to inhibit NCX activity either when the antiporter operates in the forward (Taglialatela et al., 1990b) or in the reverse mode of operation (Amoroso et al., 1997). Data on the subtype specificity of the K^+-sparing diuretic derivatives are lacking. Amiloride derivatives are reversible inhibitors of the exchanger, and the inhibition is competitive with respect to the Na^+ ion. It was hypothesized that these

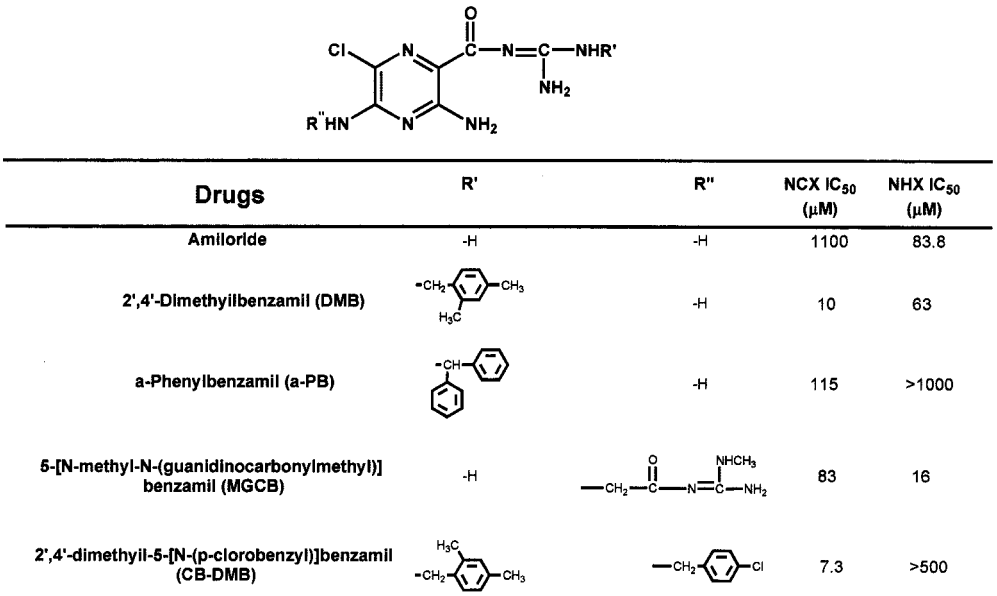

FIG. 4. Chemical structures of the two classes of amiloride derivatives and their inhibitory concentrations on NCX and Na^+/H^+ exchanger activity.

derivatives act as Na^+ analogs, interacting at an Na^+-binding site on the carrier, presumably, the region to which the third Na^+ binds, and reversibly tie up the transporter in an inactive complex (Kaczorowski et al., 1985).

Because of their pharmacological properties, these amiloride derivative compounds have been shown to interfere with the release of neurotransmitters from the brain, under both physiological and pathophysiological conditions. As a result, DMB is able to prevent ouabain-induced DA release from tuberoinfudibular neurons (Taglialatela et al., 1990a). The same compound was demonstrated to increase anoxia-induced D-^{3}H-aspartate release from hippocampal slices (Amoroso et al., 1993). Amiloride analogs have also enabled researchers to detect the NCX involvement in the process of anterior pituitary release; in fact, prolactin secretion, induced by the inhibition of the Na^+/K^+ ATPase, is remarkably blocked by DMB (Di Renzo et al., 1995). The role played by NCX in glial and neuronal damage, induced by anoxic conditions in vitro and by pMCAO in vivo, has also been revealed in studies performed with this class of agents. In fact, the blockade of NCX by CB-DMB enhances LDH release, induced by chemical hypoxia, and completely reverts the protective effect exerted by the removal of Na^+ ions on glioma cells exposed to chemical hypoxia. Likewise, DMB and CB-DMB worsen delayed neuronal death, observed in cerebellar granule cell cultures after glutamate exposure (Andreeva et al., 1991). Moreover, studies of an in vivo model of cerebral ischemia have demonstrated that CB-DMB, intracerebroventricularly infused with an osmotic minipump for 24 h after the beginning of the pMCAO, increases the volume of the ischemic region (Pignataro et al., 2004b).

2. Diarylaminopropylamine Derivatives: Bepridil and Aprindine. Bepridil is a diarylaminopropylamine derivative having both antianginal and antiarrhythmic effects (Figs. 2 and 5). Bepridil has multiple inhibitory effects on ionic currents, including the L-type (Yatani et al., 1986) and T-type Ca^{2+} currents (Cohen et al., 1992), the delayed-rectifier K^+ current, the transient outward current (Berger et al., 1989), as well as the K^+ current, activated by intracellular Na^+ (Mori et al., 1998). In addition to these pharmacological properties, bepridil can also block NCX activity with an IC_{50} value of 8.1 μM (Watanabe and Kimura, 2001). This inhibitory action is dependent on the mode of operation of the antiporter. In fact, NCX operating in the forward manner is more strongly inhibited than in the reverse manner of action. Although this type of pharmacological inhibition is similar to that of 3′,4′-dichlorobenzamil, it is different from the isothiourea derivative, KB-R7943 (2-[2-[4-(4-nitrobenzyloxy) phenyl] ethyl]isothiourea methanesulfonate) mode of action (see below), which inhibits, preferentially, NCX activity in the reverse mode (Watano et al., 1996). The site of action of bepridil may be located on the cytoplasmic side of the exchanger, since the intracellular treatment with trypsin attenuates its inhibitory action (Watanabe and Kimura, 2001).

