Functional and Structural Properties of the NCKX2 Na^+-Ca^{2+}/K^+ Exchanger: A Comparison with the NCX1 Na^+/Ca^{2+} Exchanger

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Abstract

Na^+/Ca^{2+}-K^+ exchangers (NCKX), alongside the more widely known Na^+/Ca^{2+} exchangers (NCX), are important players in the cellular Ca^{2+} toolkit. But, unlike NCX, much less is known about the physiological roles of NCKX, while emergent evidence indicates that NCKX has highly specialized functions in cells and tissues where it is expressed. As their name implies, there are functional similarities in the properties of the two Ca^{2+} exchanger families, but there are specific differences as well. Here, we compare and contrast their key functional properties of ionic dependence and affinities, as well as report on the effects of KB-R7943 – a compound that is widely used to differentiate the two exchangers. We also review structural similarities and differences between the two exchangers. The aim is to draw attention to key differences that will aid in differentiating the two exchangers in physiological contexts where both exist but perhaps play distinct roles.

Keywords

Na^+/Ca^{2+} exchanger • Na^+/Ca^{2+}-K^+ exchanger • NCX • NCKX • KB-R7943 • SLC24

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8.1 Introduction

Calcium plays a ubiquitous role in eukaryotic intracellular signalling. To maintain a high signal-to-noise ratio, cells maintain their resting Ca^{2+} at very low levels relative to the extracellular environment through the concerted action of buffering cytosolic Ca^{2+}, sequestration in organelles and extrusion – the latter is most essential for long-term homeostasis. Two mechanisms exist to extrude Ca^{2+}, the

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ATP-driven plasma membrane Ca^{2+} ATPase (PMCA) in humans represented by four genes *ATP2B1-4* (Strehler and Zacharias 2001) and Na^+-driven Na^+/Ca^{2+} exchangers (NCX) in humans represented by three genes *SLC8A1-3* (Quednau et al. 2004) and the later discovered (see below) Na^+/Ca^{2+}-K^+ exchangers (NCKX) in humans represented by five genes *SLC24A1-5* (Schnetkamp 2004).

PMCA has a high affinity for Ca_i^{2+}, less than 0.5 μM, while Na^+/Ca^{2+} exchangers have a lower affinity for Ca_i^{2+} (in the range of 1–5 μM); however, Na^+/Ca^{2+} exchangers have a higher turnover rate than PMCA (Blaustein and Lederer 1999). Hence, what is thought to differentiate these two mechanisms physiologically is that PMCA regulates resting Ca_i^{2+}, while Na^+/Ca^{2+} exchangers handle the larger, more dynamic fluxes of Ca^{2+} which are most prominent in excitable tissue. But while both plasma membrane Ca^{2+} pump and Na^+/Ca^{2+} exchange were known to the scientific community since the 1960s, it was a relatively later discovery which revealed that retinal rod outer segments express a unique Na^+/Ca^{2+} exchanger which co-transports K^+ with Ca^{2+} (Schnetkamp et al. 1989; Cervetto et al. 1989). The subsequent cloning of multiple members of this family – NCKX – which have a tissue distribution that is often overlapping with NCX (Li and Lytton 2002; Papa et al. 2003; Minelli et al. 2007) has raised the question of whether these two distinct proteins play redundant roles in Ca^{2+} regulation and cellular physiology. It is also clear that in many tissues functional expression of both NCX and NCKX was observed within the same cell (Roberts and Bose 2011; Yang et al. 2011; Kiedrowski et al. 2004; Li et al. 2006; Pan et al. 2008).

The purpose of this chapter is to compare functional as well as structural features of NCKX against NCX, to highlight similarities in their function and address differences, with the hope of drawing more attention to the distinction between the two, so that future studies can help us to further understand the important roles that these two families of plasma membrane Ca^{2+} transporters play in normal cell function and in pathophysiology.

8.1.1 Roles of NCX and NCKX in Cell Physiology

While numerous studies have investigated the physiological roles of NCX, especially in myocardial cells (for review, see Blaustein and Lederer (1999)), relatively little detail is known of the in situ contributions of NCKX outside of retinal photoreceptors (Schnetkamp 1995); in recent years, however, novel specific roles for NCKX proteins have been emerging in pigmentation in epidermal melanocytes and the retinal pigment epithelium (Lamason et al. 2005; Vogel et al. 2008), motor learning and memory (Li et al. 2006) and olfaction (Stephan et al. 2011). In the brain, where both NCX and NCKX are abundantly expressed, the investigation of the role of NCX2 (*SLC8A2*) by genetic ablation revealed a deficit in neuronal Ca_i^{2+} clearance, associated with a lowering of the threshold for induction of long-term potentiation (LTP) in the hippocampus – a process that is thought to be critical for the formation of new spatial memories – hence, animals deficient in NCX2 displayed enhanced learning and memory performance relative to controls (Jeon et al. 2003). On the other hand, knockout of NCKX2 (SLC24A2) – which is also highly expressed in the hippocampus – also perturbed Ca^{2+} fluxes in neurons, but led to elimination of LTP expression and an associated deficiency in spatial working memory (Li et al. 2006). These studies indicate that in fact NCX and NCKX could serve distinct roles, potentially through mechanistic differences in their modes of operation. However, to begin to address differences in the modes of operation and physiological functions of NCX and NCKX in tissues or cells where both are expressed, it would be desirable to have specific pharmacological tools which can be used to antagonize the function of one set of proteins while leaving the other functional and importantly that the elimination of function can be implemented on a timescale relevant to the operation of NCX and NCKX in Ca_i^{2+} clearance.

