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Na^+-dependent Inactivation of the Retinal Cone/Brain Na^+/Ca^{2+}-K^+ Exchanger NCKX2*

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The *SLC24* gene family Na^+/Ca^{2+}-K^+ exchangers (NCKX) are bidirectional plasma membrane transporters whose main function is the extrusion of Ca^{2+} from the cytosol. In this study, we used human embryonic kidney 293 cells expressing human retinal cone/brain NCKX2 to examine its Na^+ affinity and kinetic parameters of Ca^{2+} transport. With the use of the ionophore gramicidin to control alkali cation concentrations across the plasma membrane, application of high intracellular Na^+ promoted large NCKX2-mediated increases in intracellular free Ca^{2+} in the 15–20 μM range; this also resulted in inactivation of NCKX2 transport, the first description of this novel kinetic state. The affinity of NCKX2 for internal Na^+ was found to be sigmoidal, with a Hill coefficient of 2.6 and K_d = 50 mM. The time-dependent ($t_{1/2} \sim 40$s) inactivation of NCKX2 required high intracellular Na^+ levels (K_d > 50 mM) as well as high occupancy of the extracellular Ca^{2+}-binding site. Also reported are two residues whose substitution resulted in an increase in internal Na^+ affinity to values of ~19 mM; these mutants also displayed enhanced inactivation, suggesting that inactivation requires binding of Na^+ to its intracellular transport sites. These findings are the first report of a regulatory kinetic state of Ca^{2+} transport via NCKX2 Na^+/Ca^{2+}-K^+ exchangers that may play a prominent role in regulation of Ca^{2+} extrusion in cellular environments such as neuronal synapses that experience frequent and dynamic Ca^{2+} fluxes.

Among the multitude of proteins that handle Ca^{2+} fluxes across the plasma membrane of excitable tissue, Na^+/Ca^{2+} exchangers play a prominent role in maintaining intracellular Ca^{2+} homeostasis. These proteins belong to one of two gene families: *SLC8*, the members of which mediate electrogenic exchange of three Na^+ ions for one Ca^{2+} ion (1), and *SLC24*, the members of which mediate electrogenic exchange of four Na^+ ions for one Ca^{2+} ion and one K^+ ion (2). The *SLC8* gene family is composed of three distinct proteins: the Na^+/Ca^{2+} exchanger (NCX)[3] NCX1, which has widespread tissue distribution in animals, with strong expression in the heart and spleen; and NCX2 and NCX3, which are restricted to the brain and skeletal muscle (3–5). On the other hand, the *SLC24* gene family is composed of five members. The Na^+/Ca^{2+}-K^+ exchanger (NCKX) NCKX1 was first reported in retinal rod outer segments and is the isoform on which most extensive physiological studies have been performed (6–8). NCKX2 transcripts have been reported in the brain and retina (9–12), and recent emergent evidence indicates that this isoform is important for regulation of Ca^{2+} levels in synaptic terminals (13–15). NCKX3 and NCKX4 transcripts are also found in the brain, but have also been localized to smooth muscle of the aorta, uterus, and intestine (16, 17). Recently, NCKX5 transcripts have been reported to be rich in melanin-containing tissues, *viz.* the eye and skin (18).

Most of the available studies on NCKX structure have been carried out on NCKX2. The NCKX proteins studied to date share a common predicted structure of two sets of five and six hydrophobic domains; the current topological model suggests an arrangement of five and five transmembrane segments separated by a large cytosolic loop (19). They also have a cleaved signal peptide that is essential for plasma membrane targeting, leaving a large extracellular loop at the N terminus (the C-terminal loop is also extracellular) (20). NCKX and NCX proteins share sequence homology in only two transmembrane regions forming internal repeats termed the α1 and α2 repeats. Residues within the α1 and α2 repeats of human NCKX2 have been shown to be important for the transport function of NCKX2 and for its affinity for both Ca^{2+} and K^+ (21–23); in addition, these transmembrane segments are in close proximity in three-dimensional space (24).