In in vitro and in vivo models of hypoxia and ischemia, respectively, bepridil, by blocking NCX, enhances glial and neuronal injury elicited by chemical hypoxia and pMCAO, respectively (Amoroso et al., 2000; Pignataro et al., 2004b). In addition, in rats' striatal spiny neurons, bepridil has been used to investigate membrane potential changes induced by oxygen glucose deprivation or by excitatory amino acids (Calabresi et al., 1999). The results of these experiments, performed in preparations of brain slices, confirmed that the activation of NCX is able to exert a protective role during oxygen glucose deprivation (Calabresi et al., 1999). In contrast, this compound has been shown to improve the functional recovery of the

spinal cord white matter after anoxia and traumatic compression (Li et al., 2000). Hence, NCX operating in the reverse mode seems to play a relevant role in cellular calcium overload and in reversible damage after the induction of anoxic and traumatic injury to the dorsal column white matter tracts. Aprindine, another diarylaminopropylamine derivative, belongs to the class of I-B antiarrhythmic agents widely used for treating atrial and ventricular tachyarrhythmia. This drug inhibits several ionic currents such as L-type Ca^{2+}, Na^{+}, and delayed rectifier K^{+} currents. Aprindine inhibits NCX current with an IC_{50} value of 49 μM and with a Hill coefficient of 1.3. Its inhibitory NCX action site has not yet been clarified. However, since the deletion of amino acids 247 to 671 in the f loop does not interfere with its NCX inhibitory properties, it most likely acts at a locus different from that of amiodiarone (Watanabe et al., 2002) (see below).

3. Isothiourea Derivatives. KB-R7943, a 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulfonate derivative (Figs. 2 and 5), was identified by Shigekawa's group (Iwamoto and Shigekawa, 1998b) because they screened a compound library for the inhibition of Na^{+}-dependent Ca^{2+} uptake. A particular feature of this compound is that it inhibits the antiporter with a different potency, depending on NCX mode of operation. In fact, in intact cells, when the NCX operates in the reverse mode, the IC_{50} value needed for NCX inhibition is 1.1 to 2.4 μM, whereas when NCX operates in the forward mode, the IC_{50} value is much higher (IC_{50} value > 30 μM) (Iwamoto et al., 1996b). In addition, this compound seems to have a different ability to block NCX activity depending on the gene product involved. In fact, NCX3 inhibition requires concentrations that are 3-fold lower than those necessary to inhibit NCX1 and NCX2 (Iwamoto and Shigekawa, 1998b).

KB-R7943 interacts with the exchanger molecule extracellularly at the α-2 repeat, between the TMS7 and TMS8 of the exchanger and, more specifically, at the Val-820, Gln-826, and Gly-833 levels (Iwamoto et al., 2001; Shigekawa et al., 2002). Actually, site-directed mutagenesis and chimera exchange studies have recently demonstrated that when Val-820 and Gln-826 in NCX1 are replaced with Gly (V820G) and with Val (Q826V), two amino acids present in the same position in NCX3 molecule, the sensitivity of NCX1 to KB-R7943 increases by 3-fold (Iwamoto et al., 2001; Shigekawa et al., 2002). However, when Gly-833 is mutated, the exchanger becomes practically insensitive to the drug (Iwamoto et al., 2001). Furthermore, if the Ala-809 of NCX3 is substituted with Val, which is the corresponding NCX1 amino acid, the inhibitory property of KB-R7943 decreases by 3-fold. This evidence suggests that the three amino acids Val-820, Gln-826, and Gly-833, present in the α2-repeat region, play a critical role in the KB-R7943 molecular action (Fig. 2). Interestingly, its derivative KB-R7898 (2-[2-[4-(3,4-dichlorobenzyloxy)phenyl]ethyl]isothiourea methanesulfonate), in which two electron donor groups ($-Cl$) are inserted instead of one electron attractor group ($-NO_2$) and one $-H$ atom, is able to block all three NCX gene products (NCX1, NCX2, and NCX3) (Shigekawa et al., 2002). Thus, this structure-activity relationship demonstrates that the structural modification in the benziloxy moiety is critical for its inhibitory activity.

In light of these peculiar pharmacological properties, in the last 7 years, KB-R7943 has aroused a great deal of interest among investigators working on NCX activity. It has been demonstrated that KB-R7943 exerts a neuroprotective effect in anoxic conditions. Specifically, in rat hippocampal slices, this drug preserves CA1 neurons from hypoxic-hypoglycemic injury (Schroder et al., 1999). This rescuing action has been attributed to the inhibition of the Na^{+}/Ca^{2+} exchanger operating in the reverse mode. Analogously, in rats bearing pMCAO, KB-R7943 reduces infarct volume. This neuroprotective action of KB-R7943 can be theoretically attributed to its more selective inhibitory action on NCX3, since this isoform is consistently present in those brain regions involved in pMCAO-induced damage (Papa et al., 2003). However, this neuroprotective effect does not seem the result of NCX blockade, since other antiporter inhibitors, such as GLU-XIP, CB-DMB, and bepridil, do indeed aggravate brain injury (Tortiglione et al., 2002; Pignataro et al., 2004b). To explain the neuroprotective effect of KB-R7943 in pMCAO, it should be noted that this drug is also able to produce a remarkable and long-lasting hypothermic effect (Pignataro et al., 2004b), which itself exerts a relevant neuroprotective action in cerebral ischemia (Yanamoto et al., 2001). In addition, recent reports have shown that KB-R7943, besides its peculiar NCX blocking properties, also exerts an inhibitory effect on several other ionic transport mechanisms, such as L-type VGCC. Moreover, KB-R7943 inhibits receptor-operated ion channels, such as NMDA (Matsuda et al., 2001), whose blockade may also lead to neuroprotective actions (Paule et al., 2003). Finally, since this drug, at low concentrations, can depress Ca^{2+} transients in heart tubes from mouse embryos, whose NCX1 gene is knocked out, its specificity as an NCX inhibitor has been further questioned (Reuter et al., 2002).

4. Ethoxyanilines. In 2001, the newly synthesized 2-[4-[(2,5-difluorophenyl)methoxyl-phenoxy]-5-ethoxyaniline derivative, SEA0400 (2-[4-[(2,5-difluorophenyl)-methoxyl-phenoxy]-5-ethoxyaniline), was reported as being the most potent (IC_{50} value = 5–92 nM) NCX inhibitor available at the time (Matsuda et al., 2001) (Figs. 2 and 5). More recent studies, however, demonstrated that it was also able to interfere with Ca^{2+} movement across the cell membrane (Reuter et al., 2002). This compound, similarly to KB-R7943, inhibits the antiporter's Na^{+} efflux-Ca^{2+} influx mode of opera-

