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Sodium-calcium exchangers (NCX): molecular hallmarks underlying the tissue-specific and systemic functions

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Abstract NCX proteins explore the electrochemical gradient of Na^+ to mediate Ca^{2+} -fluxes in exchange with Na^+ either in the Ca^{2+} -efflux (forward) or Ca^{2+} -influx (reverse) mode, whereas the directionality depends on ionic concentrations and membrane potential. Mammalian NCX variants (NCX1–3) and their splice variants are expressed in a tissue-specific manner to modulate the heartbeat rate and contractile force, the brain's long-term potentiation and learning, blood pressure, renal Ca^{2+} reabsorption, the immune response, neurotransmitter and insulin secretion, apoptosis and proliferation, mitochondrial bioenergetics, etc. Although the forward mode of NCX represents a major physiological module, a transient reversal of NCX may contribute to EC-coupling, vascular constriction, and synaptic transmission. Notably, the reverse mode of NCX becomes predominant in pathological settings. Since the expression levels of NCX variants are disease-related, the selective pharmacological targeting of tissue-specific NCX variants could be beneficial, thereby representing a challenge. Recent structural and biophysical studies revealed a common module for decoding the Ca^{2+} -induced allosteric signal in eukaryotic NCX variants, although the phenotype variances in response to regulatory Ca^{2+} remain unclear. The breakthrough discovery of the archaebacterial NCX structure may serve as a template for eukaryotic NCX, although the turnover rates of the transport cycle may differ $\sim 10^3$ -fold among NCX variants to fulfill the physiological demands for the Ca^{2+} flux rates. Further elucidation of ion-transport and regulatory mechanisms may lead to selective pharmacological targeting of NCX variants under disease conditions.

Keywords NCX · Antiporter · Structure–activity relationships · Tissue-specific regulation · Alternating access · Catalytic capacity · Sodium · Calcium

Abbreviations

CALX	<i>Drosophila melanogaster</i> NCX ortholog
CAX	Ca^{2+} /anion exchanger
CBD	Ca^{2+} binding domain
E_{Ca}	Equilibrium potential of Ca^{2+}
E_{NCX}	Equilibrium potential of NCX
FRCRCFa	NCX inhibitory cyclic hexapeptide
FRET	Fluorescence resonance energy transfer
I_1 -inactivation	Na^+ -dependent inactivation of NCX
I_2 -inactivation	Ca^{2+} -dependent inactivation of NCX
NCLX	Mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger
NCKX	$\text{Na}^+/\text{Ca}^{2+}$ - K^+ exchanger
NCX	$\text{Na}^+/\text{Ca}^{2+}$ exchanger
n_{H}	Hill coefficient
NMR	Nuclear magnetic resonance
SAXS	Small-angle X-ray scattering
SLC8	Solute carrier 8 gene family
SLC24	Solute carrier 24 gene family
SR	Sarcoplasmic reticulum
TM	Trans-membrane segment
VCX	Vacuolar $\text{Ca}^{2+}/\text{H}^+$ exchanger
LTCC	L-type voltage-dependent Ca^{2+} channel
XIP	NCX inhibitory peptide

Typical hallmarks of SLC8 (NCX) transporters

Genes, alternative splicing, and tissue-specific expression of NCX variants

The $\text{Na}^+/\text{Ca}^{2+}$ exchanger proteins represent an antiporter system that utilizes the electrochemical gradient of Na^+ to

In memoriam John Reeves

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catalyze Ca^{2+} -extrusion from the cytosol or organelle matrix [15, 78, 125]. The SLC8 (NCX) gene family is one of five families belonging to the CaCA (Ca^{2+} /cation antiporter) superfamily [26, 94, 125]. The phylogenetic tree of the SLC8 gene family and its place in the CaCA superfamily has been extensively reviewed [94, 115, 116] and will not be covered here. Briefly, members of the CaCA superfamily share similar topology, comprising two clusters; each cluster contains five or six transmembrane helices (TM), and two clusters are joined by a cytoplasmic loop of varying lengths [91, 94, 110, 125, 151, 154]. The CaCA proteins possess a conserved sequence motif in each cluster (α_1 and α_2 segments), which are involved in the ion transport events [91, 94, 125, 151].

The recently discovered crystal structure of archaeobacterial *Methanococcus jannaschii* (NCX_Mj) represents a long-awaited breakthrough [91] and offers new opportunities for

systematically testing the specific molecular mechanisms underlying the alternating access in the antiporter system. The molecular weight of eukaryotic NCX proteins (typically containing 930–970 amino acids) is more than three times higher than that of archaeobacterial NCX_Mj, which contains “only” 301 amino acid residues [91]. Although the crystal structure of eukaryotic NCX is still unavailable, recent evidence suggests that the total number of helices as well as their packing in the membrane might be quite similar in eukaryotic and archaeobacterial NCX [91, 132], meaning that the size differences among phylogenetically distant NCXs are due to regulatory domains associated with eukaryotic NCX proteins (Fig. 1).

Mammals express the *SLC8A1* (NCX1), *SLC8A2* (NCX2), and *SLC8A3* (NCX3) genes; NCX1 and NCX3 undergo alternative splicing [94, 107, 125]. Recent evidence indicates

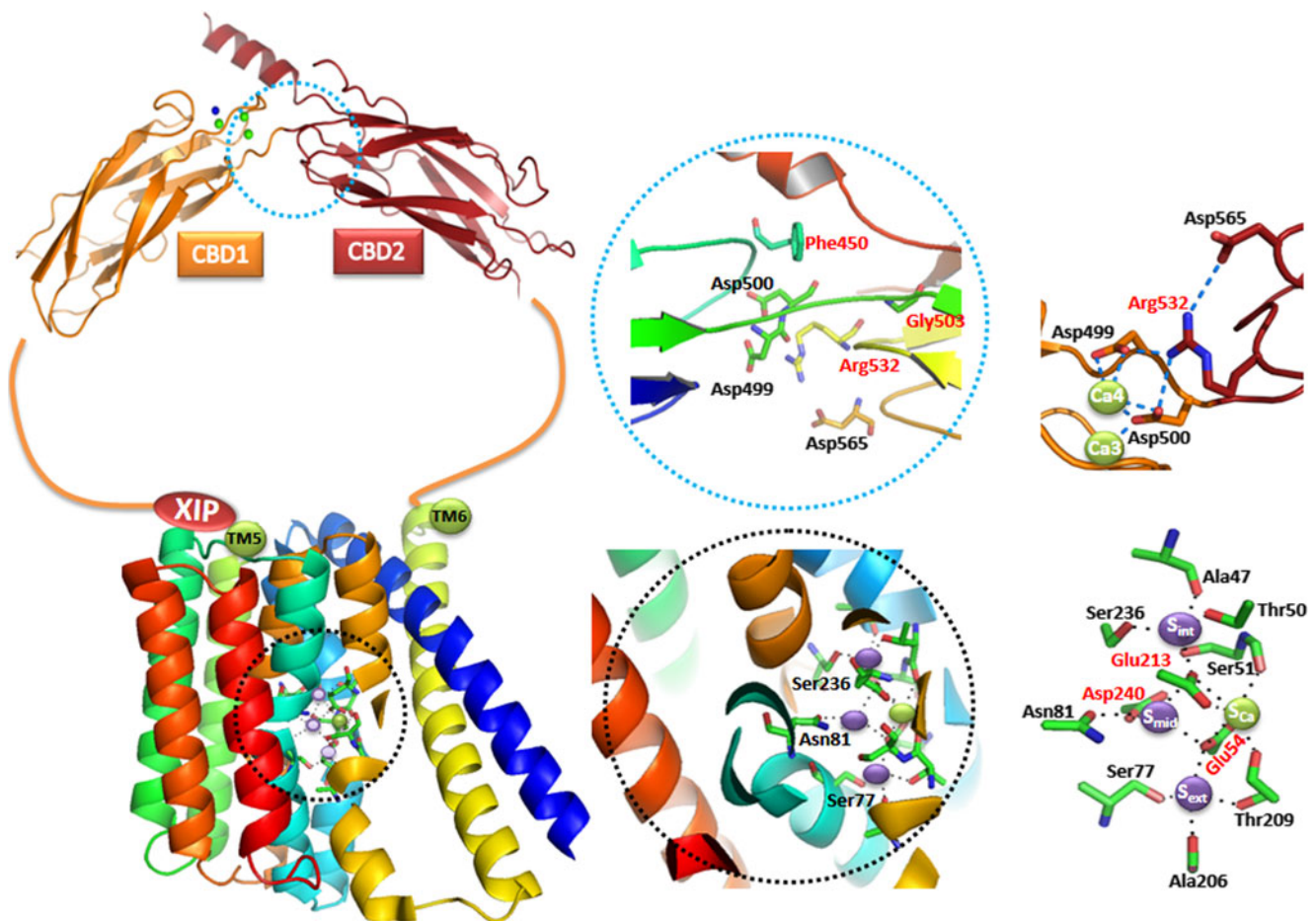


Fig. 1 NCX structure. Archaeobacterial and eukaryotic NCX contain ten trans-membrane helices (TM1–10). The cytosolic regulatory f-loop (~500 amino acids) contains two Ca^{2+} -binding CBD domains, CBD1 and CBD2, which are connected with the TM-5 and TM-6, respectively, though the long linkers. The auto-inhibitory segment, XIP (20 amino acids), is connected to the C-terminal of TM-5. The interdomain Ca^{2+} -sensing module at the interface of the two CBD domains involves two amino acids on CBD1 (Asp499 and Asp500) and CBD2 (Arg532 and Asp565). Occupation of Ca3–Ca4 sites by Ca^{2+} may shift a dynamic