8.1.2 Small Compound Inhibitors of Na^+/Ca^+ Exchangers

One of the first small compound inhibitors developed to target NCX was KB-R7943, an isothiurea derivative (Iwamato et al. 1996). Because this initial study reported that KB-R7943 preferentially inhibits the Ca^{2+} influx mode of the bidirectional NCX, numerous studies have since used the compound to demonstrate beneficial effects of application of the drug under posited pathological conditions (typically under perturbed ionic conditions) in which NCX is expected to predominantly mediate unabated Ca^{2+} influx into the cytosol, leading to toxic Ca_i^{2+} accumulation (reviewed in Amran et al. (2003)). However, it is clear by now that KB-R7943 is far from a specific NCX inhibitor; many ion channels and transporters have been shown to be inhibited by the compound at concentrations equivalent to, or lower than, those used to inhibit the Ca^{2+} influx mode of NCX (e.g. Barrientos et al. 2009 and references therein). Moreover, the extent to which KB-R7943 inhibits NCX is variable under different experimental conditions (Iwamoto et al. 1996; Linck et al. 1998; Elias et al. 2001). Additionally, questions have been raised as to the apparent paradox of the compound acting more potently on the Ca^{2+} import mode of transport over the Ca^{2+} efflux mode (Iwamoto et al. 1996; Kimura et al. 1999); further insight has been gained on the mechanisms of action of KB-R7943 since these early experiments, and it is now evident that the compound acts on specific kinetic states of NCX (Bouchard et al. 2004; Lee et al. 2004), discussed further below. Other, more potent, compounds have been developed to target NCX since the development of KB-R7943; SEA0400, for example, is effective at much lower concentrations (Matsuda et al. 2001) and appears to be substantially more selective (Tanaka et al. 2002), but nonetheless has been shown to have non-specific actions (Reuter et al. 2002).

Thus far, no commercially available small compound has been found to appreciably inhibit the function of NCKX proteins, except for 3′, 5′ dichlorobenzamil (Nicol et al. 1987), tetracaine and L-cis diltiazem (Schnetkamp et al. 1989), all of which are non-specific. Since it was reported that KB-R7943 acts selectively to inhibit NCX over NCKX (Iwamoto et al. 2001), other investigators have used sensitivity to the compound as a diagnostic to attribute Na^+/Ca^{2+} exchange activity in a given preparation to NCX over NCKX (Czyz and Kiedrowski 2002; Kiedrowski et al. 2004; Wu et al. 2008). On the other hand, studies have reported on KB-R7943-sensitive Ca^{2+} transport in invertebrate spermatozoa (Su and Vacquier 2002; Islam et al. 2006), as well as mammalian platelets (Takano et al. 2001), and that the putative target of KB-R7943 in those cells is of the NCKX type. This has prompted us to re-examine the effect of KB-R79473 on NCKX; herein, we test the compound on NCKX2 as well as NCKX1, since, previously, only NCKX2 had been directly tested for sensitivity to KB-R7943 (Iwamoto et al. 2001). We also present data demonstrating directly the key functional difference between NCX and NCKX in K^+ dependence and transport, as well as affinity for Na^+ and Ca^{2+}. This is followed by a comparative review of structural features of these two exchangers, with NCKX2 serving as the model NCKX as it is the best studied member of the family in terms of structure-function relationships and NCX1 as the best studied member of the NCX family.

8.2 Functional Comparison of NCX1 and NCKX2

To clearly demonstrate functional differences between NCX and NCKX, we have undertaken measurements of K^+ dependence and Na^+ and Ca^{2+} affinity under defined conditions of heterologous expression in HEK293 cells; for NCKX2, we used the human clone (Prinsen et al. 2000), while for NCX1 we used the canine clone (Nicoll et al. 1990). Details of our assays have been previously published elsewhere (Szerencsei et al. 2001; Kang et al. 2005a; Altimimi and Schnetkamp 2007; Altimimi et al. 2010).

8.2.1 K^+ Dependence and Transport

Figure 8.1 illustrates the key difference between NCX and NCKX – the absolute requirement for K^+ as a co-transported substrate in the latter. As in