tion. However, at variance with the isothiourea derivative, it predominantly blocks NCX1 (IC_{50} value = 56 nM), it has a lower affinity for NCX2 (IC_{50} value = 980 nM), and it has no effect on NCX3 (Iwamoto et al., 2004). It has recently been found that multiple mutations occurring at the Phe-213 level, located at the end of TMS5, significantly alter the exchanger's sensitivity to the SEA0400-induced blockade, whereas they do not modify KB-R7943 activity (Iwamoto et al., 2004). In addition, by replacing Gly-833, located at the α2-repeat level, with Cys, the SEA0400 and KB-R7943 sensitivity is eliminated. Therefore, whereas Phe 213 is crucial for SEA0400 inhibitory action, Gly-833 is a target for both SEA0400 and KB-R7943. On the other hand, the α2-repeat and TMS5 are not the only SEA0400 molecular action sites. In fact, mutants of the amino acids 224, 226, 228, and 231, belonging to the XIP region in which the Na^+-dependent inactivation is completely eliminated, display a reduced sensitivity to SEA0400 and KB-R7943 inhibitory actions (Iwamoto et al., 2004). In contrast, the NCX1 mutants bearing Glu instead of Phe-223 (F223E), in which the Na^+-dependent inactivation is accelerated, show an increased sensitivity to SEA0400 and KB-R7943. Such variable effects have led to the conclusion that the inhibitory effect of the isothiourea derivative and of the ethoxyaniline analog is better exerted during the Na^+-dependent inactivation (Iwamoto et al., 2004).

On the basis of such potent and selective action, some studies have been performed to clarify the role played by NCX in anoxic/ischemic injury. This compound has been shown to attenuate dose dependently astrocyte damage induced by reperfusion injury in vitro and to reduce infarct volume after a transient middle cerebral artery occlusion in rat cerebral cortex and striatum (Matsuda et al., 2001). SEA0400 neuroprotective effect was attributed to its pharmacological capability of inhibiting prevalently NCX1 activity. However, the specificity of SEA0400 on NCX activity has recently been questioned, since it can also interfere with Ca^{2+} movement across the cell membrane, in the heart tubes of mouse embryos whose NCX1 was knocked out (Reuter et al., 2002). On the other hand, it should be considered that SEA0400 exerts its neuroprotective action in an animal model of cerebral ischemia in which the neuronal damage is mainly caused by the reperfusion period with a Ca^{2+} paradox-like injury accompanied by an exaggerated ROS production and apoptotic cell death (Matsuda et al., 2001). Therefore, the SEA0400 inhibition of Ca^{2+} transients could result in a neuroprotection.

5. Quinazolinone Derivatives. Most recently, in experiments designed to develop new and potent NCX inhibitors, a new compound belonging to quinazolinone family named SM-15811 (4-phenyl-3-[(*N*-benzyl)-4-pyperidine]-3,4-dihydro-2(1*H*)-quinazolinone) has been found to inhibit Na^+-induced Fura-2-monitored Ca^{2+} increase. This drug is provided with a high affinity for the exchanger with an IC_{30} value of 17 nM (Hasegawa et al., 2003).

6. Benzofuran Derivatives. Amiodarone is an antiarrhythmic agent, belonging to the class of benzofuran derivatives (Fig. 2) that is able to block several plasma membrane voltage-gated ionic channels (Watanabe and Kimura, 2000). So far, this compound has been found to inhibit bidirectional NCX current direction independently with an IC_{50} value of 3.3 μM and a Hill coefficient of 1 (Watanabe and Kimura, 2000). No information is available on NCX isoform selectivity of these derivatives. The large intracellular domain, the f loop, has been identified as the major interactive site between amiodarone and the exchanger molecule (Kimura et al., 2002). In fact, the trypsin-induced f loop proteolysis decreases the blocking effect of the drug.

7. Imidazoline Derivatives: Cibenzoline. Cibenzoline, a diarylcyclopropylimidazoline derivative, can inhibit voltage-operated Na^+, Ca^{2+}, and K^+ channels and K^+ currents elicited by the muscarinic receptor (M current). It has recently been shown that this compound is also able to inhibit NCX currents with an IC_{50} value of 95 μM (Kimura et al., 2002).

8. Phenylalkylamines: Verapamil and Methoxyverapamil. The phenylakylamine derivative, i.e., verapamil, and its methoxy derivative, i.e., methoxyverapamil, besides inhibiting L-subtype of voltage-sensitive Ca^{2+} channels, can also block NCX activity with a K_i of 50 μM. Interestingly, both the hydrophobic drug methoxy-verapamil, which penetrates into the cytoplasm, and its hydrophilic parent compound verapamil, which hardly enters the cell, can inhibit the antiporter. This observation suggests that phenylalkylamines can block the exchanger from the extracellular and/or from the intracellular side (Erdreich and Rahamimoff, 1984).

9. Oxime Derivatives. The oxime derivative, 2,3-buthenedione monoxime, was originally developed to counteract organophosphorous poisoning of acetylcholinesterase. Initially, it was shown that this compound could inhibit VGCC and transient outward K^+ currents in neurons (Coulombe et al., 1990; Huang and McArdle, 1992). However, more recently, it has been reported that this compound can also inhibit I_{NCX} in a concentration-dependent and direction-independent manner. The IC_{50} value is 3.3 μM with a Hill coefficient of 1. Similar features of NCX inhibition were found with the well known oxime pralidoxime (Watanabe et al., 2001). Although the mechanism of inhibition has not yet been fully clarified, the phosphorylation of the antiporter does not seem to be involved in this blocking action (Watanabe et al., 2001).

10. Acridines: Quinacrine. Quinacrine, an acridine derivative, displays a peculiar action on the exchanger activity. In fact, it can either inhibit or stimulate NCX, depending on the experimental conditions. Indeed, when quinacrine is present in the extracellular medium, both the Na^+-dependent Ca^{2+} uptake and the Na^+-depen-