equilibrium of disorder-to-order transition (population shift) due to charge neutralization and coordination, thereby constraining conformational freedom of the two-domain CBD tandem, rigidifying the NCX1 f-loop, and triggering allosteric signal transmission to the membrane domains. The crystal structure of NCX_Mj reveals an ion-binding cluster with four ion-binding sites, one for Ca^{2+} (S_{Ca}), and three for Na^{+} (S_{ext} , S_{mid} , S_{int}), posed in a diamond-shaped pattern. Twelve amino acid residues contribute to ligation of ions, whereas Asp240, Glu213, and Glu54 play a critical role in coordinating and transport of ions

that NCLX protein (Slc8b1), previously assigned to the NCKX family (the SLC24 gene family mediating the Na^+ / Ca^{2+} exchange coupled with the K^+ co-transport), is a long-wanted mitochondrial Na^+ / Ca^{2+} exchanger [122] owing to a unique feature that enables it to transport either the Na^+ or Li^+ ion (but not K^+) in exchange for Ca^{2+} . The properties of NCKX [140] and NCLX [24, 121] were recently summarized and will not be discussed here. Three mammalian genes (NCX1–3) and their splice variants are expressed in a tissue-specific manner to mediate Ca^{2+} fluxes in many cell types [93, 125]. Namely, NCX2 and NCX3 have been found in the brain and skeletal muscles, whereas NCX1 is universally disseminated. At least 17 NCX1 and 5 NCX3 proteins are produced through alternative splicing, whereas no splice variants of NCX2 have been yet identified. The splice variants arise from a combination of six small exons (A, B, C, D, E, and F), whereas a mutually exclusive exon (either A or B) appears in each given splice-variant [86, 89]. Interestingly, the cardiac and neuronal variants contain exon A, whereas the kidney, stomach, and skeletal muscle variants include exon B (Fig. 2). The structural organization of A–F exons has physiological relevance. The splice-variant segment is exclusively allocated within the CBD2 domain [57] and modifies the response kinetics, dynamic range, and affinity of allosteric sensors [43, 47], thereby exhibiting positive, negative, or no response to regulatory Ca^{2+} [18–22]. For example, Ca^{2+} activates the brain (AD), cardiac (ACDEF), and kidney (BD) variants, although the Ca^{2+} -induced relieve of Na^+ -dependent inactivation is observed only in the cardiac and brain variants [38, 39]. In contrast, the regulatory Ca^{2+} inhibits *Drosophila* NCX (CALX1.1), but it has no effect on the CALX1.2 variant [66, 114].

Ion-transport mechanisms and alternating access

NCX proteins represent a carrier-type “exchange-only” mechanism (otherwise known as antiporter or counter transport system) [79, 82, 85, 143], where a transmembrane electrochemical gradient of Na^+ is utilized to couple stoichiometric and electrogenic ion exchange ($3\text{Na}^+:\text{Ca}^{2+}$) for each transport cycle [15, 130]. According to the unifying concept of an alternating access mechanism [41, 143], NCX might undergo a major conformational change involving an alternative exposure of transport sites to extracellular and intracellular sides of the membrane, where the ion binding to the transport-site pocket must promote a major conformational change, associated with alternative access [41, 78, 143]. The NCX proteins can mediate the partial reactions and the Na^+/Na^+ and $\text{Ca}^{2+}/\text{Ca}^{2+}$ exchanges as a part of the $\text{Na}^+/\text{Ca}^{2+}$ exchange cycle, although the physiological relevance of these partial reactions is unclear [15, 78, 79].

Kinetic studies reveal that the $\text{Na}^+/\text{Ca}^{2+}$ exchange cycle can be described as separate movements of 3Na^+ and Ca^{2+} ions through the exchanger [80, 109], whereas the Na^+ -translocation accounts for a voltage-sensitive step of the exchange cycle [63, 81–83, 85]. Consistent with this, the recently derived crystal structure of archaeobacterial NCX_Mj revealed three binding sites for Na^+ and one binding site for Ca^{2+} , where the simultaneous occupation of all four binding sites by 3Na^+ and Ca^{2+} ions is thermodynamically forbidden [91]. Since the ion-transport pathways of archaeobacterial and eukaryotic NCX proteins show striking similarities [75, 91, 132], phylogenetically distant NCX orthologs might share common mechanisms to drive alternative access. Electrostatic interactions of 3Na^+ and Ca^{2+} ions with the ion-binding

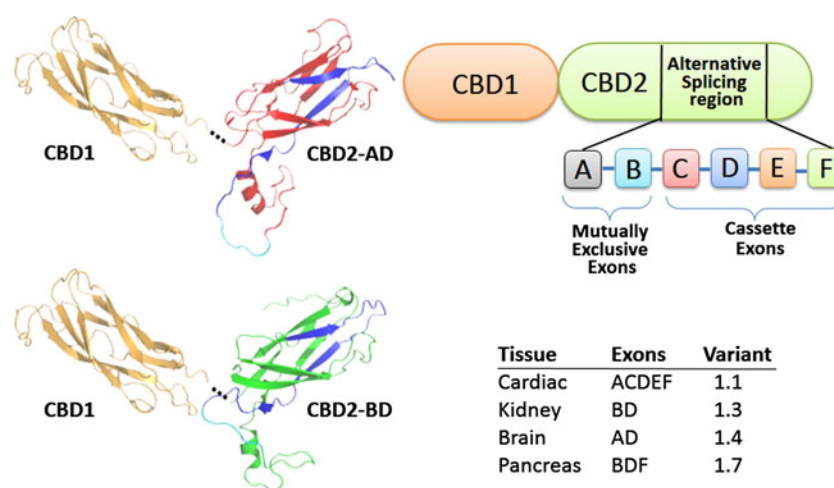


Fig. 2 Splice variants of NCX. The splice segment is exclusively located on CBD2. Tissue-specific splice variants arise from a combination of six small exons A, B, C, D, E, and F, whereas a mutually exclusive exon (either A or B) shows up in every splice variant. The NMR structures of CBD1 (PDB 2FWS, orange), CBD2-AD (PDB 2FWU, red), and CBD2-

BD (PDB code 2KLT, green) structures were superimposed on the template of the CBD12-E454K crystal structure (PDB 3US9) to show the position of the splice segment. Residues encoded by exons A and B are shown in blue and residues encoded by exon D are cyan

pocket of NCX might play a crucial role in generating critical transitions along the transport cycle [41, 88, 91]. Kinetic observations are consistent with the notion that the unloaded ion-binding domain bears two negative charges ($z=-2$), meaning that the ground-state E.Na3 species are positively charged (and voltage-sensitive) and the E.Ca species are electroneutral [81–85]. This working hypothesis is very attractive to test in light of the newly derived X-ray structure of NCX_Mj [91].

Turnover rates of the ion-transport cycle in phylogenetically distant NCX variants

It is widely accepted that mammalian NCX variants represent a “high-capacity/low-affinity” system capable of rapidly extruding large amounts of cytosolic Ca^{2+} in a relatively short time in order to maintain the proper dynamic balance between the Ca^{2+} entry and exit modes (e.g., the size of the Ca^{2+} transient amplitude and its decay kinetics in cardiomyocytes) [8, 9]. Mammalian NCX proteins are low-abundant proteins (<0.1 % of the total plasma membrane protein) owing to the high rates (2,500–5,000 s^{-1}) of single-cycle turnover either in intact cardiomyocytes [109], excised patches of cardiomyocytes [63], or isolated sarcolemma vesicles [6]. These high rates of ion transport are compatible with the site density of 250–2,500 exchangers/ μm^2 in cardiomyocytes, corresponding to $\sim 10^6$ – 10^7 copies of NCX per cell [6, 63, 106, 109]. Interestingly, there are at least 50-fold differences in the turnover rates among NCKX proteins [33, 136, 140]. Moreover, taking into account the protein expression levels and the ion flux activities of NCX_Mj, the calculated turnover rate of the transport cycle might be at least 10^3 times slower in archaeobacterial NCX_Mj than in mammalian NCX. More extensive experimentation is required for quantifying the turnover rate of a single transport cycle in NCX_Mj and the relevant kinetic mechanisms governing its catalytic capacity. This issue is especially interesting in light of the striking structural similarities in ion transport pathways owing to the mammalian and archaeobacterial NCX variants [75, 91, 132]. Again, these differences in the catalytic capacity may be physiologically relevant for mammalian NCX proteins since these proteins are “committed” to rapidly extrude the cytosolic Ca^{2+} from the cell within a limited time slot in order to fulfill the physiological demands of functionally diverse cell types.