NCKX-mediated exchange of Ca^{2+} is characterized by a unique requirement for Na^+ over any other alkali cation (25, 26). However, to date, the residues responsible for Na^+ binding and transport are unknown. Hence, we sought to devise an assay that will allow us to control internal Na^+ to measure the affinity of the exchanger for Na^+. Additionally, although all Na^+/Ca^{2+} exchangers characterized to date are capable of mediating bidirectional transport of Ca^{2+} across the plasma membrane, most methods that have been devised to measure

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[3] The abbreviations used are: NCX, Na^+/Ca^{2+} exchanger; NCKX, Na^+/Ca^{2+}-K^+ exchanger; HEK293, human embryonic kidney 293 cells; TAPS, 3-[tris(hydroxymethyl)methyl]aminopropanesulfonic acid; HEDTA, *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid; PMCA, plasma membrane Ca^{2+}-ATPase; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; Tg, thapsigargin; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Na^+/Ca^{2+} exchange in intact cells rely on measuring the reverse (Ca^{2+} influx) mode of exchange. For this study, we sought to examine the biophysical properties of the forward (Ca^{2+} efflux) mode of exchange of human NCKX2, which is the mode of transport that is of physiological relevance. Finally, we set out to evaluate the contributions of other cellular Ca^{2+}-handling mechanisms in our assay system. From the results we obtained, we discovered that NCKX2 is a highly efficient Ca^{2+} extrusion mechanism, but displays a previously unknown inactive kinetic state that is both time- and Na^+-dependent.

EXPERIMENTAL PROCEDURES

The experimental procedures have been described in detail elsewhere (22, 27), and only modifications are included herein. All chemical reagents used were from Sigma unless specified otherwise.

Assaying for NCKX Function—Human embryonic kidney 293 (HEK293) cells transiently expressing the short splice variant of Myc-tagged NCKX2 (10, 21) were trypsinized, resuspended in Dulbecco's modified Eagle's medium (Invitrogen, Burlington, Ontario, Canada) supplemented with 12 μM fluo-3 acetoxymethyl ester or fluo-4FF acetoxymethyl ester (Molecular Probes, Burlington), and incubated at room temperature for 20 min. The cells were then pelleted by centrifugation at 300 $\times$ *g* for 1 min and washed with a medium of buffered 150 mM LiCl (150 mM LiCl, 0.1 mM EDTA, 20 mM HEPES, 0.25 mM sulfinpyrazone (to reduce dye leakage), and 6 mM D-glucose, pH 7.4). The cells were resuspended in buffered 150 mM LiCl, and 50-μl aliquots (containing $\sim 10^5$ cells) were added separately to cuvettes containing 1950 μl of test medium. The test medium was buffered 150 mM NaCl (150 mM NaCl, 0.1 mM EDTA, 20 mM HEPES, 0.25 mM sulfinpyrazone, and 6 mM D-glucose, pH 7.4) or buffered 150 mM KCl (150 mM KCl, 0.1 mM EDTA, 20 mM HEPES, 0.25 mM sulfinpyrazone, 6 mM D-glucose, and 0.5 mM dithiothreitol, pH 7.4). (The same stocks of test media were used to prepare mixed test media of different ionic compositions, *i.e.* 75 mM LiCl and 75 mM KCl was prepared by adding equal amounts of buffered 150 mM LiCl and buffered 150 mM KCl.) For the experiments carried out in buffered 150 mM KCl, pH 8.8 (see Fig. 2*A*), TAPS was used in place of HEPES. For each experiment, the cuvette containing Ca^{2+} indicator dye-loaded NCKX2-transfected HEK293 cells was placed in the 25 °C thermostatted cuvette housing of an SLM Series 2 luminometer (SLM Instruments, Urbana, IL), and fluorescence was continuously measured under constant stirring and upon the addition of various ion concentrations as described below. To clamp the alkali cation concentrations across the plasma membrane, the channel-forming alkali cation ionophore gramicidin (2 μM) was added to the cuvette 3 min prior to the start of the experiment; other drug treatments (as described below) were also added at the same time as gramicidin. The first addition thereafter was 350 μM $CaCl_2$, which caused an instantaneous step increase in fluorescence due to Ca^{2+} binding to leaked Ca^{2+} indicator dye; this level of fluorescence remained constant throughout the duration of experimentation and was also observed in mock-transfected Ca^{2+} indicator dye-loaded HEK293 cells (data not shown) and hence was subtracted during analysis of fluorescence signals. On the other hand, in experiments in which EDTA was added to the cuvette to chelate external Ca^{2+}, an instantaneous step decrease in fluorescence occurred, which signified chelation of Ca^{2+} from leaked Ca^{2+} indicator dye in the cuvette; in those cases, the value of the step increase in fluorescence at the beginning of the experimental recording period was added to the fluorescence values at the point at which EDTA was introduced in the cuvette. At the end of each experiment, 0.02% saponin was added in the presence of saturating $CaCl_2$ to saturate the Ca^{2+} indicator dye and to obtain a maximal fluorescence signal, which was used to normalize the fluorescence values for the respective experiment. In the experiments carried out with 10 μM external Ca^{2+}, the Ca^{2+} was buffered by the addition of 1 mM HEDTA and 8 mM Ca^{2+}/HEDTA to the cuvette; to chelate external Ca^{2+} in this case, 5 mM EDTA was added to the cuvette.