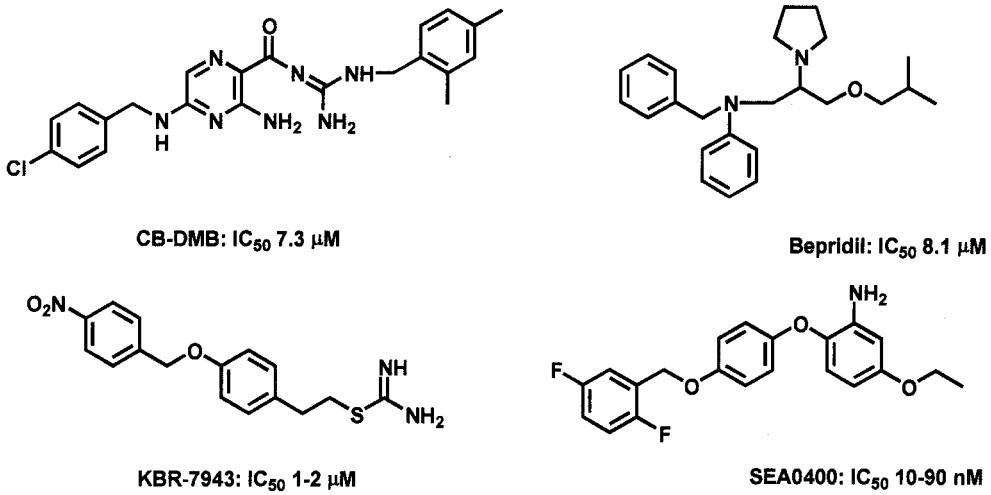

FIG. 5. Chemical structures of the most widely used NCX blockers.

dent Ca^{2+} efflux are inhibited with a K_i of 50 μM. In contrast, if quinacrine is preincubated in conditions of Na^+ loading and then removed from the medium, the exchanger activity is stimulated (de la Pena and Reeves, 1987).

11. Opiate Derivatives. Evidence that opiate agonists and antagonists inhibit cardiac NCX has been provided (Khananshvili and Sarne, 1992; Khananshvili et al., 1995). Interestingly, opioid stereoisomers, devoid of analgesic activity, are provided with the same NCX blocking properties as their opioid isomers. Such observation suggests that the NCX inhibition is independent of opiate receptor interaction (Khananshvili and Sarne, 1992; Khananshvili et al., 1995). This is further sustained by the fact that the opiate antagonist naloxone inhibits NCX1 exchanger (cardiac) in a dose-dependent manner. This effect achieves a complete blockade without competing with extravesicular Ca^{2+}. The naloxone action site seems to be located on the extravesicular surface of the membrane, since its nonpermeable analog, methylnaloxone, is still able to block NCX (Khananshvili and Sarne, 1992; Khananshvili et al., 1995).

12. Anesthetics. Volatile anesthetics, mainly the ones belonging to the halogenate class, halothane, isofluorane, and enflurane, have been shown to inhibit the activity of NCX at concentrations relevant to anesthesia. A peculiar feature of these volatile anesthetics is that not all of them exert the same level of inhibitory potency. For instance, enflurane is known to be the most specific agent working against NCX (Haworth and Goknur, 1995).

13. Remarks on Structure-Activity Relationships of Heterocycles. The analysis of the structure-activity relationships of most of the heterocycles treated in the present review reveals that a drug provided with NCX blocking action ought to possess the following chemical characteristics: a pharmacophore portion consisting of a six-atom aromatic ring coupled with an electron donor group through a methylene bridge; a spacer connecting the aromatic ring with the electron donor group consisting only of a methylene group. In fact, the presence of a group that is longer than the methylene (i.e., ethylene) reduces the NCX inhibitory activity; and the electron donor group must bear a long lateral chain containing at least one aromatic radical (Fig. 5).

D. Antisense Oligodeoxynucleotides versus Na^+/Ca^{2+} Exchanger Isoforms

The availability of NCX gene sequence information has enabled researchers to find alternative and selective pharmacological approaches that, by modulating NCX gene products (NCX1, NCX2, and NCX3) activity, have helped to identify their functions and, consequently, to develop specific therapeutic strategies. An antisense oligodeoxynucleotide directed against 19 nucleotides in the 3′-untranslated region of NCX has been shown to inhibit the NCX, both in the forward and reverse modes of operation (Egger and Niggli, 1999) (Fig. 6). The efficacy of this antisense strategy has been mainly directed against NCX1 isoforms expressed in cardiac myocytes (Slodzinski and Blaustein, 1998), in rat pancreatic β-cells (Slodzinski and Blaustein, 1998), in mouse distal convoluted tubular cells (Lipp et al., 1995), and in arterial myocytes (Takahashi et al., 1995). In particular,

NCX1 antisense oligos (AS) :	5'-TGAGACTTCCAATTGTT-3'
NCX1 non sense oligos (NS) :	5'-TAGTACCTTCTATGAGT-3'
NCX1 sense oligos (S) :	5'-AACAATTGGAAGTCTCA-3'
NCX2 antisense oligos (AS) :	5'-TCGGAGTAGCTTC-3'
NCX2 non sense oligos (NS):	5'-ACGATCAGGATCC-3'
NCX2 sense oligos (S):	5'-GAAGCTACTCCGA-3'
NCX3 antisense oligos (AS) :	5'-GCCATACACAAGAC-3'
NCX3 non sense oligos (NS):	5'-TTGACTGCTGGTGT-3'
NCX3 sense oligos (S):	5'-GTCTTGTGTATGGC-3'

FIG. 6. Oligodeoxynucleotide sequence of chimeric oligos targeting NCX mRNA.

after the treatment of cardiac myocytes with phosphorothioated oligodeoxynucleotides (0.5 μM) for 7 days, the antisense strategy induces a 50% knockdown of the NCX1 protein (Takahashi et al., 1995). By virtue of this pharmacological inhibition, investigators have been able to estimate an NCX protein half-life ($t_{1/2}$) of 33 h in cardiac myocytes. The NCX1 antisense inhibition showed that cultured cardiac myocytes exhibit neither Ca^{2+} transients (Takahashi et al., 1995) nor $I_{Na/Ca}$ current. Furthermore, the antisense oligodeoxynucleotides strategy prevents both the increase in $[Ca^{2+}]_i$, upon extracellular Na^+ removal and the increase in diastolic Ca^{2+} levels (Takahashi et al., 1999). Similarly, in primary cultured arterial myocytes, chimeric phosphorothioated antisense oligodeoxynucleotides increase resting $[Ca^{2+}]_i$ and also prevent $[Ca^{2+}]_i$ increases that ouabain elicits by decreasing $[Na^+]_e$ and by increasing $[Na^+]_i$ (Slodzinski et al., 1995, 1998).