The directionality of NCX-mediated Ca^{2+} flux

The $\text{Na}^+/\text{Ca}^{2+}$ exchange can occur either in the forward (Ca^{2+} -efflux) or reverse (Ca^{2+} -influx) mode. At constant RT/F (~ 25 mV), the directionality of net Ca^{2+} flux depends on cytosolic and extracellular $[\text{Na}^+]$ and $[\text{Ca}^{2+}]$ and on the membrane potential [8–10, 15]. At fixed levels of extracellular $[\text{Na}^+]_o$ and $[\text{Ca}^{2+}]_o$ and resting membrane potential ($E_m =$

-80 mV), the electrochemical driving force for electrogenic ($3\text{Na}^+/\text{Ca}^{2+}$) exchange is $E_{\text{NCX}} = -32$ mV.

$$E_{\text{NCX}} = 3E_{\text{Na}} - 2E_{\text{Ca}} = (RT/F) \ln \left\{ \frac{([\text{Na}]_o)^3 [\text{Ca}]_o}{([\text{Na}]_i)^3 [\text{Ca}]_i} \right\}$$

Thus, at resting E_m , the Ca^{2+} -efflux is a thermodynamically favored mode ($E_{\text{NCX}} > E_m$), where the $\text{Na}^+/\text{Ca}^{2+}$ exchange changes the directionality when E_{NCX} and E_m become equal. The dynamic swings in $[\text{Na}^+]_i$, $[\text{Ca}^{2+}]_i$, and in membrane potential (e.g., in cardiomyocytes) modify the thermodynamic driving force, which actually governs the directionality of the net Ca^{2+} movements [8, 15]. Therefore, 10–20-fold changes in cytosolic $[\text{Ca}^{2+}]_i$ and varying the membrane potential (from -90 mV to $+50$ mV) in ventricular cardiomyocytes modify the thermodynamic driving force (E_{NCX}) and thus, the directionality of the $\text{Na}^+/\text{Ca}^{2+}$ exchange along the action potential [8, 9]. Moreover, at fixed extracellular $[\text{Na}^+]$ levels, even two-fold changes in cytosolic $[\text{Na}^+]_i$ can significantly affect the E_{NCX} profile (and thus, the directionality) along the action potential since, according to the equation (see above), the changes in $[\text{Na}]_i$ are powered in the third degree. Although the Ca^{2+} -efflux is a predominant mode of NCX under default conditions, the reverse mode (Ca^{2+} -influx) may become functionally important under both physiological and pathophysiological conditions [10, 15, 79]. For example, in cardiac disease, a prolonged APD (action potential duration) and elevated $[\text{Na}^+]_i$ promote Ca^{2+} -entry through the reverse mode of NCX, thereby mediating life-threatening arrhythmogenic currents [8–10, 15].

NCX interaction with specific ion-transport systems

The coupling of the reverse mode NCX with specific ion-transport systems may mediate the net fluxes of Na^+ and/or Ca^{2+} , which may effectively integrate and regulate important physiological events on the cellular and systemic levels [105, 126, 137, 155]. Notably, the physiological relevance of the reverse mode NCX has been controversial for many years, but the contribution of NCX-mediated Ca^{2+} -influx in EC-coupling, vascular constriction, and synaptic transmission has been underscored in recent publications [120, 126, 137]. According to these reports, the Ca^{2+} -influx through the NCX protein is coupled with the Na^+ -efflux either through the voltage-sensitive or store-operated Na^+ conducting channels, where a rapid increase in $[\text{Na}^+]_i$ within the restricted space of a putative “ Na^+ microdomain” drives the NCX-mediated Ca^{2+} -entry into the cell. More specifically, in smooth muscle cells, the Ca^{2+} -influx through NCX1 can be coupled with Na^+ transport via store-operated channels, TRPC6 and Orai1, where TRPC6, Orai1, and NCX1 are colocalized with α -2 Na^+/K^+ ATPases in the plasma membrane clusters near junctional SR [16, 126, 155–158]. The open question is: How do microdomains govern the net fluxes of Na^+ and/or Ca^{2+} from

a channel or exchanger (the source) to its target transducer or pump (the sink), while preventing “loss” of ions to the bulk cytosol? [126, 155]. To address this question, one needs detailed information about the architecture of the plasma membrane–SR junction including the position and site-density of relevant ion-transport systems as well as knowledge of the physical factors (e.g., viscosity) limiting the ion diffusion nearby the membrane space.

“Secondary” (kinetic) modulation of NCX

Kinetic modulation of NCX is mediated by interaction of cytosolic Ca^{2+} , Na^+ , and H^+ ions with sites that are not directly involved in Ca^{2+} and Na^+ transport (translocation), thereby representing “secondary” or allosteric regulation of NCX [35–38, 59–63, 100]. Owing to hefty and speedy swings in cytosolic $[\text{Ca}^{2+}]$ during the action potential, the allosteric activation of NCX by Ca^{2+} is especially important in excitable tissues [8–10, 131]. For example, in cardiomyocytes, NCX-mediated ion-currents are elevated ~25-fold when the cytosolic $[\text{Ca}^{2+}]_i$ level rises from 0.1 to 2 μM , showing that the level is highly dependent on $[\text{Ca}^{2+}]_i$ with an unusually high degree of cooperativity [21, 49]. Rapid removal of cytosolic Ca^{2+} in patch-clamp experiments results in slow inactivation (I_2 state) of NCX, thereby exhibiting hallmark kinetic differences among tissue-specific NCX variants, which perhaps have physiological relevance [65, 100, 131]. Recent studies demonstrated that high-affinity Ca3 – Ca4 sites on the CBD1 domain represent a primary Ca^{2+} sensor [27, 28, 118, 119], whereas the specific interdomain interactions between the two CBD domains result in slow dissociation of “occluded” Ca^{2+} from Ca3 – Ca3 sites [44–48]. These interdomain interactions are “secondarily” modified by a tissue-specific splice segment located on CBD2 [43, 44], which may represent tissue-specific differences in slow inactivation (I_2) of matching NCX variants [38, 39].

In patch-clamp experiments, a rise in cytosolic $[\text{Na}^+]$ rapidly stimulates eukaryotic NCX (due to Na^+ interaction to transport sites), followed by hallmark slow inactivation of the exchanger, which levels off at certain steady-state levels of ion current (I_1 inactivation state) [59–62]. The amplitude and kinetics of I_1 inactivation characteristically differ among NCX variants [38, 39], whereas the elevation of cytosolic $[\text{Ca}^{2+}]$ relieves Na^+ -dependent inactivation [59–62, 65]. The Na^+ -dependent inactivation of NCX cannot be explained by direct interaction of Na^+ with Ca^{2+} binding sites on CBD domains, because Na^+ has no effect on Ca^{2+} binding to these regulatory sites in the isolated preparations of CBD1, CBD2, or CBD12 [21, 23, 44, 47]. It was suggested that the “regulatory Na^+ site” may be located on the “catenin-like domain” at the terminals of the cytosolic f-loop [57], but there are no data that support this. Most probably, the Na^+ -dependent inactivation involves the ion-transport sites and the XIP region, as

originally suggested by Hilgemann and collaborators [59–62, 65, 131], although the underlying structure–activity relationships remain unclear. Anyway, Ca^{2+} binding to the CaI site of CBD2 relieves Na^+ -dependent inactivation [27, 28, 118, 119], although it is presently unclear how the allosteric signal is transmitted from CBD2 to transport sites.

Eukaryotic NCX is extremely sensitive to mild cytosolic acidification (a pH decrease from 7.2 to 6.9 results in nearly 90 % inactivation of NCX), thereby emphasizing the physiological relevance of NCX “proton block” under acidosis and ischemia conditions [35–37]. In general, protons may interact with transport and/or regulatory domains, although there is no evidence that within the physiological range of pH the protons affect the ion binding affinity and/or transport rates. According to Doering and Lederer, there are two components that contribute to proton inhibition of NCX in patch-clamp experiments, one of which is fast but the other is slow and requires cytoplasmic Na^+ [36, 37]. In isolated preparations of CBDs, Na^+ has no effect on the competition of Ca^{2+} and H^+ for Ca^{2+} -binding to CBD12 [21, 23, 44], which is consistent with Na -independent proton block of NCX. The mechanism underlying Na -dependent proton-inhibition of NCX remains unclear. Most probably, the protonation of CBD sites prevents the Ca^{2+} -dependent displacement of inhibitory Na^+ from the transport sites, since protons can effectively compete for Ca^{2+} binding to CBDs, thereby causing a dramatic shift in $[\text{Ca}]_i$ -dependent activation of NCX in cardiomyocytes [21]. Thus, CBDs may function as a dual Ca^{2+} /pH sensor whereas, under acidosis/ischemia conditions, the interaction of protons with CBDs decreases Ca^{2+} affinity for allosteric activation and thus shuts down the NCX activation to prevent NCX-mediated arrhythmogenic ion-currents [21].