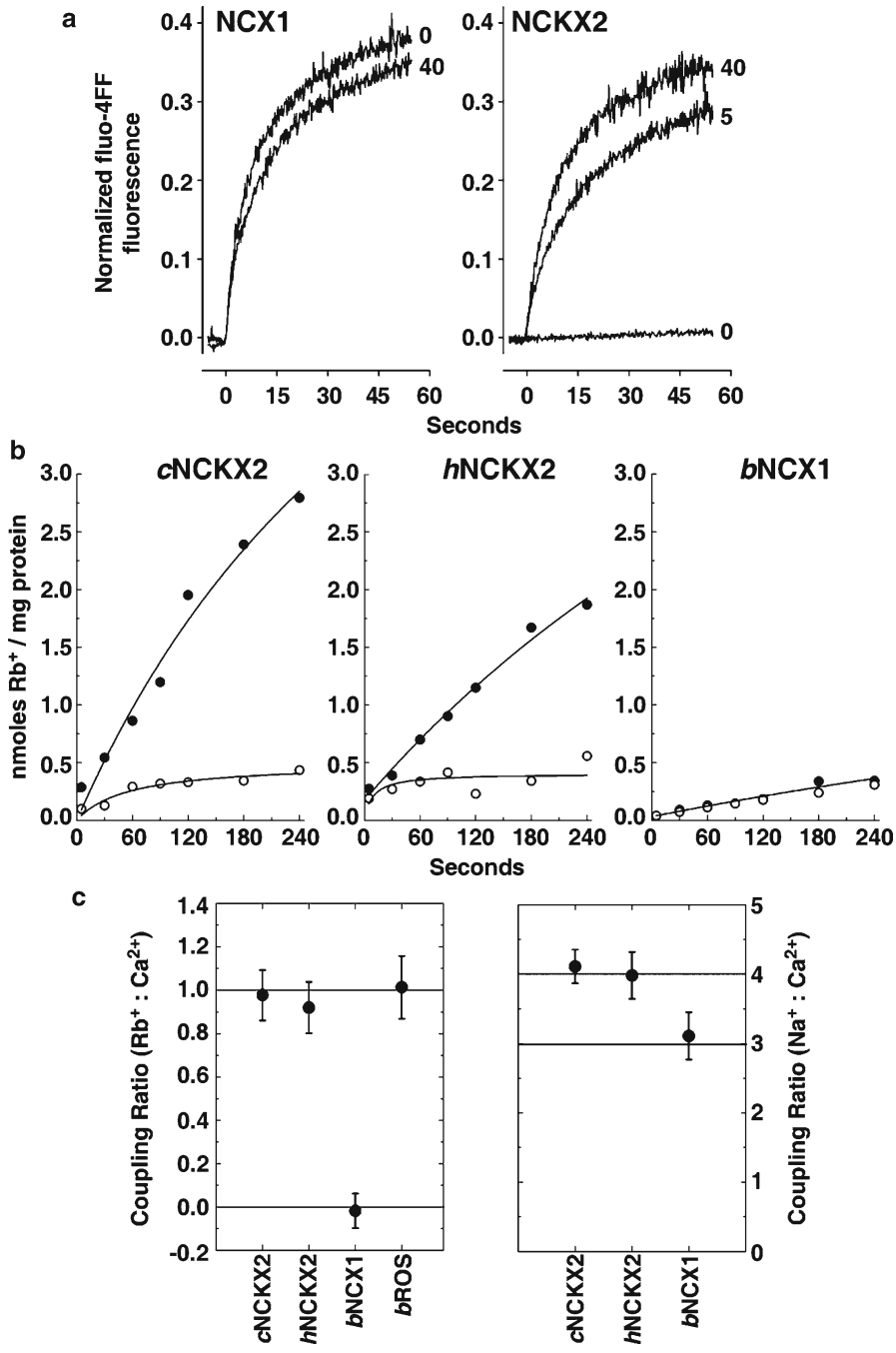

Fig. 8.1 *K^+ dependency and transport stoichiometry of NCX1 and NCKX2 expressed in HEK 293 cells*: (**a**) Ca^{2+} influx through reverse Na^+/Ca^{2+} exchange was initiated at time zero by addition of 250 μM $[Ca^{2+}]_o$ in the presence of the indicated $[K^+]_o$ (mM). Experimental conditions and protocols are as described in the text and in more detail in Kang et al. (2005a). (**b**) Ca^{2+}-dependent ^{86}Rb uptake in High Five cells expressing chicken NCKX2, human NCKX2 and bovine NCX1. ^{86}Rb uptake was initiated at time zero by addition of ^{86}Rb, 0.4 mM RbCl in the presence of 0.4 mM EDTA (*open circles*) or 0.4 mM $CaCl_2$ (*filled circles*) in a medium containing 150 mM choline chloride and 20 mM HEPES (pH 7.4). (**c**) Rb/Ca and Na/Ca coupling ratios were obtained in High Five cells expressing chicken NCKX2, human NCKX2, bovine NCX1 or bovine rod outer segments (ROS which express NCKX1) as described in detail in Szerencsei et al. (2002). Average values (± standard deviation) are illustrated representing 8–15 separate experiments (panels (**b**) and (**c**) were taken with slight modifications from Szerencsei et al. (2001), with permission from the publisher)

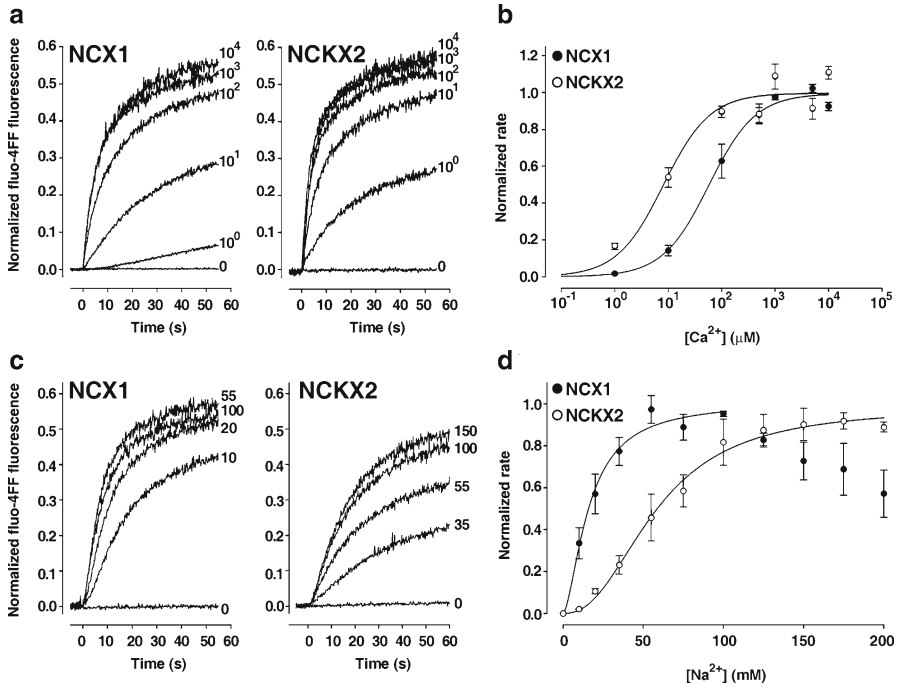

Fig. 8.2 *Na^+ and Ca^{2+} dependencies of NCX1 and NCKX2 expressed in HEK293 cells*. (**a**) Ca^{2+} influx through reverse Na^+/Ca^{2+} exchange was initiated at time zero by addition of the indicated $[Ca^{2+}]_o$ (μM). Experimental conditions and protocols are as described in the text and in more detail in Kang et al. (2005a). (**b**) Average initial rate of Ca^{2+} influx (± standard error of the mean for all subsequent error bars) is plotted versus $[Ca^{2+}]_o$ for three experiments. (**c**) Ca^{2+} influx through reverse Na^+/Ca^{2+} exchange was initiated at time zero by addition of the indicated $[Na^+]_i$ (mM). Experimental conditions and protocols are as described in the text and in more detail in Altimimi et al. (2010). (**d**) Average initial rate of Ca^{2+} influx is plotted versus $[Na^+]_i$ for three experiments