RESULTS

Assaying for NCKX2 Reverse Exchange and Dependence on Internal Na^+—Typical reverse exchange (Ca^{2+} influx) assays in whole cells rely on bathing the cells in a medium free of Na^+ while monitoring intracellular Ca^{2+} increases. During the course of our experimentation, we noticed that the activity of NCKX, as assessed by the magnitude of induced Ca^{2+} influx, decayed with time as NCKX-transfected cells were placed in Na^+-free medium. Along with the observation that the Ca^{2+} influx responses of NCKX-transfected HEK293 cells originating from different stocks were quite variable, this indicated that the discrepancies were likely due to variations in cytosolic Na^+. Therefore, we set out to modify our assay to allow us to verify that the reverse exchange mode of NCKX is indeed dependent on internal Na^+. To this end, we employed the cation channel-forming ionophore gramicidin to clamp monovalent cation concentrations across the plasma membrane and to allow us to control internal Na^+ concentrations (27, 28), thereby circumventing the variability in assaying reverse exchange in intact cells.

NCKX2-transfected HEK293 cells were loaded with the cell-permeant form of the Ca^{2+} indicator fluorescent dye fluo-3 and resuspended in buffered 150 mM LiCl. The cells were then placed in a cuvette containing buffered 150 mM KCl and treated with 2 μM gramicidin, and 350 μM $CaCl_2$ was added to the cuvette 3 min later, which caused a step increase in fluorescence that remained constant (due to leaked dye; see "Experimental Procedures"). (Also note that all cuvette media contained 0.1 mM EDTA, and hence, the addition of 350 μM $CaCl_2$ gave a final free Ca^{2+} concentration in the cuvette of 250 μM.) After 30 s, 75 mM NaCl was added to the cuvette, which caused a rapid increase in fluorescence (internal free Ca^{2+}) that reached a plateau level within 10 s (Fig. 1*A*). The same aforementioned procedure was repeated, but in a cuvette with buffered 150 mM NaCl in place of buffered 150 mM KCl. In this case, the addition of KCl (40 mM) was required to initiate reverse exchange and to elevate intracellular Ca^{2+} (Fig. 1*B*), consistent with the established absolute requirement of NCKX for K^+. NCKX2-transfected HEK293 cells were placed in a cuvette with buffered 150 mM LiCl and treated with gramicidin; after the addition of 350 μM $CaCl_2$, 40 mM KCl was added to the cuvette, but the fluorescence remained at the base line until the addition of 75 mM