Structure–function relationships

NCX_Mj as a template structure for the CaCA superfamily

This structure describes the outward-facing (extracellular) conformation of NCX_Mj with ten trans-membrane helices, TM1–10 (Fig. 1). The ion-binding pocket contains four ion-binding sites: one for Ca^{2+} (S_{Ca}), and three for Na^+ (S_{ext} , S_{mid} , S_{int}), arranged in a diamond-shaped configuration. Two apparent passageways allow separate access for Na^+ and Ca^{2+} ions to a central ion-binding pocket, where 12 residues contribute to Na^+ and Ca^{2+} ligation (four in TM2 and TM7, and two in TM3 and TM8). Similar passageways for ion access and ion-pockets might exist in NCX_Mj [91], mammalian NCX [75, 132], and NCKX [2, 140] proteins, although the specific structural differences might be responsible for the differences in the ion-binding selectivity and turnover rates among the related proteins.

In contrast to previous argumentations, recent experimental approaches imply a ten-helix structure with a similar packing of TMs and ion-transport pathways in the archaeobacterial and eukaryotic NCX variants [75, 132]. Therefore, the basic mechanisms underlying ion transport might be similar in phylogenetically distant NCX variants, but the catalytic capacity or regulation can widely differ among NCX orthologs. This tentative conclusion seems to be encouraging, since a better understanding of the basic ion transport mechanisms in NCX_Mj might help in elucidating physiologically more relevant features of mammalian NCX proteins such as the dynamic features of regulation and pharmacological targeting of tissue-specific NCX variants under altered conditions.

In sharp contrast with eukaryotic NCX, the cytosolic loop between TM5 and TM6 is extremely short in NCX_Mj, meaning that this loop cannot be a prototype for a large cytosolic f-loop (~500 amino acids) of eukaryotic NCX bearing the regulatory CBD domains (Fig. 1). Most importantly, eight helices of NCX_Mj (TM2–5 and TM7–10) generate a tightly packed hub (which is perpendicularly inserted into the membrane), whereas two long and tilted helices (TM1 and TM6) are limply packed in front of a rigid eight-helix core [91]. This structure originally predicted the sliding of TM1 and TM6 toward the rigid core helices, which is a hallmark feature representing a major conformational change associated with alternating access. However, it remains unclear how partial occupation of four sites by Ca^{2+} or 3 Na^+ ions drives the sliding of TM1/TM6 to initiate alternative access.

In 2013, the crystal structures of inward (cytosol)-facing conformations were resolved for three members of the $\text{Ca}^{2+}/\text{H}^+$ (CAX) exchanger (which also belongs to the CaCA superfamily), showing astonishing structural similarities to outward-facing NCX_Mj [91, 110, 151, 154]. This breakthrough provided valuable information on the trajectory of helix movements associated with alternative access in NCX and CAX proteins, although the dynamic features of conformational changes underlying the helix sliding remain unclear. An interesting outcome of these new structures is that, in both NCX and CAX, the gap between TM2 and TM7 might hasten the sliding of the gating bundles (TM1 and TM6) in the inward-facing apo state (in order to prevent ion-leakage). According to this scenario, ion binding to a yet undefined site may result in the gap's closure, thereby allowing the sliding motion of the gating bundle. Thus, the open question is how the ion-protein interactions drive the alternative exposure of the ion-binding pocket.

One possibility is that the ion-selectivity and electrostatic properties of a four-site ion-binding cluster (S_{ext} , S_{mid} , S_{int} , and S_{Ca} sites arranged in a diamond-shaped configuration) dictate the conformational transitions associated with alternative access. More specifically, based on crystal data, it is reasonable to assume that the S_{ext} and S_{int} sites have high selectivity to the Na^+ ion, whereas the S_{mid} and S_{Ca} sites are

less selective to Na^+ and thus, these sites can be occupied either by the Ca^{2+} or Na^+ ions [91]. If so, the flickering of the Ca^{2+} or Na^+ ions between the “vacant sites” (S_{mid} and S_{Ca}) may involve specific interactions of charged amino acids (E54, E213, and D240) with ligated ions, which thermodynamically favor the gap closure between TM2 and TM7 to promote alternative access. The challenge is to identify the relevant ion-protein interactions that result in alternating exposure of the ion-binding pocket.

Strikingly, the NCX, NCKX, and CAX families contain structurally related hallmark segments, α_1 (on TM2 and TM3) and α_2 (on TM7 and TM8) [91, 110, 140, 151, 154], thereby suggesting that the principal mechanism underlying ion-transport and alternating access might be similar among Ca-CA proteins. In addition, it is unclear to what extent the fine nuances of the α_1 and α_2 sequence contribute to differences in the ion-transport rates owing to NCX variants. Notably, all eukaryotic NCX variants (including CALX) contain a huge cytosolic f-loop between TM5 and TM6, bearing a number of regulatory domains [27, 28, 57, 107, 125], which are completely absent in NCX_Mj [91]. At the N terminus of the f-loop is located a positively charged auto-inhibitory XIP sequence (20 amino acids), exhibiting an α -helix structure [90, 101], where this region is involved in both Na^+ and PIP_2 regulation [65, 90]. The cytosolic loop-f contains Ca^{2+} binding regulatory domains, CBD1 and CBD2, which are connected in a head-to-tail fashion to form a CBD12 tandem [12, 13, 57, 58]. CBD1 contains a primary allosteric Ca^{2+} sensor (Ca3–Ca4 sites) [27, 28, 119], the affinity and kinetics of which are modulated by a spliced segment located on CBD2 [12, 43, 58].

Structure-dynamic features of regulatory CBD domains

High-resolution X-ray and nuclear magnetic resonance (NMR) studies of isolated CBD1 and CBD2 domains revealed an immunoglobulin-like β -sandwich structure with seven antiparallel β -strands containing four Ca^{2+} binding sites (Ca1–Ca4) on CBD1 and two Ca^{2+} sites (CaI–CaII) on CBD2 [12, 57, 108]. In the cardiac, brain, and kidney variants, the Ca3 and Ca4 sites of CBD1 have high affinity for Ca^{2+} binding ($K_d=0.05\text{--}0.2\text{ }\mu\text{M}$), whereas the remaining two sites of CBD1, namely, Ca1 and Ca2, exhibit low affinity ($K_d>20\text{ }\mu\text{M}$) for Ca^{2+} binding [23, 43, 44, 47, 57]. In the cardiac and brain variants, the CaI site of CBD2 binds Ca^{2+} with a K_d value of $2\text{--}10\text{ }\mu\text{M}$, whereas the CaII site of CBD2 exhibits lower affinity ($K_d>20\text{ }\mu\text{M}$) for Ca^{2+} binding [13, 44, 47, 58]. Mutant analysis of full-size NCX revealed that, in the cellular system, only three of the six Ca^{2+} sites (Ca3 and Ca4 on CBD1 and CaI on CBD2) contribute to $[\text{Ca}^{2+}]$ -dependent regulation of NCX [27, 28, 118, 119]. Namely, in the cellular system, the Ca3–Ca4 sites govern the “affinity” of the primary allosteric sensor ($K_{0.5}\sim 0.3\text{ }\mu\text{M}$), whereas the CaI site is

involved in $[\text{Ca}^{2+}]$ -dependent alleviation of Na^{+} -dependent inactivation, showing a $K_{0.5}$ value of $\sim 10 \mu\text{M}$ [28, 118, 119]. Most probably, the low-affinity sites (Ca1, Ca2, and CaII) are Mg^{2+} rather than Ca^{2+} sites, which are constitutively occupied by Mg^{2+} under physiologically relevant ionic conditions [23, 25, 46]. Moreover, occupation of Ca1–Ca2 sites by Mg^{2+} decreases the affinity of the primary sensor (Ca3–Ca4 sites), whereas the occupation of the CaII site by Mg^{2+} increases the affinity of the CaI site [23, 46, 47]. The rationale behind this is that this occupation maintains the properties of regulatory Ca^{2+} sensors within a physiologically relevant range, thereby exhibiting K_d values of $0.2\text{--}10 \mu\text{M}$ and Ca^{2+} off-rates of $0.02\text{--}150 \text{ s}^{-1}$ [47].

There is an increasing body of evidence for synergistic interactions between CBDs, either in the isolated CBD12 or intact NCX [23, 58, 76, 139], where the short interdomain linker (501-HAGIFT-506) encodes unique information governing the flexibility of CBD movements and Ca^{2+} -driven coupling for decoding and transmission of allosteric signals [23, 45]. For example, in order to couple Ca^{2+} -driven conformational transitions in CBDs, it is obligatory to have a glycine residue at position 503 not only in isolated CBD12 [45] but also in intact NCX [99]. Recently derived crystal structures of an isolated two-domain tandem (CBD12) revealed a unique interface between the CBD domains, whereas the occupation of the Ca3–Ca4 sites by Ca^{2+} generates interdomain salt-bridges in which R532 (located in CBD2) tethers D565 from CBD2 with D499 and D500 from CBD1 [48, 153]. Interestingly, these bifurcated salt-bridges are obligatory for slow Ca^{2+} dissociation and for Ca^{2+} -induced restriction of CBD movements (Fig. 1), thereby representing a hallmark feature for regulatory coupling of CBDs [23, 45–47]. Namely, the sequential dissociation of two Ca^{2+} ions from Ca3–Ca4 sites involves a rapid dissociation of the first Ca^{2+} ion, followed by a slow dissociation of the second (occluded) Ca^{2+} ion (the off-rate of which is 20–50 times slower than for the first Ca^{2+} ion) [23, 45, 46]. The slow off-rates of occluded Ca^{2+} (measured by stopped-flow) differ among the cardiac, kidney, and brain variants ($k_s = 0.02\text{--}0.5 \text{ s}^{-1}$) [43, 47] and correlate with the slow inactivation kinetics (I_2), observed for matched NCX variants in patch-clamp experiments [38, 39].