most functional studies on NCX and NCKX, we measured Na_i^+-dependent Ca^{2+} influx or reverse Na^+/Ca^{2+} exchange. Ca^{2+} influx mode was initiated in NCX1-transfected, fluo-4FF-loaded HEK293 cells by the addition of Ca_o^{2+} in the presence or absence of K_o^+ in a buffered medium where Li^+ is the major constituent cation; there was no difference in initial rate of Ca^{2+} influx between the presence or absence of K^+ (Fig. 8.1a). NCKX2-transfected HEK293 cells in the same Li^+ medium, on the other hand, did not show Ca^{2+} influx on addition of Ca_o^{2+} until the addition of K_o^+ at time zero (Fig. 8.1a). While the free $[Ca^{2+}]_i$ measurements shown in Fig. 8.1a illustrate K^+-dependence, they do not signify K^+-cotransport, which is the hallmark of NCKX; Fig. 8.1b shows Ca^{2+}-activated Rb^+ (a substitute for K^+) co-transport in two different NCKX clones, chicken and human, compared against bovine NCX, where it is clear that only NCKX mediates Ca^{2+}-activated transmembrane Rb^+ transport (from Szerencsei et al. (2001)). Figure 8.1b also demonstrates a direct determination of the stoichiometry of transport in NCKX with 1 K^+ ion coupled to the transport of 1 Ca^{2+} ion, in exchange for 4 Na^+ ions, while NCX transports 1 Ca^{2+} ion in exchange for 3 Na^+ ions (from Szerencsei et al. (2001)).

8.2.2 Ca^{2+} Affinity

To compare Ca^{2+} affinity of the two exchangers, Ca^{2+} influx mode was initiated in NCX1 or NCKX2-transfected, fluo-4FF-loaded HEK293 cells by the addition of different $[Ca^{2+}]_o$ in the presence of K_o^+ in a buffered medium where Li^+ is the major constituent cation (Kang et al. 2005a; Altimimi and Schnetkamp 2007) and changes in

free $[Ca^{2+}]_i$ were monitored (Fig. 8.2a). Note that at 1 μM $[Ca^{2+}]_o$ NCX1-mediated Ca^{2+} influx was comparatively very low and commenced after a lag of a few seconds. This is indicative of the catalytic requirement of NCX1 for $[Ca^{2+}]_i$, which binds to domains within its large intracellular loop to activate the exchanger (Hilgemann et al. 1992). NCKX2 on the other hand does not appear to require $[Ca^{2+}]_i$ to activate the exchanger, as seen in Fig. 8.2a; addition of 1 μM Ca_o^{2+} results, without any lag, in rapid influx at a rate 15–20% of Vmax. The K_m values for $[Ca^{2+}]_o$ derived under these conditions were 58 ± 8 μM and 8 ± 3 μM for NCX1 and NCKX2, respectively (Fig. 8.2b).

8.2.3 Na^+ Affinity

Na^+ affinity of the exchangers was measured using an assay based on the use of gramicidin as a means of controlling $[Na^+]_i$ (Altimimi et al. 2010); the assays illustrated in Fig. 8.2c, d were carried out in the presence of 0.5 mM Ca_o^{2+} in a buffered medium where K^+ was the major constituent cation and Ca^{2+} influx was initiated by the addition of Na^+. From the exemplar traces illustrated in Fig. 8.2c, it is also apparent that Ca^{2+} influx in NCX1-transfected HEK293 cells follows a sigmoidal trajectory, indicative of the lag required for catalytic Ca_i^{2+} to fully activate NCX1 by binding to its intracellular loop; this does not appear for NCKX2-mediated Ca^{2+} influx. From these measurements, we find that NCX1 under these conditions has a higher affinity for Na_i^+ than NCKX2; the K_m values are 16 ± 2 mM and 58 ± 3 mM, respectively (Fig. 8.2d).

These properties may dictate differences in the modes of operation of NCX and NCKX in their native environments and may be at play in tissues and cells where both are expressed in the same compartments. The catalytic requirement of NCX for Ca_i^{2+} to fully activate the exchanger, which factors into our measurements of Ca^{2+} and Na^+ affinity herein, may limit its contribution under basal conditions when $[Ca^{2+}]_i$ is at low levels – near resting $[Ca^{2+}]_i$. Given that NCKX does not require Ca_i^{2+} for its activation, NCKX may be positioned as the "intermediate" Ca^{2+} extrusion mechanism in between PMCA which is posited to be fully operational at resting $[Ca^{2+}]_i$ and NCX which is fully operational when $[Ca^{2+}]_i$ reaches higher levels required to occupy its Ca^{2+}-binding domains. In this context, it is pertinent to highlight the study of Kim et al. (2005) who dissected the contributions of plasma membrane Ca^{2+} transporters to Ca^{2+} clearance in neuronal axon terminals at the calyx of Held when $[Ca^{2+}]_i$ loading was modest, in the range of < 2 μM; NCKX made the biggest contribution to Ca_i^{2+} clearance at 42%, followed by NCX and PMCA at 26% and 23%, respectively.