In rat pancreatic β-cells, equipped with two NCX1 alternative splicing products, viz., NCX1.3 and NCX1.7 (Van Eylen et al., 2001a), the treatment with oligodeoxynucleotides, which are used to knockdown the exchanger, is able to yield the following two effects: firstly, it reduces the increase in $[Ca^{2+}]_i$ induced by membrane depolarization resulting from high K^+ or hypoglycemic sulfonylurea tolbutamide; and secondly, it profoundly alters the oscillatory pattern induced by high glucose concentrations (Van Eylen et al., 1998).

In addition, the antisense strategy allows researchers to design exon specific oligodeoxynucleotides that target different isoforms of the same NCX gene product. In fact, in immortalized renal distal convoluted tubule cells, expressing three alternative spliced NCX isoforms, NCX1.2, NCX1.3, and NCX1.6, the use of exon-specific oligodeoxynucleotides targeting NCX isoforms permitted to clarify the hypothesis that only the splicing variants NCX1.2 and NCX1.3 can mediate Na^+-dependent Ca^{2+} extrusion by NCX, whereas NCX1.6 and NCX1.7 cannot (White et al., 1998).

This approach, a potentially therapeutical tool, has also been used in primary culture of brain neurons (Ranciat-McComb et al., 2000) and astrocytes (Takuma et al., 1996). An antisense oligodeoxynucleotide directed against a conserved sequence of all three gene products, NCX1, NCX2, and NCX3, increases resting $[Ca^{2+}]_i$ in cortical neurons and slows the rising decay of $[Ca^{2+}]_i$, induced by NMDA, without discriminating one gene from the other (Ranciat-McComb et al., 2000). Analogously, in cultured astrocytes, NCX antisense oligodeoxynucleotides boost the ionomycin-induced increase in $[Ca^{2+}]_i$ and block SNP and 8-bromo cyclic GMP reducing effects on Na^+-induced Ca^{2+} signal (Takuma et al., 1996).

Remarkably, because phosphorothioate oligodeoxynucleotides can significantly penetrate and accumulate within the brain after being intraventricularly administered (Yaida and Nowak, 1995), their use has led to a better understanding of the role played by each NCX gene product during brain ischemia. Hence, in rats bearing pMCAO, it has been noted that oligodeoxynucleotides, by blocking NCX1, NCX2, and NCX3, can induce different effects on the development of brain ischemia (Pignataro et al., 2004a). In fact, after pMCAO, the neuroprotective effect exerted by NCX is prevalently due to NCX1 and NCX3 products (Pignataro et al., 2004a).

VII. Activators

The availability of pharmacological agents capable of stimulating the activity of NCX, either in the reverse or in the forward mode of operation, may represent a useful strategy to adopt in some pathophysiological conditions, such as cardiac or brain ischemia. The pharmacological stimulation of the antiporter could, in fact, contribute to the re-establishment of intracellular Na^+ and Ca^{2+} ion homeostasis.

A. Inorganic Cations

1. Monovalent Cations: Li^+. Li^+, the lightest of alkaline cations, whose carbonate and citrate salts are currently used as antimaniac and mood-stabilizing drugs, stimulates the Na^+_i-dependent Ca^{2+} uptake of all three NCX gene products with low affinity. Its extent of stimulation is, however, somewhat smaller in NCX1 than in NCX2 and NCX3 (Iwamoto and Shigekawa, 1998b). In fact, the amount of Li^+-induced Na^+_i-dependent Ca^{2+} uptake in NCX1 and in NCX3 transfected cells reaches 145 and 270%, respectively, compared with that of the control measured in the absence of Li^+. Interestingly, NCX1 chimera exchangers, containing a major portion of the α-2 repeat from NCX3, exhibit a level of sensitivity to Li^+ that is almost identical to that of wild-type NCX3. Reciprocally, the NCX3 mutants, in which the α-2 repeat was substituted with the corresponding NCX1 region, show a reduced level in Li^+ sensitivity similar to that of wild-type NCX1. This demonstrates that α-2 repeat seems to be exclusively responsible for the Li^+-induced NCX stimulation in both NCX1 and NCX3. In addition, single mutation studies have demonstrated that the amino acids Val-820 and Gln-826 of NCX1, along with the amino acids Ala-809 and Val-815 of NCX3, are responsible for the exchanger's different susceptibility to Li^+ (Iwamoto and Shigekawa, 1998b; Iwamoto et al., 1999) (Fig. 2). Recently, some groups of investigators have provided evidence that a long-term Li^+ exposure robustly protects cultured cerebellar, cortical, and hippocampal rat neurons against glutamate-induced excitotoxicity (Nonaka et al., 1998), suppresses the protein and mRNA expression of the two pro-apoptotic factors p53 and BAX, and increases the antiapoptotic mediator Bcl-2 (Chen and Chuang, 1999). Therefore, these last two antiapoptotic properties seem to explain the neuroprotective effect exerted by the alkaline cation. However, the possible interplay between the activation of NCX and the Li^+ neurobeneficial effect

still remains to be demonstrated. In fact, there is a 15-fold difference between the Li^+ concentration needed for a neuroprotective effect and that needed for NCX activation in the reverse mode of operation (Iwamoto et al., 1999).

B. Redox Agents

As mentioned previously, changes in the redox state are capable of stimulating the NCX activity. Therefore, the simultaneous presence of reducing compounds, such as GSH, DDT, Fe^{2+}, and O_2^- superoxide, and of oxidizing agents, such as Fe^{3+}, H_2O_2, GSSG, and O_2, is able to stimulate NCX activity (Reeves et al., 1986). When this property of redox agents was first highlighted, it was proposed that these agents could activate the exchange activity by promoting thiol-disulfide interchange in the protein carrier (Reeves et al., 1986). Particularly, it was hypothesized that the stimulation of NCX came from the reduction of a disulfide bond and from the formation of a new disulfide bond (Reeves et al., 1986). More recently, the cysteine residues involved in this disulfide bond have been identified as Cys-14, Cys-20, and Cys-780. They are located extracellularly, for Cys-780 is connected either to Cys-14 or to Cys-20 (Santacruz-Toloza et al., 2000). However, the analysis of mutated exchangers has indicated that cysteines are not responsible for the stimulation of the exchange activity induced by a mixture of redox agents (Fe-DTT) (Santacruz-Toloza et al., 2000). Therefore, it has been suggested that the stimulation of wild-type exchanger by Fe-DTT is mainly due to the removal of the Na^+-dependent inactivation process (Santacruz-Toloza et al., 2000).