A fundamental question is how is the information about Ca^{2+} binding to the primary allosteric sensor on CBD1 (Ca3–Ca4 sites) decoded, diversified, and propagated to ion-transport domains, whereas the positive, negative, or no sustained response to regulatory Ca^{2+} is realized in diverse NCX variants. The SAXS [57, 58], FRET [76], and NMR [139] were explored to resolve the dynamic mechanisms underlying the Ca^{2+} -induced conformational transitions in the isolated preparations of the two-domain CBD12 tandem. Advanced ensemble optimization method (EOM) SAXS analyses revealed that, as a result of Ca^{2+} binding to the Ca3–Ca4 sites, more constrained conformational states become highly

populated at a dynamic equilibrium in the absence of global conformational transitions in the CBDs' alignment [46]. This conclusion is consistent with NMR analyses describing a similarly extended shape for both the apo and Ca^{2+} -bound states of CBD12, where Ca^{2+} binding results in rigidified motions of CBDs [139]. Moreover, EOM-SAXS data can rationalize crystallographic data, showing that the interdomain angles between CBDs in Ca^{2+} -bound form are nearly identical in NCX1.4 [48] and CALX1.1 [153] owing to positive and negative responses to regulatory Ca^{2+} , respectively. Since the structural organization of the CBD interface is highly homologous (if not identical) among NCX and CALX orthologs (showing a positive, negative, or no sustained response to regulatory Ca^{2+}), it is reasonable to conclude that a primary mechanism for decoding the allosteric signal is very common among diverse NCX phenotypes [48]. The question is how the allosteric signal from Ca^{2+} binding regulatory domains (CBD1 and CBD2) to ion-transport sites of NCX (located 80–90 Å away) is propagated in specific cases when the negative, positive, or no response to regulatory Ca^{2+} is instigated. This issue is especially interesting in light of principle mechanisms proposed for transmission of allosteric signals in multi-domain proteins [96, 148].

Up- and downstream signaling cascades

Metabolic regulation of NCX holds particular promise due to its pathophysiological relevance and potential outlook for devising selective blockers and activators possessing clinical relevance. NCX is regulated by small cytosolic effectors such as PIP_2 , phosphoarginine, ATP, and putative endogenous inhibitors [4, 7, 22, 35, 50]. Since these issues were extensively reviewed in the past, only the recent contributions will be covered here.

NCX, MAPK-cascade, and related regulatory pathways

To evaluate the MAPK-dependent regulation of NCX, recent work examined the expression levels and activity of specific NCX1–3 isoforms in PC12 cells under conditions in which the ERK1/2, JNK, and p38 MAPKs were shut down, pharmacologically blocked, or activated with NGF [141]. These studies demonstrated isoform-dependent regulation of NCX in a MAPK-cascade-specific manner. Namely, upon NGF stimulation, both ERK1/2 and p38 upregulated NCX1 and NCX3, whereas only p38 had the capacity to downregulate NCX2. Notably, in NGF-untreated cells (where NCX1 and NCX3 were controlled by JNK and ERK1/2, respectively), NCX2 was totally MAPK-independent. Moreover, p38 does not regulate the basal expression levels of any NCX1–3 isoform in PC12 cells, whereas in cardiomyocytes p38 upregulates NCX1 [141]. These findings may be useful for selective

pharmacological targeting of predefined NCX isoforms. This issue is especially interesting in conjunction with brain injuries (e.g., brain ischemia and stroke), where the specific expression profiles of NCX isoforms are observed under pathophysiological conditions [17–19].

The involvement of NCX in NO-induced cellular toxicity in neuroblastoma, astrocytes, and microglia cells has been recently documented [145, 146]. Interestingly, in neuroblastoma and astrocyte cells, NO stimulates reverse mode NCX presumably through a cGMP/protein kinase G (PKG)-dependent mechanism, accompanied by elevated $[Ca^{2+}]_i$ levels, ROS production, and phosphorylation of ERK, JNK, and p38 MAPK, which finally ends in apoptosis. In contrast, in microglia, NO stimulates forward mode NCX, which results in ER Ca^{2+} depletion and ER stress associated with apoptotic cell death [117, 145, 146]. The relevant mechanisms could be of special interest in view of the role of NO in neurodegenerative disorders (including Alzheimer's and Parkinson's diseases), because the Ca^{2+} and NO signaling pathways may synergistically interact with each other to promote neurotoxicity. An open-ended problem concerning the mechanisms underlying NO-mediated NCX activation is that the actual phosphorylation of NCX by PKG has not yet been demonstrated at any level of an experimental system (in vitro, in situ, or in vivo). It is possible that NO-mediated NCX activation involves phosphorylation–dephosphorylation of yet an unidentified player (e.g., phospholemman), which may directly interact with NCX to modulate its activity. Further resolution of the underlying molecular mechanisms may help in devising selective blockers for specific neuronal NCX variants, which could be beneficial for suppressing neurodegenerative progression.

A recent report reveals that reverse mode NCX (Ca^{2+} entry) activates the plasma membrane PKC α of endothelial cells to promote VEGF-induced ERK1/2 phosphorylation and angiogenesis [3]. Besides the endothelial cells, the reverse-mode NCX was reported to be compulsory for ERK1/2 activation in neuroblastoma cells [144, 145]. The significance of these findings is that NCX may activate ERK1/2 downstream of thrombin and angiopoietin, two pathways that are associated with tumor angiogenesis and evasion of anti-angiogenic therapy. This research area becomes especially interesting in light of the fact that the relevant regulatory pathways may represent major therapeutic targets in anti-angiogenic cancer and chiefly contribute to drug resistance in VEGF and related signaling pathways. Therefore, it is challenging to look for and identify new drug candidates that selectively inhibit tissue-specific NCX isoforms, with the goal of hampering drug-resistant cancer forms.

NCX and phospholemman

Phospholemman (PLM) is a 72-amino acid phosphoprotein with a single transmembrane domain, which is highly

expressed in cardiomyocytes to regulate Na^+/K^+ -ATPase [124], Na^+/Ca^{2+} exchange [30, 31], and L-type Ca^{2+} channel [52] activities; thus, it can affect the heart rate and/or the contractility force. Phosphorylation of Ser68 by PKA is an important regulatory mode, resulting in NCX inhibition and activation of Na^+/K^+ -ATPase in cardiomyocytes [31, 32, 124]. Notably, the cytoplasmic tail of PLM interacts with two short segments on NCX located on the f-loop, whereas the PLM-dependent regulation is independent of Ca^{2+} interaction with CBDs [31].

Under catecholamine stress conditions (when PLM is phosphorylated at Ser68 by PKA), inhibition of Na^+/K^+ -ATPase by PLM is relieved, whereas NCX activity is suppressed [30–32, 124]. Therefore, under stress conditions, phosphorylated PLM may decrease arrhythmia risks (by suppressing Ca^{2+} and Na^+ overload through Na^+/K^+ -ATPase activation), and phosphorylated PLM may preserve the inotropic capacity of muscle contractility (by decelerating Ca^{2+} efflux through NCX inhibition). Interestingly, PLM-S68E mutations in adult cardiomyocytes lead to NCX inhibition, which therefore represents the potential capacity for enhancing $[Ca^{2+}]_i$ -transients and contractility [31–33]. Thus, this approach may be used for specific inhibition of NCX under disease-related conditions, whereas the targeting of PLM-Ser68 could be beneficial for improving cardiac performance under stress.

Physiological functions

Although NCX variants are associated with diverse regulatory machinery to fit the tissue-specific physiological demands of Ca^{2+} homeostasis, the partial contributions of NCX variants to specific cells and systemic functions remain largely unclear. The present article focuses only on selected topics covering the recent advances.

Knockout mouse models of NCX

Early studies with the NCX knockout models provide information on NCX contributions to physiological functions on a systemic level [67, 128, 134]. Recent studies with organ-specific knock-out mouse models revealed more specific contributions of NCX variants to heart contractility, arrhythmia, and ischemic damage [50, 113, 120, 157], to smooth muscle vasoconstriction and blood pressure regulation [155, 156, 158], and to brain activities (hippocampal long-term potentiation in learning, cerebral reperfusion damage, stroke, preconditioning, etc.) [17–19, 73, 74, 141]. Interestingly, the total deletion of the *Slc8a1* gene results in NCX1-null embryos that cannot perform a spontaneous heart beat and consequently die at early stages of development, whereas those mice with cardiac-specific knockout of NCX1.1 can live to adulthood

[50, 67, 128]. The accumulating data suggest that NCX variants contribute to specific systemic functions and organ protection. For example, ablation of NCX1 protects against heart ischemia–reperfusion injury [113], whereas mice lacking the brain NCX2 (the major isoform of the brain) exhibit an enhanced capacity for learning and memory [74].