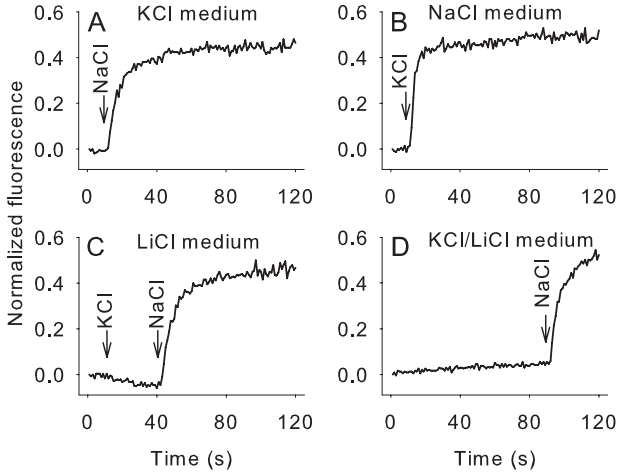

FIGURE 1. **Reverse exchange (Ca^{2+} import) mode of human NCKX2 and absolute requirement for internal Na^+.** *A*, fluo-3-loaded HEK293 cells transfected with human NCKX2 were placed in a cuvette with buffered 150 mM KCl (see "Experimental Procedures") and treated with the channel-forming alkali cation ionophore gramicidin (2 μM) 3 min before data collection. At the *arrow*, 75 mM NaCl was added (preceded by the addition of 350 μM $CaCl_2$ 30 s earlier for a final concentration of 250 μM external free Ca^{2+}), and the fluorescence was continuously monitored. *B*, fluo-3-loaded NCKX2-transfected HEK293 cells were placed in a cuvette with buffered 150 mM NaCl and treated with 2 μM gramicidin. At the *arrow*, 40 mM KCl was added (in the presence of 250 μM external free Ca^{2+}) to induce reverse exchange. *C*, fluo-3-loaded NCKX2-transfected HEK293 cells were placed in a cuvette with buffered 150 mM LiCl and treated with 2 μM gramicidin. At the *first arrow*, 40 mM KCl was added (in the presence of 250 μM external free Ca^{2+}); note that base-line fluorescence level was not altered significantly. After the passage of another 30 s, 75 mM NaCl was added to the cuvette, which induced the rise in fluorescence from the cells. *D*, fluo-3-loaded NCKX2-transfected HEK293 cells were placed in buffered 75 mM LiCl and 75 mM KCl and treated with 2 μM gramicidin. At the *arrow*, 75 mM NaCl was added (in the presence of 250 μM external free Ca^{2+}) to initiate reverse exchange; note that base-line fluorescence remained unaltered prior to the addition of NaCl.

NaCl (Fig. 1*C*), at which point the fluorescence rose rapidly to around the same plateau level observed in Fig. 1 (*A* and *B*). Finally, to illustrate that the exchanger is absolutely dependent on Na^+, NCKX2-transfected HEK293 cells were placed in a cuvette with buffered 75 mM LiCl and 75 mM KCl, and treated with 2 μM gramicidin. The addition of 350 μM $CaCl_2$ caused only a small step increase in fluorescence (data not shown), which remained constant for >1 min, and the fluorescence was finally elevated by the addition of 75 mM NaCl (Fig. 1*D*). Therefore, using this modified protocol, we were able to consistently control Na^+ concentrations in the medium and thereby assess the affinity of the exchanger for Na^+. In all subsequent experiments, NCKX2-transfected HEK293 cells were resuspended in buffered 150 mM LiCl, placed in cuvettes with 150 mM KCl, and treated with 2 μM gramicidin (unless specified otherwise). Reverse exchange was then initiated by the addition of NaCl subsequent to the addition of 350 μM $CaCl_2$; this concentration of Ca^{2+} was chosen to promote maximal Ca^{2+} influx through the exchanger while minimizing influx due to capacitative Ca^{2+} entry following internal Ca^{2+} store depletion (discussed below). Activation of capacitative Ca^{2+} entry in HEK293 cells typically requires the addition of millimolar concentrations of Ca^{2+} (29, 30), consistent with our observations (data not shown). Another modification we made in subsequent experiments was related to the observation that fluorescence amplitude levels attained using the described gramicidin clamp method were significantly higher than those previously obtained with intact cells (22), suggesting that internal Ca^{2+} was elevated to a greater extent in the presence of the gramicidin clamp than in its absence. Hence, we tested two other intracellular Ca^{2+} indicators from Molecular Probes: fluo-4FF, with a reported K_d of 9.7 μM, and fluo-5N, with a K_d of 90 μM. On the basis of comparison of fluorescence increases among these various Ca^{2+} indicator dyes and transfection efficiencies we routinely obtained (70–80%), we estimated that the fluorescence value of 0.5 obtained with fluo-4FF (normalized to the total amount of dye present in all cells) corresponded to intracellular free Ca^{2+} in the range of 15–20 μM. Therefore, in all subsequent experiments, we employed the cell-permeant form of the Ca^{2+} indicator dye fluo-4FF, which gave the best signal resolution without saturation of the fluorescence increase signals.

Isolating the Activity of NCKX2 from Other Ca^{2+}-handling Mechanisms—The addition of $CaCl_2$ and Na^+ to mock-transfected HEK293 cells in buffered 150 mM KCl did not result in an increase in internal Ca^{2+} (data not shown), indicating that HEK293 cells do not harbor endogenous Na^+/Ca^{2+} exchange activity. However, like all eukaryotic cells, they nevertheless have other mechanisms for intracellular Ca^{2+} regulation, *viz.* the plasma membrane Ca^{2+}-ATPase (PMCA), the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), and mitochondria. The contributions of these mechanisms to regulation of intracellular free Ca^{2+} in our assay system were examined. The PMCA activity was decreased by carrying out the assay in KCl medium with the pH adjusted to 8.8; SERCA was inhibited with 0.4 μM thapsigargin (Tg; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); and mitochondrial Ca^{2+} buffering was disabled with the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) at 2 μM. All drugs were added to the cells in the cuvette at the beginning of the experiment along with gramicidin, *i.e.* 3 min prior to initiating reverse exchange to allow the Tg-induced rise in internal Ca^{2+} to return to the base line.