8.2.4 Comparison of the Effect of KB-R7943 on NCX and NCKX

The commonly used drug KB-R7943 was tested on NCX1 and NCKX2, as well as dolphin NCKX1 (Cooper et al. 1999). Transfected HEK293 cells were placed in a medium of Li^+ and treated with various concentrations of KB-R7943 10s prior to addition of Ca^{2+} in the case of NCX, or addition of K^+ (in the presence of Ca_o^{2+}) for NCKX1 and NCKX2. Figure 8.3a illustrates exemplar Ca^{2+} influx traces for all three exchangers. We noted a marked difference of the effect of KB-R7943 on the initial rates of Ca^{2+} influx compared with steady state free $[Ca^{2+}]_i$ levels in the case of NCX1, where even at the highest concentration of the drug tested, the rate of Ca^{2+} influx appeared unchanged from control (Fig. 8.3b). However, there was an abrupt plateau phase in the free $[Ca^{2+}]_i$ signal in NCX1-transfected cells, appearing only at the higher concentrations tested – 30 μM (not shown) and 50 μM (Fig. 8.3a). These results are quantified both in terms of effect of KB-R7943 on initial rates (Fig. 8.3b) as well as on the steady-state level of free Ca_i^{2+} achieved by the three exchangers (Fig. 8.3c).

From these results, it is clear that under our assay conditions, KB-R7943 produces only a modest effect on NCX1 and is only effective at relatively high concentrations. While our assay here used ensemble fluo-4FF measurements of free $[Ca^{2+}]_i$ in HEK293 cells, an equally low efficacy of KB-R7943 on NCX1 has been

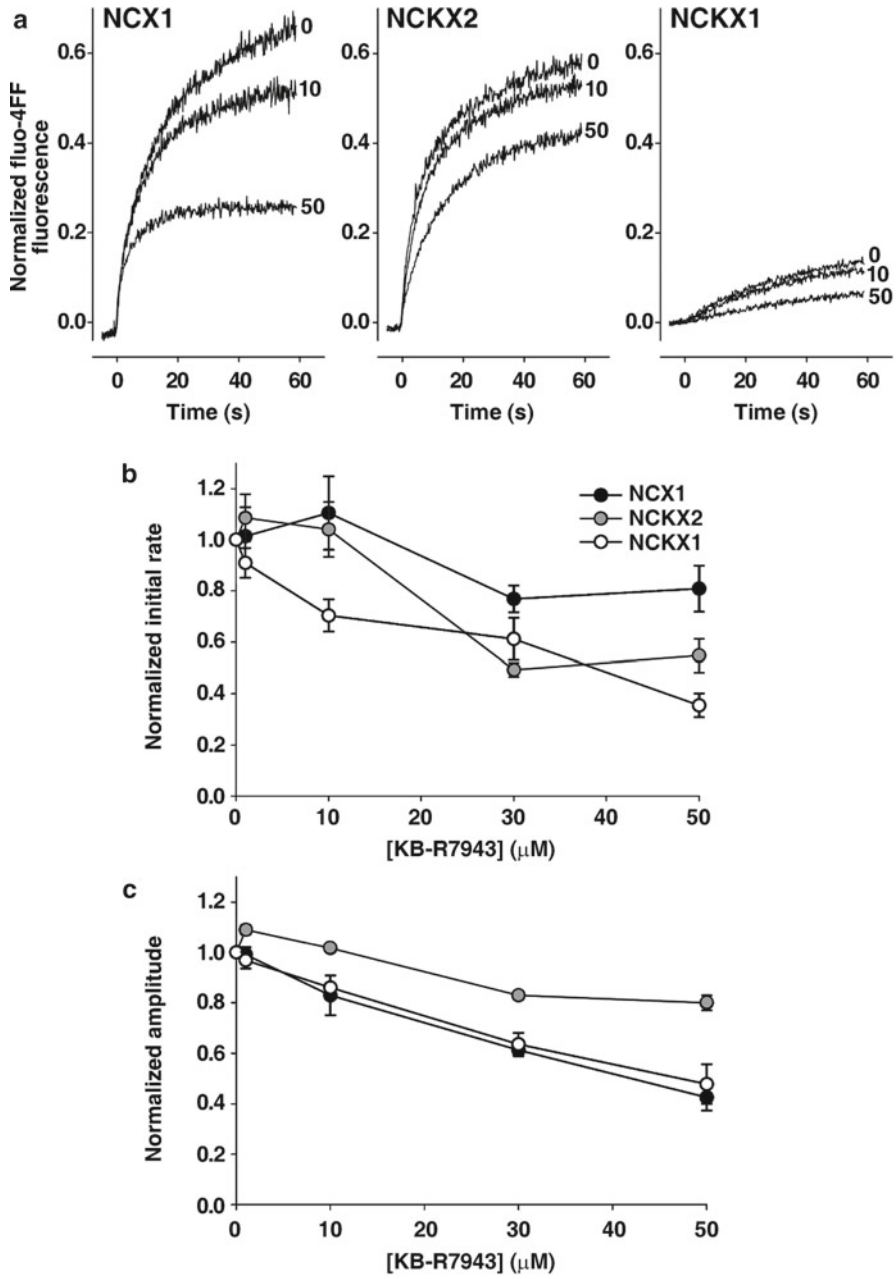

Fig. 8.3 *Effect of KB-R7943 on NCKX1, NCKX2 or NCX1 expressed in HEK293 cells*. (**a**) Ca^{2+} influx through reverse Na^+/Ca^{2+} exchange was initiated at time zero by addition of 250 μM $[Ca^{2+}]_o$ in the presence of the indicated concentrations (in μM) of KB-R7943. (**b**) Initial rate of Ca^{2+} influx as a function of KB-R7943 concentration. (**c**) Steady-state level of increase in $[Ca^{2+}]_i$ as a function of KB-R7943 concentration

reported previously in a study that employed radioactive $^{45}Ca^{2+}$ flux in both NCX1-transfected BHK cells (20% inhibition by KB-R7943 at 30 μM) and membrane vesicles isolated from those cells (30% inhibition by KB-R7943 at 10 μM) (Linck et al. 1998). One possible explanation for the relatively low potency of KB-R7943 inhibition observed in our measurements is that the time of