Since redox changes in NCX activity have been implicated in several aspects of cell physiology and pathophysiology, it is possible to speculate that agents capable of stimulating NCX might constitute a possible therapeutic strategy in those pathological conditions in which oxidative stress is involved. In this regard, evidence that the stimulation of NCX activity by the oxidant agent Fe^{3+} may exert a neuroprotective effect both in in vitro and in vivo models of hypoxia and ischemia has been provided. Thus, in C6 glioma cells, it has been demonstrated that SNP, by stimulating NCX activity through its $K_3Fe(CN)_6$ portion-containing iron, is able to significantly reduce cellular injury elicited by chemical hypoxia (Amoroso et al., 2000). This protective effect is certainly due to NCX activation as an Na^+ efflux Ca^{2+} influx pathway, since it is abolished by NCX inhibitors (Amoroso et al., 2000). That Fe^{3+} should be responsible for this SNP neuroprotective effect is demonstrated by the fact that deferoxamine, an iron ion chelator, reverses the neuroprotection (Amoroso et al., 2000). This neuroprotective effect of iron has recently been confirmed in rats bearing pMCAO. In fact, $FeCl_3$, intracerebroventricularly perfused after ischemia induction, has been shown to reduce the extension of brain infarct volume. The specificity of this effect was further supported by the counteracting neuroprotective action exerted by NCX inhibitors (Pignataro et al., 2004b).

Collectively, these results suggest that a stimulation of NCX activity during stroke might help neurons and glial cells to survive.

C. Organic Compounds

1. Agonists of G-Protein-Coupled Receptors. It has been reported that the agonists of G-protein-coupled receptors, such as α- and β-receptors, histamine, $5HT_{2c}$, and endothelin-1 and angiotensin-II receptors, are capable of stimulating NCX activity by a pathway which involves either PKA and/or PKC (Fig. 1) (Ballard and Schaffer, 1996; Smith and Armstrong, 1996; Stengl et al., 1998; Eriksson et al., 2001a,b; Woo and Morad, 2001).

2. Diethylpyrocarbonate. Recently, it has been suggested that NCX activity may be stimulated by an action occurring at the Hys-165 residue level. In fact, this histidine could be the residue modified by diethylpirocarbonate an organic compound that is shown to augment Na^+-dependent Ca^{2+} uptake (Ottolia et al., 2002).

3. Peptides. Among the peptides capable of stimulating NCX activity, only insulin and concanavalin A have been proven to exert such effect. In fact, both peptides stimulate Na^+-dependent Ca^{2+} uptake (Gupta et al., 1986; Makino et al., 1988).

VIII. Na^+/Ca^{2+} Exchanger Intervention in Physiological Conditions

The Na^+/Ca^{2+} exchanger protein may play a relevant function in different neurophysiological conditions. In neurons, the level of expression of NCX is particularly high in those sites where a large movement of Ca^{2+} ions occurs across the plasma membrane, as it happens at the level of synapses (Juhaszova et al., 1996; Canitano et al., 2002). Specifically, during an action potential or after glutamate-activated channel activity, Ca^{2+} massively enters the plasma membrane. Such phenomenon triggers the fusion of synaptic vesicles with the plasma membrane and promotes neurotransmitter exocytosis. After this event, outward K^+ currents repolarize the plasma membrane, thus leading to VGCC closure. According to the diffusion principle, Ca^{2+} ions are distributed in the cytosolic compartment, reversibly interacting with Ca^{2+}-binding proteins. Residual Ca^{2+} is then rapidly extruded by the plasma membrane Ca^{2+} ATPase and by NCX. The Na^+/Ca^{2+} exchanger becomes the dominant Ca^{2+} extrusion mechanism when $[Ca^{2+}]_i$ is higher than 500 nM, as it happens when a train of action potentials reaches the nerve terminals. It has been calculated that for these $[Ca^{2+}]_i$ values (500 nM), more than 60% of Ca^{2+} extrusion is mediated by Na^+/Ca^{2+} exchanger families. In such physiological conditions, NCX activation is consistent with its low-affinity (K_d 500 nM) and high-capacity (5×10^3 Ca^{2+}/s) function. In contrast, in resting conditions or after a single action

potential, when $[Ca^{2+}]_i$ slightly increases, requiring, therefore, a more subtle control, the high-affinity (K_d 100 nM) and low-capacity (10^2 Ca^{2+}/s) pump, plasma membrane Ca^{2+} ATPase, assumes a predominant function, thus making the involvement of NCX less relevant (Blaustein and Lederer, 1999) (Fig. 1).

A. Na^+/Ca^{2+} Exchanger: Hormonal and Neurotransmitter Release

Over the past 20 years, evidence has shown that NCX can modulate not only the synthesis and the release of neurotransmitters in the central and peripheral nervous system (Fig. 1) but also the release of anterior pituitary hormones. In fact, during NCX activation, DA synthesis increases in the dopaminergic terminals of the median eminence (Arita and Kimura, 1985). In addition, since the release of DA from tuberoinfundibular dopaminergic neurons, induced by the blockade of Na^+/K^+ ATPase, is inhibited by the NCX blockade (Taglialatela et al., 1988a,b), it has been suggested that the antiporter plays a pivotal role in controlling DA release (Taglialatela et al., 1988a,b; Annunziato et al., 1992). Interestingly, the pharmacological inhibition of NCX affects DA release in two different ways, depending on whether the antiporter operates in a forward or reverse mode. In fact, when the antiporter operates as the Ca^{2+} efflux-Na^+ influx pathway (forward mode), its pharmacological blockade stimulates the release of neurotransmitters (Taglialatela et al., 1988a,b). In contrast, when this antiporter operates as a Ca^{2+} influx-Na^+ efflux pathway (reverse mode), as a consequence of Na^+/K^+ ATPase inhibition, its pharmacological blockade prevents DA release (Taglialatela et al., 1990a). The rate of neurotransmitter release seems to be directly proportional to the rate of Na^+/Ca^{2+} exchange, as demonstrated by noradrenaline release from nerve terminals of rabbit pulmonary arteries (Magyar et al., 1987).