Recent studies with ventricular-specific NCX knockout mice show that NCX plays a new role in priming the Ca^{2+} entry in cardiomyocytes [50, 120, 127]. Namely, the rise in cytosolic $[\text{Na}^+]$ within the restricted space of diadic cleft (upon rapid depolarization of the membrane) transiently reverses NCX directionality to mediate Ca^{2+} entry, which “primes” diadic cleft so that subsequent Ca^{2+} entry through LTCC can more efficiently trigger Ca^{2+} release from the SR. An apparent drawback of this putative mechanism is that the time slot between the opening of voltage-sensitive Na^+ and Ca^{2+} channels is so short (<2 ms) that it would be hardly enough to complete even a single turnover of the NCX transport cycle. Thus, computational modeling is required for evaluating the physiological relevance of the proposed mechanism, taking into account the cleft architecture and site-density of relevant ion-transport systems.

The creation of new knockout models for distinct NCX variants is required for elucidating the important mechanisms associated with the specific tissue/organ contribution of NCX variants in systemic function and regulation of the fundamental physiological processes. For example, tissue-specific ablation of mitochondrial NCLX may provide useful information on the contribution of this protein in the systemic regulation of blood pressure, insulin, or neurotransmitter secretion, long-term potentiation of brain activity, and learning, immune response, etc.

NCX, excitation-contraction (EC) coupling, and localized Ca^{2+} -signaling

A major role of NCX in EC-coupling is to maintain a dynamic balance between Ca^{2+} entry and Ca^{2+} exit during the action potential by extruding nearly all Ca^{2+} that has entered through the LTCC into the cell during the depolarization upstroke [8–10, 15]. Thus, NCX represents a unique system that is responsible for fine-tuning and integration of rate/force relationships in cardiomyocytes [8, 9]. Interestingly, in intact cardiomyocytes, the activity of NCX is very low at resting $[\text{Ca}^{2+}]_i$, whereas $\sim 100\%$ of the maximal capacity of NCX is recruited at the $[\text{Ca}^{2+}]_i$ peak [21]. Moreover, the $[\text{Ca}^{2+}]_i$ -dependent allosteric activation of NCX has an unusually high degree of cooperativity in intact cardiomyocytes and shows a steep dependence on $[\text{Ca}^{2+}]_i$ with a Hill coefficient of $n_H=4$ –8 [21, 49, 95]. Most importantly, a time slot for this highly cooperative activation of NCX seems to be several-fold slower than the cardiac cycle, meaning that the activation of NCX by Ca^{2+} is a slow event, which does not occur on a beat-

to-beat basis [49]. Therefore, the regulation of NCX and its dynamic contribution to EC-coupling should be considered in light of multi-beat modeling. An important physiological implication of these observations could be that cardiomyocyte functioning requires a highly sensitive but delayed response of NCX to cytosolic Ca^{2+} to precisely control the dynamic changes in the steady-state rates of Ca^{2+} extrusion that occur on a multi-beat time-scale [49]. However, paradoxically, it is impossible to explain the observed high values of cooperativity for Ca^{2+} -dependent NCX activation even if one were to assume that all six Ca^{2+} binding sites of CBD12 are involved in allosteric regulation (which apparently is not the case, since only three Ca^{2+} sites of CBD12 are involved in allosteric regulation, see above). Interestingly enough, the high values of cooperativity ($n_H=4$ –8) for $[\text{Ca}^{2+}]$ -dependent allosteric activation are observed only in intact cardiomyocytes [21, 49, 95], whereas much lower degrees of cooperativity ($n_H\sim 2$) are detected in excised patches of cardiomyocytes and in an oocyte expression system [13, 27, 118, 119]. A low degree of cooperativity is also observed for Ca^{2+} binding to isolated CBD1, CBD2, and CBD12 preparations [21, 23, 43, 44]. Since all these measurements seem to be reliable, the mechanisms supporting a steep and slow activation of NCX might operate only in intact cardiomyocytes. For example, the Ca^{2+} -dependent slow oligomerization of NCX may occur only in the sarcolemma membranes of intact cardiomyocytes but not in “nonphysiological” preparations.

Recent advances in understanding, the regulatory mechanisms underlying CBD domains (see above) provide new clues regarding the dynamic regulation of EC-coupling. Namely, the CBD12 tandem represents a wide-range Ca^{2+} sensor, the dynamic properties of which are well-suited for sensing the rapid changes in $[\text{Ca}^{2+}]_i$ within sub-cellular compartments (dyadic cleft, cytosolic bulk phase, etc.). Since the LTCCs are basically located in front of the junctional SR (jSR) across a nanoscopic subspace (~ 150 Å), it is expected that when an LTCC opens, the “subspace” $[\text{Ca}^{2+}]_{SS}$ increases briefly from a diastolic level of 100 nM to ~ 10 μM [71, 72], whereas NCX near the jSR is exposed to high levels of $[\text{Ca}^{2+}]_{SS}$. Moreover, assuming that a Ca^{2+} spark is triggered by the LTCC opening, $[\text{Ca}^{2+}]_{SS}$ can reach 30–100 μM levels [71, 72, 142]. Thus, for effective response to large $[\text{Ca}^{2+}]$ swings, NCX needs an integrative feedback sensor over a range of 10^{-7} – 10^{-4} M. By having CBD1 and CBD2 domains, NCX 1.1 fully covers this sensitivity range and thus, allows differential sensing of $[\text{Ca}^{2+}]$ within the dyadic cleft, the sub-membrane cavities, and bulk cytosol [43, 44, 47]. For example, transient occupation of the CaI site on CBD2 by Ca^{2+} ($K_d\sim 5$ μM) may relieve Na^+ -dependent inactivation of NCX within the dyadic cleft, although this putative mechanism has not yet been demonstrated. Nevertheless, the low affinity CaI–C2 and CaII sites are not directly involved in Ca^{2+} -sensing, and constitutive occupation of these sites by Mg^{2+}

can adjust the affinity of the regulatory Ca3–Ca4 and CaI sites within the physiological range, thereby playing an important role in integrating the function of CBD1 and CBD2 [46, 47].

Interestingly, recent studies suggest that the high-affinity (Ca3–Ca4 sites) allosteric activation of NCX in cardiomyocytes involves slow inactivation of NCX with a time constant of ~ 20 s [49]. This inactivation kinetics displays a striking similarity to the slow dissociation of “occluded” Ca^{2+} from the cardiac CBD12, showing a rate-constant of $k_s \sim 0.05 \text{ s}^{-1}$ [43, 47]. The rationale behind this is that slow inactivation of NCX is important for dynamic regulation on a multibeat time scale, whereas NCX might better sustain a long-standing Ca^{2+} balance while contributing to the ability of cardiomyocytes to generate Ca^{2+} transients over a wide range of amplitudes [49].

NCX and Ca^{2+} -dependent pacemaker activity

Cardiac contractions are initiated and synchronized by pacemaker cells (SAN, AVN, and the bundle of His), representing a small fraction of total cardiac tissue that controls heartbeat (automaticity) in a sophisticated way. Although the heart pacemaker was discovered more than a hundred years ago, the underlying mechanisms are still under debate [87, 150]. In general, automaticity entails the existence of inward currents at diastolic potentials, and many ion-transport systems are dynamically matched and integrated to reach the threshold of the next action potential. It is thought that SR Ca^{2+} release activates the forward mode NCX (Ca^{2+} -efflux), which generates an inward current that brings the late diastolic potential to the threshold of the next action potential; however, the contribution of NCX to pacemaker activity remains a controversial issue [87, 97]. A principal question is whether NCX contributes to basal heartbeat rates and/or to regulation of basal heartbeat rates in response to global regulatory modes (e.g., adrenergic activation). Recent studies revealed that genetic ablation of NCX-mediated ion currents in vivo, ex vivo, and in isolated SAN cells disables “fight” or “flight” SAN activity without affecting the resting heart rate, meaning that NCX1.1 is required for increasing sinus rate but not for maintaining the resting heart rate [42]. However, this conclusion has been challenged by a recent report suggesting that cardiac NCX1.1 is a key player in the initiation and maintenance of a stable heart rhythm [56]. More extensive research is required to resolve these issues.

The previously unrecognized Ca^{2+} -activated K^+ channel (SK4) has been identified recently in human embryonic stem cell-derived cardiomyocytes as a key player in pacemaker activity [152]. Interestingly, in some developmental cells, the NCX-mediated inward current (in the absence of I_f current) has the capacity to gradually reduce SK4-mediated outward current, which allows inward currents to take over in reaching the threshold of the next action potential [152]. Although the

physiological relevance of this mechanism in adult heart pacemaker cells is currently unclear, it is obvious that a subtle balance between the outward SK4 currents, on the one hand, and the I_f/NCX inward currents, on the other hand, play a critical role in shaping the pacemaker activity at yearly stages of embryonic heart differentiation and development. Further research is required to resolve the underlying mechanisms involved in balancing the expression/regulation of SK4 and NCX in adult and developmental pacemaker cells.