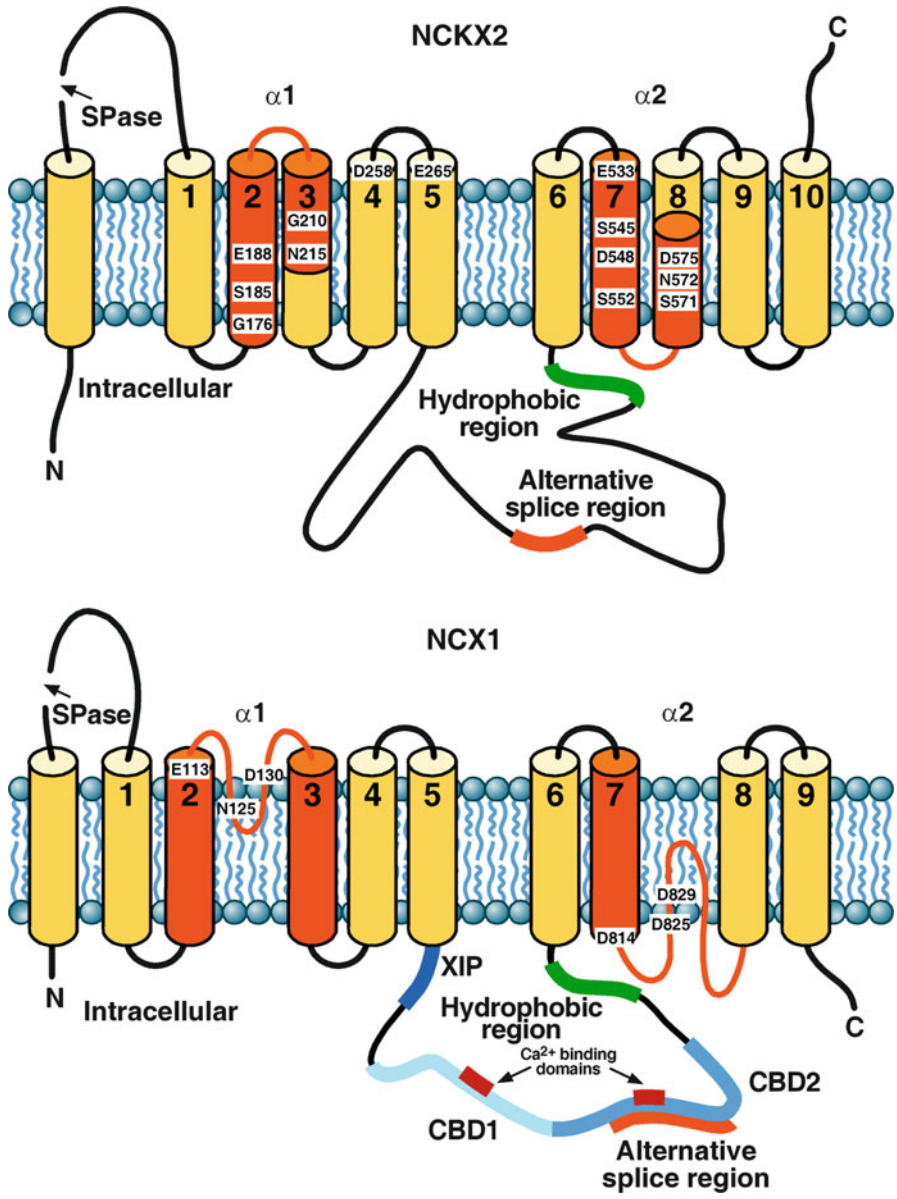

Fig. 8.4 Current topological models of NCKX2 (*top*) and NCX1 (*bottom*)

KB-R7943 pre-incubation before commencing Ca^{2+} influx through the exchanger was brief (10s in the data illustrated in Fig. 8.3); however, we tested longer pre-incubations – as long as 5 min – and found no appreciable difference in efficacy of inhibition (data not shown). Also arguing against time being the factor are the results of Linck et al. (1998), where cells were incubated with KB-R7943 for 20 min before assaying for Ca^{2+} transport.

From various studies on the effect of KB-R7943 on NCX, mechanistic insight has been gained on the structural elements important for binding (Iwamoto et al. 2001), as well as the likely kinetic state which KB-R7943 impacts (Iwamoto et al. 2004). While it was found that mutations in the α-2 repeat of the exchanger (see Fig. 8.4) abrogated sensitivity of NCX1 to KB-R7943 (Iwamoto et al. 2001), analysis of a chimeric exchanger containing the α-1 repeat of NCX1, but the α-2 repeat

of NCKX2, showed equivalent inhibition by KB-R7943 to that of wild-type NCX1; wild-type NCKX2 was not sensitive to KB-R7943 in that same study (Iwamoto et al. 2004). This suggested that earlier findings on the site of KB-R7943 interaction with NCX1 may be a result of an allosteric effect of the drug, rather than a direct effect (Iwamoto et al. 2001). The same authors also investigated the effects of KB-R7943, as well as SEA0400, on mutant NCX1 exchangers with altered regulatory properties. A prominent kinetic feature of NCX1 is Na^+-dependent inactivation, also termed I_1 inactivation, which is most evident in patch-clamp electrophysiological measurements as a decay of Na_i^+-dependent outward current to a steady state that is 10–70% of peak outward current (Hilgemann and Collins 1992). A mutant of NCX with enhanced I_1 inactivation showed greater sensitivity to KB-R7943 (as well as SEA0400), while another mutant with no apparent I_1 inactivation displayed insensitivity to KB-R7943 (Iwamoto et al. 2004). Similar findings were obtained in other detailed studies on the mechanism of SEA0400-mediated inhibition of NCX1 (Bouchard et al. 2004; Lee et al. 2004).