NCX pharmacological inhibition can also influence the release of anterior pituitary hormones, like prolactin, whose ouabain-stimulated secretion is inhibited by the rather selective NCX inhibitor DMB (Di Renzo et al., 1995).

More recently, it has been shown that NCX is also involved in the cellular events triggered by the activation of G-protein-coupled neurotransmitter receptors. Thus, it has been demonstrated that the serotonin-induced increase of the firing rate of histaminergic tuberomammilar neurons is weakened by NCX inhibitors (Eriksson et al., 2001a,b). Interestingly, the serotonin receptor 5-HT_{2C}, which mediates this effect on histaminergic tuberomammillar neurons, is also coexpressed with NCX1 (Sergeeva et al., 2003). Moreover, in the same histaminergic neurons, NCX activity plays a relevant role in orexin-induced depolarization (Eriksson et al., 2001b).

B. Effect of Knocking Out Na^+/Ca^{2+} Exchanger Genes

To determine the precise role of the different NCX genes, knockout mice have been generated lacking NCX1 or NCX2 or NCX3. Unfortunately, targeted deletion of NCX1 results in NCX1-null embryos that do not have a spontaneous beating heart and die in utero. In addition, since NCX1-deficient mice have been generated using a heart targeted promoter, α-MHC, that does not affect NCX1 gene in the brain, the functional role of this NCX1 gene in the CNS could not be explored. As for NCX2 gene, mice deficient for this major isoform in the brain exhibit an enhanced performance in several hippocampus-dependent learning and memory tasks, together with a significantly delayed clearance of elevated Ca^{2+} following depolarization (Jeon et al., 2003b). In addition, the frequency threshold for LTP and long-term depression in the hippocampal CA regions was shifted to a lowered frequency favoring LTP in these knockout mice (Jeon et al., 2003b). Very recently, mice lacking NCX3 gene have been obtained. These animals exhibit reduced motor activity, weakness of forelimb muscles, and fatigability in comparison with $NCX3^{+/+}$ mice (Sokolow et al., 2004). However, at the moment, since NCX3 is also expressed in the peripheral nervous system, it cannot be established whether these symptoms can be attributed to CNS defects or to alterations at the neuronal muscular junctions and skeletal fibers levels.

IX. Relevance of Na^+/Ca^{2+} Exchanger Activity in Pathophysiological Conditions

The disregulation of $[Ca^{2+}]_i$ and $[Na^+]_i$ homeostasis is involved in neuronal and glial injury occurring in in vitro and in vivo models of hypoxia-anoxia and in several neurodegenerative diseases.

A. Hypoxia-Anoxia

In a cellular model of glial cells, C6 glioma, the activation of NCX, as Na^+-efflux Ca^{2+}-influx pathway, obtained by $[Na^+]_e$ removal, reduces cell injury induced by chemical hypoxia. Such phenomenon suggests that the antiporter plays a protective role during this pathophysiological condition. Consistent with these results, the pharmacological inhibition of NCX activity worsens cell damage by increasing the intracellular concentration of Na^+ ions (Amoroso et al., 1997). Furthermore, the stimulation of NCX activity by redox agents results in a protective effect (Amoroso et al., 2000). Inconsistent with these results, in astrocytes, during the reoxygenation phase, the inhibition of the antiporter decreases cell toxicity (Matsuda et al., 1996, 2001), whereas NCX stimulators, such as NO donors, worsen the injury (Matsuda et al., 1996).

Conflicting reports on the role played by NCX activity in glutamate-induced neuronal damage, an in vitro model mimicking cerebral ischemia, have been published (Andreeva et al., 1991; Kiedrowski et al., 1999;

Schroder et al., 1999; Amoroso et al., 1993). For instance, studies on cerebellar granule cells, as well as on glial cells (Amoroso et al., 2000), have reported that the inhibition of NCX exacerbates delayed neuronal death elicited by glutamate (Andreeva et al., 1991). In contrast, in the same model, other investigators have demonstrated that Ca^{2+} influx, mediated via reverse mode of NCX operation, constitutes the dominant component of NMDA-induced Ca^{2+} entry and excitotoxicity (Kiedrowski, 1999). The same mechanism, an Na^+-mediated reversal of NCX activity, leads to the death of depolarized and glucose-deprived neurons (Czyz and Kiedrowski, 2002). In line with the latter hypothesis, it has been demonstrated that in rat hippocampal slices, an inhibition of NCX protects CA1 neurons against hypoxic-hypoglycemic injury (Schroder et al., 1999).

In in vivo models, reproducing human cerebral ischemia through the occlusion of the middle cerebral artery, the inhibition of NCX, induced by selective inhibitors (Pignataro et al., 2004) or by the knockout of one of the NCX isoforms (NCX2) (Jeon et al., 2003a), aggravates brain infarct, whereas the activation of the antiporter with redox agents reduces the cerebral infarctual area (Pignataro et al., 2004b). At variance with these data, Matsuda et al. (2001) reported that the inhibition of NCX, induced by putative selective NCX inhibitors, such as KB-R7943 and SEA0400, reduces brain injury in the model of transient middle cerebral artery occlusion. However, KB-R7943, besides blocking the antiporter, also produces a remarkable and prolonged hypothermic effect (Pignataro et al., 2004b) that exerts, by itself, a relevant neuroprotective action in cerebral ischemia. On the other hand, by inhibiting other cellular ionic transport mechanisms and receptors, such as NMDA receptors and L-type Ca^{2+} channels (Matsuda et al., 2001), the same drug may yield a neuroprotective effect (Lo et al., 2003). In regard to the other putative NCX inhibitor, SEA0400, it should be underlined that Matsuda et al. (2001) used an animal model of cerebral ischemia in which the neuronal damage mainly occurred during the reperfusion period with a Ca^{2+} paradox-like injury accompanied by an exaggerated ROS production and apoptotic cell death (Matsuda et al., 2001).