NCX, neurotransmitter secretion, and synaptic transmission

Although NCX's contribution to synaptic transmission was suspected for a long time, only a recent report indicated the involvement of reverse-mode NCX in synaptic activity at the parallel fiber-to-Purkinje neuron synapse in the mouse cerebellum [137]. Namely, the NCX-mediated Ca^{2+} -efflux boosts the amplitude and duration of parallel fiber Ca^{2+} transients during short bursts of high-frequency action potentials, typical of their behavior in vivo, whereas the computer-aided simulations suggest transient accumulation of intracellular $[\text{Na}^+]_i$, which is sufficient to drive NCX-mediated Ca^{2+} -efflux. It was suggested that this mechanism can feed additional Ca^{2+} influx into the parallel fibers to support synaptic transmission to Purkinje neurons for up to 400 ms after the burst [137]. The relevant mechanisms may shape the dynamics of presynaptic $[\text{Ca}^{2+}]$ swings to boost synaptic transmission with extra capacity to properly integrate and optimize the accuracy of cerebellar information transmission.

The general mechanism underlying Ca^{2+} -dependent neurotransmitter release in neurons and neuroendocrine cells involves agonist-induced stimulation, which subsequently provokes a transient increase in cytosolic $[\text{Ca}^{2+}]_i$, the amplitude and duration of which dynamically promotes and integrates exocytosis of hormone or neurotransmitter (GABA, glycine, glutamate, etc.)-containing granules [112, 123, 135, 138]. The shape of the transient Ca^{2+} signal is regulated by multiple mechanisms, although the rising phase of the agonist-induced Ca^{2+} signal is mainly governed by the amount of Ca^{2+} entry via LTCCs and/or Ca^{2+} release from the ER stores through the IP_3 receptor channels [112, 138]. Interestingly, exocytotic-like release, depending on the extracellular Ca^{2+} , can be observed when cytosolic $[\text{Na}^+]_i$ rises enough to allow the reversal of NCX [112, 135, 138]. Although NCX is involved in neurotransmitter release in both neuronal and glial tissues, NCX seems to play a major role in controlling ionic homeostasis when responding to mechanical and biochemical stimulations requiring integration of regulatory signals in the framework of a neuron-glia network [123, 135, 138]. This is true for both astrocytes and microglia, in which homeostatic responses are controlled by dynamic changes in the cytosolic $[\text{Ca}^{2+}]_i$ and $[\text{Na}^+]_i$ [112, 123, 135, 138]. The contribution of mitochondrial NCLX to Ca^{2+} -dependent proliferation of the

neuro/glial network is of special interest in terms of its contribution to the development of neurodegenerative diseases. Recent findings suggest that NCLX regulates Ca^{2+} signaling, gliotransmission, and proliferation of astrocytes and thus, links between the mitochondria and plasma membrane Ca^{2+} homeostasis to control a diverse array of astrocyte functions [123].

NCX and insulin secretion

Ca^{2+} homeostasis in β cells plays a critical role in regulating insulin secretion through multiple mechanisms involving Ca^{2+} release from ER via RyR, Ca^{2+} entry through LTCC, Ca^{2+} extrusion through NCX, and Ca^{2+} reuptake into ER via SERCA [29]. Stimulation of pancreatic β cells by glucose is associated with slow waves of membrane potential and with LTCC- and NCX-mediated oscillations of $[\text{Ca}^{2+}]_i$ [29, 53]. The β cells express NCX1.3 and NCX1.7 splice variants, the regulatory specificity of which as well as their partial contributions to Ca^{2+} homeostasis and insulin secretion remain unclear. Interestingly, overall, the overexpression of NCX1 accelerates apoptosis of β cells, whereas apoptotic cell death in β cells significantly decelerates in the heterozygous *Slc8a1* KO mouse model [129, 130]. Moreover, heterozygous *Slc8a1* KO mice exhibit enhanced glucose-induced insulin release as well as increased proliferation and resistance to abnormal changes caused by diabetes [53–55]. The selective inhibition of NCX1.7 could be useful for improving insulin secretion. On the other hand, the pharmacological inhibition of NCX1.3 (the second NCX variant expressed in β cells) seems to be undesirable since NCX1.3 is chiefly expressed in the kidney, and its inhibition may reduce the renal Ca^{2+} -reabsorption and thus, may induce systemic hypocalcemia.

The contributions of mitochondrial NCX in Ca^{2+} shuttling of pancreatic β cells was suspected for many years, but its contribution to Ca^{2+} signaling and to subsequent insulin secretion has remained controversial because the inhibitors that were used non-specifically modulate many other Ca^{2+} transporters. This certainly underscores the importance of a molecular-based approach to elucidate the role of mitochondrial NCLX in Ca^{2+} -dependent insulin release in β cells. The molecular identification of mitochondrial NCLX [121, 122] provided new molecular tools for selective ablation of this transporter. The role of mitochondrial NCLX in Ca^{2+} signaling and insulin secretion is of major interest because of the dual role of mitochondrial Ca^{2+} shuttling in metabolism and global Ca^{2+} signaling. These new molecular tools were very instrumental in showing that NCLX plays a predominant role in shaping glucose-dependent cytosolic Ca^{2+} transients regulating the temporal pattern of insulin secretion [111]. Additional analyses of the expression/regulation of NCX1.3, NCX1.7, and NCLX may elucidate the partial contributions of these variants to altered homeostasis, which may help in

pinpointing the best strategy for effective pharmacological targeting of relevant NCX variants.

NCX's relationship to diseases

NCX, arrhythmias, heart failure, and myocardial ischemia–reperfusion injury

The process of human remodeling involves the altered expression/regulation of NCX in most cases of disease development [11, 14, 40, 51, 103, 150]. In heart failure, the overexpressed levels of NCX1.1 protein can significantly contribute to reduced levels of SR Ca^{2+} load (with an associated reduction in contractile force) as well as can mediate depolarizing arrhythmogenic currents (EAD or DAD) during the action potential that arises upon the unmatched “spontaneous” release of Ca^{2+} from the SR [14, 103, 150]. In animal models, NCX contributes to arrhythmogenesis and Ca^{2+} depletion during heart failure, owing to combined volume and pressure overload whereas, in compensated hypertrophy, NCX contributes to Ca^{2+} loading [92, 127–129]. Upregulation of NCX is extremely dangerous in combination with altered expression of the other important proteins. For example, in heart failure or hypertrophy, the concomitant overexpression of NCX and the downexpression of SERCA may cause SR Ca^{2+} depletion (with contractile malfunctions), whereas an affiliated overexpression of NCX and the downexpression of the K1-potassium channel may disturb the balance between the inward and outward currents during the repolarization, causing life-threatening arrhythmias [8–11, 103, 113, 127]. Although disorders in the expression/regulation of NCX accompany the clinical settings of arrhythmias, heart failure, and myocardial ischemia–reperfusion injury, it is not trivial to match the contributions of NCX to clinical states [11, 51, 92, 103, 150]. A major problem is that heart remodeling involves numerous proteins, the expression levels of which are controlled by many personal variables including the history and complexity of disease development and drug treatment.

NCX, cerebral ischemia, and stroke

A general feature of cerebral ischemia is the overload of Na^+ and Ca^{2+} [4, 34]. This is a very harmful situation because the elevated levels of Ca^{2+} induce necrosis and/or apoptosis of vulnerable neurons, whereas the elevated levels of $[\text{Na}^+]_i$ result in cell swelling and microtubular disorganization. In animal models of cerebral ischemia, consequently resulting in permanent vascular occlusion, the pharmacological activation of NCX reduces brain damage, whereas the NCX blockers worsen the infarct lesion [4, 98, 149]. This conclusion was supported in experiments with transgenic mouse

models, whereas the ablation of NCX1, NCX2, or NCX3 protein leads to a worsening of brain damage after focal ischemia [17–19, 73]. An important message is that the pharmacological activation of neuronal NCX variants could be a beneficial approach for decreasing brain ischemia-associated damage.

NCX, blood pressure regulation, and hypertension

The transgenic mouse model, expressing altered levels of smooth muscle NCX1, demonstrated that NCX1 chiefly contributes to diverse signaling pathways involved in activating vascular smooth muscle contraction in response to stretch (i.e., myogenic response) and in activating certain G protein-coupled receptors [16, 155]. Moreover, overexpression of vascular NCX1 has been linked with human primary pulmonary hypertension and with several salt-dependent hypertensive animal models [155–158]. It was suggested that specific mechanisms governing arterial NCX1 expression and local levels of sub-sarcolemma $[Na^+]_i$, induced by Na^+/K^+ ATPase and TRPC6, assist in regulating arterial vasoconstriction and blood pressure [16, 155, 157]. According to this proposal, the Ca^{2+} -influx through NCX1 is coupled with Na^+ transport via store-operated channels, TRPC6, and Orai1, whereas TRPC6, Orai1, and NCX1 are clustered with α -2 Na^+/K^+ ATPases in cell-membrane “microdomains” in the vicinity of junctional SR [16, 156–158]. This putative cluster may integrate the local $[Na^+]_i$, Ca^{2+} signaling, and arterial tone to control vascular resistance. Interestingly, NCX1 and TRPC6 are vastly overexpressed in de-endothelialized mesenteric arteries in ouabain-induced hypertensive rats, Milan hypertensive strain rats, and Dahl salt-sensitive hypertensive rats [159].