When examining the effect of KB-R7943 on NCKX2, we noted that the drug had only a small effect on the steady-state level of free $[Ca^{2+}]_i$ attained at ~1 min from initiation of Ca^{2+} influx. However, analysis of the initial rate of change in free $[Ca^{2+}]_i$ revealed a more pronounced inhibition produced by KB-R7943 at 30 and 50 μM. NCKX1, on the other hand, appeared to be more sensitive to KB-R7943; there was equivalent inhibition to that seen in NCKX2 when examining initial rates of change in free $[Ca^{2+}]_i$, and the steady-state level of $[Ca^{2+}]_i$ was also decreased to the same extent as that seen in NCX1-transfected HEK293 cells (Fig. 8.3b).

In light of the mechanistic insight gained for the effects of KB-R7943 and SEA0400 on NCX1, it is plausible that the inhibition we observed with NCKX1 (or on the rate of change in $[Ca^{2+}]_i$ mediated by NCKX2) is due to some interaction of KB-R7943 with specific kinetic states of NCKX. In HEK293 cells, we found that NCKX2 displayed kinetic features reminiscent of Na^+-dependent inactivation described for NCX1, but under the conditions of our assay, inactivation was only evident for the Ca^{2+} extrusion mode. Like I_1 inactivation in NCX1, NCKX2 inactivated with exposure to high $[Na^+]_i$ was relieved by decreasing $[Ca^{2+}]_o$ (which favours outward-facing conformation exchangers, thereby decreasing high Na_i^+ exposure), and mutants with increased Na_i^+ affinity displayed inactivation at lower $[Na^+]$ than wild-type NCKX2 (Altimimi and Schnetkamp 2007).

In summary, we found that KB-R7943 is a poor agent for unambiguously differentiating the contributions of NCX from NCKX for several reasons: (a) its efficacy in inhibiting NCX is variable under different assay conditions; (b) it may in fact impact the function of NCKX, depending on isoform present; while we have tested the compound here on NCKX1 and NCKX2, it is not yet known what the effects of the compound are on NCKX3-5, and (c) as found by many other investigators, KB-R7943 can interfere with many other ion channels and transporters, many of which transport Ca^{2+}, further complicating the assignment of perturbations in Ca^{2+} signalling caused by the compound as the result of inhibition of NCX.

8.3 Comparing NCX and NCKX Sequences

Both NCX and NCKX are intrinsic membrane proteins. The predominant structural motifs of the membrane-spanning domains of such proteins are invariably alpha-helical segments of ~20 residues that traverse the membrane (transmembrane segments or TMS). Hydrophobicity analysis of all NCX and NCKX sequences reveals the presence of twelve hydrophobic segments that could constitute TMS. The first is located at the N-terminus and thought to be a cleavable signal peptide. By placing tags before and after the putative cleavage site, we determined that the putative signal peptide of NCKX2 was only partially cleaved, resulting in two populations of NCKX2 protein: one full-length and one with the signal peptide cleaved as evidenced by a characteristic two-band pattern seen in Western blots (Kang and Schnetkamp 2003). The situation with

NCKX1 was more complex, as partial signal peptide cleavage was seen for dolphin NCKX1 but not for chicken NCKX1. Additionally, deletion of the signal peptide in both NCKX2 and dolphin NCKX1 prevented trafficking to the plasma membrane (Kang and Schnetkamp 2003). The consequence of partial cleavage of the signal peptide is that after expression of various NCKX1 and NCKX2 cDNAs in cell lines, a significant fraction of expressed NCKX protein is localized within the cell rather than in the plasma membrane. It remains to be established whether this is an artefact of overexpression of NCKX cDNA in cell lines or if NCKX proteins may function in intracellular organelles as well. This intriguing possibility is made more likely by the observation that NCKX5, critical for pigmentation in epidermal melanocytes and the retinal pigment epithelium, is not found in the plasma membrane but localized exclusively within the cell, most likely in the trans-Golgi network, although its precise function remains to be elucidated (Lamason et al. 2005; Ginger et al. 2008).

Little sequence conservation is observed for the signal peptides of the five NCKX isoforms, whereas the remaining eleven hydrophobic segments are the only sequence elements that show significant sequence conservation among all NCKX isoforms. These eleven hydrophobic segments are grouped in two sets of five and six putative TMS, respectively, and separated by a large hydrophilic loop thought to be located in the cytoplasm. This large hydrophilic loop is not or is poorly conserved among NCKX isoforms and ranges from more than 400 residues in mammalian NCKX1 to approximately 100 residues in NCKX5. Very little has been elucidated about the role of the large cytoplasmic loop in NCKX function except that it is not directly involved in either cation transport or cation selectivity, that is, the TMS domains are both necessary and sufficient for Na^+/Ca^{2+}-K^+ exchange transport (Szerencsei et al. 2000). In the same study, we also showed that the transport properties observed for mammalian NCKX1 were very similar to those observed for a distantly related NCKX cloned from *C. elegans*. The highest degree of sequence conservation was observed in four of the eleven hydrophobic segments which make up the two so-called alpha repeats which are thought to have arisen from an ancient gene duplication event (Schwarz and Benzer 1997). This suggests that the alpha repeats contain most of the residues important for cation binding and cation transport. Much of the work in our laboratory over the past ten years has focused on elucidating the role of the TMS in NCKX ion transport function and determining a topological model for their arrangement.