The role played by NCX in those neurons and glial cells involved in cerebral ischemia should be differentiated according to the anatomical regions involved in the ischemic pathological process. In particular, it is conceivable that, since in the penumbral region ATPase activity is still preserved, NCX may likely operate in a forward mode. As a result, by extruding Ca^{2+} ions, the exchanger favors the entry of Na^+ ions. Therefore, the inhibition of NCX in this area reduces the extrusion of Ca^{2+} ions, thus enhancing Ca^{2+}-mediated cell injury. In contrast, in the ischemic core region, in which ATP levels are remarkably low and Na^+/K^+ ATPase activity is reduced, intracellular Na^+ ions massively accumulate because of Na^+/K^+ ATPase failure. Hence, the intracellular Na^+ loading promotes NCX to operate in the reverse mode as an Na^+ efflux-Ca^{2+} influx pathway. In conclusion, the NCX pharmacological inhibition in this core region further worsens the necrotic lesion of the surviving glial and neuronal cells as the loading of intracellular Na^+ increases (Pignataro et al., 2004b).

B. White Matter Degeneration after Spinal Cord Injury, Brain Trauma, and Optical Nerve Injury

Several reports suggest that NCX plays a major role in mediating Ca^{2+}-induced white matter damage induced by anoxia or trauma. During these noxious stimuli, myelinated axons lose K^+, whereas intra-axonal Na^+ concentrations increase after Na^+ entry, primarily through voltage-gated tetrodotoxin-sensitive channels. Hence, elevated axoplasmic Na^+ and axolemmal depolarization promote a neurodetrimental Ca^{2+} overload mediated primarily by NCX operating in the reverse mode (Stys and Lopachin, 1998). In fact, drugs capable of inhibiting NCX activity, such as bepridil and amiloride derivatives, reduce white matter damage in different experimental models of white matter injury: rat optic nerve anoxia (Stys et al., 1990; Stys and Lopachin, 1998), spinal cord injury (Li et al., 2000), and stretch-injured axons (Wolf et al., 2001).

Overall, these studies emphasize the relevance of NCX modulation in an attempt to prevent white matter degeneration in different models of axonal injury.

C. Na^+/Ca^{2+} Exchanger and Neuronal Apoptosis

The possibility that NCX is a substrate for caspases was suggested by the demonstration that in Western blot analysis the full-length 120-kDa NCX1 protein copurifies with an active proteolytic fragment of 70 kDa (Philipson et al., 1988); this latter segment is likely to derive from a proteolytic cleavage at the level of two close sites of the NCX intracellular f loop (Gabellini et al., 1995). More recently, Nicotera, Carafoli, and colleagues (Schwab et al., 2002) claimed that NCX1 can be cleaved by caspase 3 in cerebellar granule cells undergoing apoptosis, thus suggesting that NCX possesses consensus sites for caspases. As a result, the NCX cleavage operated by caspases might participate in the events leading neurons to switch from apoptosis to necrosis (Schwab et al., 2002). In fact, when cellular Ca^{2+} efflux is hindered by NCX failure, a Ca^{2+} overload occurs, shifting the balance of neuronal death from apoptosis to necrosis (Schwab et al., 2002).

D. Aging

The impairment of Ca^{2+} homeostasis in neuronal cells is considered to be the major triggering event that leads to the development of brain aging (Annunziato et al., 2002). Studies performed on the cerebro-cortex nerve endings of aged rats have shown that the activity of NCX is markedly reduced in the forward and in the reverse mode of action (Michaelis et al., 1984; Canzoniero et

al., 1992). NCX decline seems to be the consequence of a reduced affinity of the antiporter for Ca^{2+} ions (Michaelis et al., 1984). Nevertheless, during the aging process, NCX is not the only membrane extrusion system that is impaired. In fact, the V_{max} for Ca^{2+} activation of the Ca^{2+}-activated Mg^{2+}-dependent pump is also reduced (Michaelis, 1989).

E. Alzheimer's Disease

A large bulk of studies have shown that the neurotoxicity exerted by the amyloid-β (A-β) protein is intimately related to intracellular Ca^{2+} concentrations. Indeed, the attenuation of $[Ca^{2+}]_i$ increase by Ca^{2+} channel blockers (Weiss et al., 1994), growth factors (Mattson et al., 1993), and cytochalasins (Furukawa and Mattson, 1995) results in a reduction of neural damage induced by the A-β protein. It has recently been demonstrated that exposure to the A-β protein partially reduces Na^+-dependent Ca^{2+} accumulation in plasma membrane vesicles deriving from the human frontal cortex of patients affected by Alzheimer's disease (Wu et al., 1997). These findings have suggested that A-β directly interacts with the hydrophobic surface of the NCX molecule, thus interfering with plasma membrane Ca^{2+} transport.

X. Conclusions and Future Perspectives

An increasing amount of evidence seems to suggest that NCX plays a pivotal role in maintaining intracellular Na^+ and Ca^{2+} homeostasis during pathophysiological conditions in the brain. Indeed, it still remains to be fully clarified whether it is the suppression or the activation of the exchanger to yield potentially beneficial effects on a number of neurodegenerative diseases, such as ischemia, AD, aging, and white matter trauma. A number of conflicting results on the modulating effects of the exchanger have highlighted such differences. For instance, the severity and extension of brain injury may vary depending on whether the exchanger is activated or inhibited. Specifically, in animal models of cerebral ischemia, consequent to permanent vascular occlusion, the pharmacological activation of NCX reduces brain damage, whereas drugs provided with inhibitory properties aggravate the infarct lesion. Yet, in models of white matter injury, the inhibition of NCX activity gives rise to a reduction of cellular injury.

Data derived from the antisense oligodeoxynucleotide strategy seem to indicate that the possible development of agents able to selectively interact with each of the three NCX gene products might achieve a more promising therapeutic goal. Unfortunately, although efforts to synthesize organic compounds that target the different NCX gene products have been made, no highly specific agents have yet been designed. A further obstacle underlying the pharmacological modulation of brain NCX activity is represented by the difficulty of compounds to cross the blood-brain barrier. In this regard, promising strategies entail the conjugation of organic and peptidergic drugs with compounds that are substrates for transporters present on the cell membranes of blood-brain barrier and neurons (Rochat and Audus, 1999).

In conclusion, although the studies concerning the role played by NCX in the pathological mechanisms of brain injury during neurodegenerative diseases have had a late beginning compared with those concerning heart disease, the availability of pharmacological agents able to selectively modulate each NCX subtype activity and antiporter mode of operation will enable researchers to gain fundamental insights into its pathophysiological role and, thus, more tangible perspectives on how to treat such neurological disorders.

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