NCX, apoptosis, and proliferation

It is well established that the Ca^{2+} -dependent regulation of apoptotic and proliferative processes is a general mechanism in many cell types, although the particular contributions of specific Ca^{2+} -transport systems (and their variants) in regulating cell apoptosis/proliferation under normal and disease conditions remain unresolved. Previous studies have shown that distinct NCX variants contribute to altered $[Ca^{2+}]_i$ and $[Na^+]_i$ homeostasis in neurodegenerative disorders [4, 17–19, 123, 138] and diabetes [53–55, 123]. Altered expression/regulation of specific NCX variants is a hallmark feature of disease-related remodeling of microglia migration and proliferation, meaning that the underlying mechanisms could be relevant in pathophysiological settings [112, 135]. Thus, NCX variants control the balance between apoptosis and proliferation under normal and disease conditions. Mitochondrial NCLX also regulates Ca^{2+} -dependent proliferation of astrocytes, which could be related to neurodegeneration [123].

Recent studies have shown that NCX plays an essential role in regulating Ca^{2+} and Na^+ homeostasis, cell migration, and proliferation of human gastric myofibroblasts, whereas all three isoforms (NCX1–3) contribute in handling ionic homeostasis and cellular functions [77]. The physiological significance of these findings is that gastrointestinal myofibroblasts are contractile, electrically non-excitable, transitional cells that participate in extracellular matrix formation and thus, contribute to ulcer healing, chronic inflammation, and tumor development. Inhibition of predefined NCX variant(s) could be a therapeutic target in combating hyperproliferative gastric diseases.

Pharmacological implications

Undoubtedly, there is a cumulative request by both researchers and clinicians for effective pharmacological targeting of disease-related NCX variants owing to the effect of high potency, selectivity, and the bioavailability of potential drugs at the cellular and systemic levels. Notably, both the selective blockers and activators of NCX variants are desired for intervening in diverse pathological scenarios. For example, activation of NCX1, NCX2, or NCX3 isoforms could be beneficial for brain ischemia or stroke [4, 17–19, 74, 75], whereas a potent inhibition of NCX (e.g., by SEA0400) can prevent dopaminergic neurotoxicity in the mouse model of Parkinson's disease [1]. Potentially, the cardiac NCX blocker may have an anti-arrhythmic effect as well as improve cardiac contractility (e.g., in heart failure), but it may also worsen conditions such as recovery from ischemia or relaxation abnormalities [5, 14, 67, 103, 147]. The search for marginal anti-arrhythmic strategies is motivated by the shortcomings of currently available drugs as well as by new prospects arising from the screening and identification of new lead-structures for selective and effective targeting. In general, the overall outcome might be rather positive in comparison with its potential drawbacks, whereas the NCX blocker could be a part of a multi-target strategy for devising new drugs [5, 14, 20].

Drawbacks of currently available NCX blockers

During the last four decades, several organic compounds have been identified that affect NCX activity, but it appears that they also affect other ion-transport systems as well. For example, amiloride or bepridil analogs and isothiourea derivatives (KB-R7943) are non-selective inhibitors of NCX, but they exhibit relatively low potency (IC_{50} =5–10 μ M) for inhibition [5, 20, 68–70, 147]. Positively charged peptide inhibitors, XIP [90, 101], and FRCRCFa [64, 84, 102, 152] exhibit relatively high potency (IC_{50} <1 μ M) for inhibition in patch-clamp experiments (when added from the cytosolic side), but they are not selective to NCX isoforms and are unusable in

most physiological experiments due to poor permeability into the cell membrane when added to an extracellular medium. SEA0400 exhibits remarkably high potency for inhibition [1, 104], although experiments with *Slc8a1* knockout mice have shown that SEA0400 is a non-selective NCX blocker as well [5, 133]. Recently developed ethoxyaniline, quinazolinone, thiazolidine, phenoxy pyridine, acylacetamide, benzofuran, and imidazoline derivatives (including SN-6 and YM-244769) exhibit improved pharmacological properties for NCX inhibition, although the development of selective blockers for tissue-specific NCX variants on the basis of these derivatives remains doubtful [5, 69, 147].

More advanced approaches are required to generate a new generation of NCX blockers/activators. There are perhaps two foremost motives why effective pharmacological targeting of NCX variants is unavailable at present: (1) The molecular and cellular mechanisms governing the regulatory diversity of isoform/splice variants are still poorly understood; (2) No analytical systems are available for in vitro high-throughput screening (HTS) of small “drug-like” compounds. Isolated protein preparations of CBD12 constructs obtained from the cardiac, brain, kidney, and pancreas NCX [43], in combination with fluorescent assays for Ca^{2+} binding [44], could be a useful analytical approach for serial HTS analyses of NCX variants, aiming for primary identification and development of potential drug candidates.

General perspectives toward future NCX pharmacology

The resolution of the structure–activity relationships underlying the interaction between the two CBD domains is especially interesting in terms of developing coherent strategies for selective targeting of NCX variants. The rationale behind this is that the wide-range of the Ca^{2+} sensing machinery is modulated by alternatively spliced segments on CBD2, thereby making CBD12 a highly versatile Ca^{2+} sensor [47]. This enables NCX to efficiently respond to and regulate a plethora of Ca^{2+} -dependent signals and processes in distinct tissues and cellular compartments, consistent with the ubiquitous tissue distribution of NCX1. Drugs targeting the regulatory CBDs, rather than the ion translocation sites, have potential to target tissue-specific NCX variants since the alternative splicing region of NCX lies within CBD2. More specifically, drugs targeting the domain’s interface are of interest because the splicing region is adjacent to that area. More specifically, drugs directed at the interface can enhance NCX activity via domain stabilization or inhibit NCX by disrupting specific interactions between the two CBD domains, such as the salt-bridge network. Using computer-added virtual screening, numerous compounds may be found and thus, in vitro HTS tests are required to detect potential candidates. The identification of Ca^{2+} occlusion as a biochemical hallmark of interdomain interactions in CBD12 can serve as a selection criterion for

drug discovery. Stopped-flow kinetics analyses are convenient because they are rapid and require minute amounts of protein sample [43–47], making this technique suitable for screening. In addition, knowledge regarding the structural consequences of Ca^{2+} binding allows the use of SAXS for defining the structural outcomes exerted by compounds binding to CBD12 [45–48]. Importantly, SAXS is much less time and resource consuming than is X-ray crystallography and allows analyses of dynamic conformational states, making it appropriate for screening drug-like compounds.

Future perspectives

Since 2006, significant progress has been made in better understanding the molecular and cellular mechanisms underlying NCX regulation in health and disease, and much more progress is expected in forthcoming years. Substantial progress has been achieved in deciphering the molecular basis for allosteric regulation of the NCX family, whereas the equilibrium, kinetic, and structural characterization of CBD12 isoforms, variants, orthologs, and their mutants have provided important mechanistic insights and a conceptual framework for better understanding the mechanisms underlying the allosteric regulation of mammalian NCX. Recent breakthroughs provided fundamental information on the crystal structure of archaeobacterial NCX_Mj, which may serve as a template structure not only for eukaryotic NCX proteins, but also for numerous proteins belonging to the CaCA superfamily of Ca^{2+} -transport proteins. The significance of these findings is that the general ion-transport mechanism may involve the sliding of the TM1/TM6 cluster, although it remains unclear how ion interaction with the binding pocket drives the alternating excess (sliding) of the TM1/TM6 cluster and what are the dynamic features of the involved helix movements underlying alternative access. Despite these similarities between archaeobacterial and eukaryotic NCX, up to 10^3 -fold differences in the turnover rate occur among phylogenetically distant NCX orthologs, the underlying mechanisms of which are challenging to resolve.

The present achievements provide new hopes for better understanding the molecular basis of ion transport and regulation in NCX proteins as well as for the rational design of a new generation of drugs. Application of advanced molecular approaches, including the silencing/overexpression of specific NCX variants in cellular systems and organ-specific KO mouse models provided useful information about the contribution of NCX proteins to physiological processes and diseases. It is expected that selective pharmacological targeting of predefined NCX isoforms/variants has the ability to rescue specific cell types from apoptosis, malfunction, or irreparable harm. The application of adequate in vitro procedures is required for HTS and testing of large libraries of “drug-like”

synthetic compounds. Although this long-wanted intervention has not yet been realized and definitely requires long-term collaboration between academic staffs and industrial partners, the needed scientific knowledge and technological tools are now available to challenge the rational development of potential drug candidates for predefined NCX variants.

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