8.3.1 Topological Models of NCX and NCKX

We have proposed a topological model for human NCKX2 (Fig. 8.4) based on a combination of results from two methods: (1) determining the accessibility of substituted cysteine residues to small externally applied hydrophilic cysteine-modifying reagents (e.g. MTSET) and (2) inserting glycosylation sites in the short loops connecting putative TMS (Kinjo et al. 2003). This model places the alpha repeats in an inverted configuration while the short C-terminal loop faces the extracellular space. Although actual sequence similarity between NCX and NCKX is extremely limited to two short stretches of ~35 residues that make up the core of each of the two alpha repeats, the hydropathy analysis of NCX1 reveals a very similar pattern of eleven hydrophobic segments. However, the current topological model of NCX1 (Nicoll et al. 1999; Iwamoto et al. 2000) differs considerably from that of NCKX2 (Fig. 8.4) due to the presence of two re-entrant loops, one in each of the two alpha repeats. The first re-entrant loop is located in the region linking TMS2 to TMS3, while the second re-entrant loop replaces TMS8, inverting the orientation of the two TMS closest to the C-terminal and thus placing the C-terminus of NCX1 in the cytoplasm.

Unlike the case for NCKX proteins, many studies have addressed regulatory features imposed on Na^+/Ca^{2+} exchange transport by distinct sequences contained in the large cytosolic loop of NCX1, for example, the binding domain of the XIP peptide and sequences responsible

for Na^+-dependent inactivation and secondary activation by cytosolic Ca^{2+}. Both NMR and X-ray crystal structures have been obtained for these domains (see other chapters in this volume). We have described Na^+-dependent inactivation for NCKX2 that shares some characteristics with Na^+-dependent inactivation seen in NCX1 (i.e. occupancy of the cation transport sites by Na^+), but it remains to be established if sequence elements in the cytosolic loop of NCKX2 participate in this process (Altimimi and Schnetkamp 2007).

8.3.2 Residues Important for NCX- and NCKX-Mediated Cation Transport

We carried out scanning mutagenesis of the two alpha repeats of NCKX2 to identify residues important for Na^+/Ca^{2+}-K^+ exchange transport. We also examined all aspartate and glutamate residues found in the TMS as such acidic residues are commonly found to be critical for Ca^{2+} and Na^+ transport (Kang et al. 2005a, b; Winkfein et al. 2003). All of these residues were examined for changes in total activity (V_{max}) and changes in K_m for Na^+, while a subset was examined for changes in the K_m for Ca^{2+} and K^+ (a full scan is currently in progress). Some of the most important residues are highlighted in Fig. 8.4. It is probably no surprise that these residues are mostly negatively charged residues or polar residues that could provide cation-coordinating oxygen atoms, but also include two glycine residues. Moreover, all the residues involved are conserved in most if not all NCKX sequences currently in the database. Furthermore, all the residues depicted here as located in the membrane interior are conserved between NCX and NCKX sequences with one notable exception. An aspartate residue is found in all NCKX sequences at the position equivalent to D575 in human NCKX2, whereas an asparagine is found in all NCX sequences at this position. We showed that the D575N (or C) substitution in human NCKX2 renders the mutant NCKX2 protein independent of K^+ (as is the case with NCX proteins), and we suggest this is an essential residue for K^+ binding to NCKX (Kang et al. 2005b). For most of the residues shown here that are conserved between NCX1 and NCKX2, the activity of mutant NCX1 in which any of these residues were replaced was very low in comparison to wild type and did not permit an analysis of shifts in K_m's for Ca^{2+} or Na^+. We proposed that E188 and D548 in NCKX2 (and the equivalent E113 and D814 in NCX1) are the two main Ca^{2+}-coordinating residues based on three observations: (1) these two residues are conserved in all NCX and NCKX sequences, (2) they are the only two acidic residues in NCKX2 for which removal of the charge led to a complete abolition of transport (<0.2%) and (3) the charge-conservative E188D and D548E substitutions resulted in mutant NCKX2 proteins that displayed the largest shifts in Ca^{2+} K_m (Kang et al. 2005a). The more peripherally located acidic residues (e.g. D258, E265, E533) are conserved in most NCKX sequences, while NCX has different conserved acidic residues in more peripheral locations that can affect Ca^{2+} K_m values (Iwamoto et al. 2000). Such residues may not be directly involved in the Ca^{2+} binding site of NCX or NCKX but may influence K_m values by increasing the local [Ca^{2+}] due to electrostatic attraction. The two glycine residues shown in Fig. 8.4a (G176 and G210) are very sensitive to substitution as even the very conservative Gly to Ala substitution results in a greater than 90% inhibition of the V_{max} (Winkfein et al. 2003; Altimimi et al. 2010). Critical glycine residues often are in positions that either require hingelike movement of two helical segments or indicate helix-helix contacts.

8.4 Conclusions

Na^+/Ca^{2+} exchangers, NCX, and Na^+/Ca^{2+}-K^+ exchangers, NCKX, both play important roles in physiology as part of the cellular Ca^{2+} toolkit. While it may be convenient to lump the two mechanisms as one that mostly mediates Ca^{2+} extrusion via Na^+/Ca^{2+} exchange, the data and review of the literature we presented here hopefully will have convinced the reader that these

two Ca^{2+} transporters are in fact quite distinct, both structurally and functionally. Especially when considering cells in which both NCX and NCKX are expressed, we believe that the differences between NCX and NCKX could serve mechanistically distinct functions. With this in mind, we hope that future studies will continue to shed light on the specific physiological roles that NCX and NCKX play in cellular physiology.

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