Application of 0.4 μM Tg to gramicidin-treated NCKX2-transfected HEK293 cells resulted in a slight increase in the amplitude of fluorescence attained after inducing Ca^{2+} influx via reverse exchange compared with NCKX2-transfected HEK293 cells treated with gramicidin alone (Fig. 2*A*). Increasing the pH of the assay medium resulted in greater enhancement of the reverse exchange amplitude of the fluorescence increase as well, but the most marked enhancement of reverse exchange activity was observed with cells treated with 2 μM FCCP. The amplitude of fluorescence attained with cells treated with FCCP reached a value of ~0.5 of maximal fluorescence compared with ~0.2 for untreated cells (Fig. 2*A*). These results suggest that all these mechanisms contribute to some extent to regulation of cytosolic Ca^{2+} in HEK293 cells, and therefore, to isolate the activity of the exchanger, we must take into account the contribution of those mechanisms. We decided to closely examine the interplay of Ca^{2+} handling among NCKX2, SERCA, and mitochondria, but decided against carrying out our assays in pH 8.8 media (to inhibit PMCA), as that would preclude the use of gramicidin because it would lead to strong alkalinization of the cytosol. Moreover, it was unclear whether the observed enhancement of the fluores-

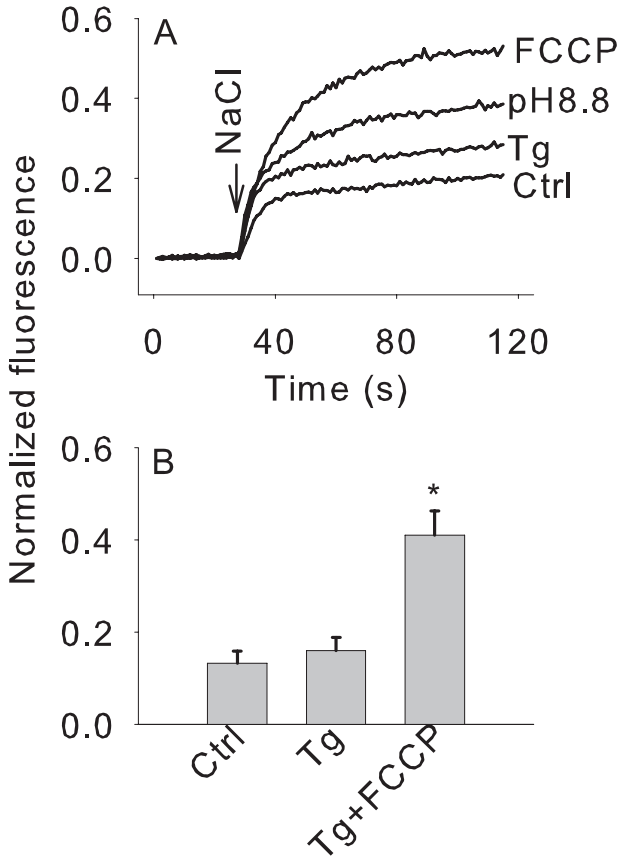

FIGURE 2. **Contribution of other cellular Ca^{2+}-handling mechanisms.** *A*, fluo-4FF-loaded NCKX2-transfected HEK293 cells were placed in a cuvette with buffered 150 mM KCl and treated with 2 μM gramicidin, and 75 mM NaCl was added at the *arrow* (in the presence of 250 μM external free Ca^{2+}; control (*Ctrl*)). In a second cuvette, NCKX2-transfected HEK293 cells from the same suspension were treated as described for the control with the addition of 0.4 μM Tg to inhibit the SERCA. In a third cuvette, NCKX2-transfected HEK293 cells from the same suspension were treated as described for the control with the addition of protonophore FCCP (2 μM) to prevent mitochondrial Ca^{2+} accumulation. In a fourth cuvette, NCKX2-transfected HEK293 cells from the same suspension were treated as described for the control, except that they were placed in a cuvette with 150 mM KCl adjusted to pH 8.8 to decrease PMCA activity. The traces shown are representative of results obtained in three other experiments. *B*, shown is a comparison of the final amplitudes of fluorescence attained by the addition of 75 mM NaCl to fluo-4FF-loaded NCKX2-transfected HEK293 cells placed in buffered 150 mM KCl with 250 μM external free $CaCl_2$ and treated with 2 μM gramicidin with cells additionally treated with 0.4 μM Tg with or without 2 μM FCCP. *Error bars* represent S.D. *, significantly greater than control ($p < 0.05$, Student's *t* test, $n = 3$).

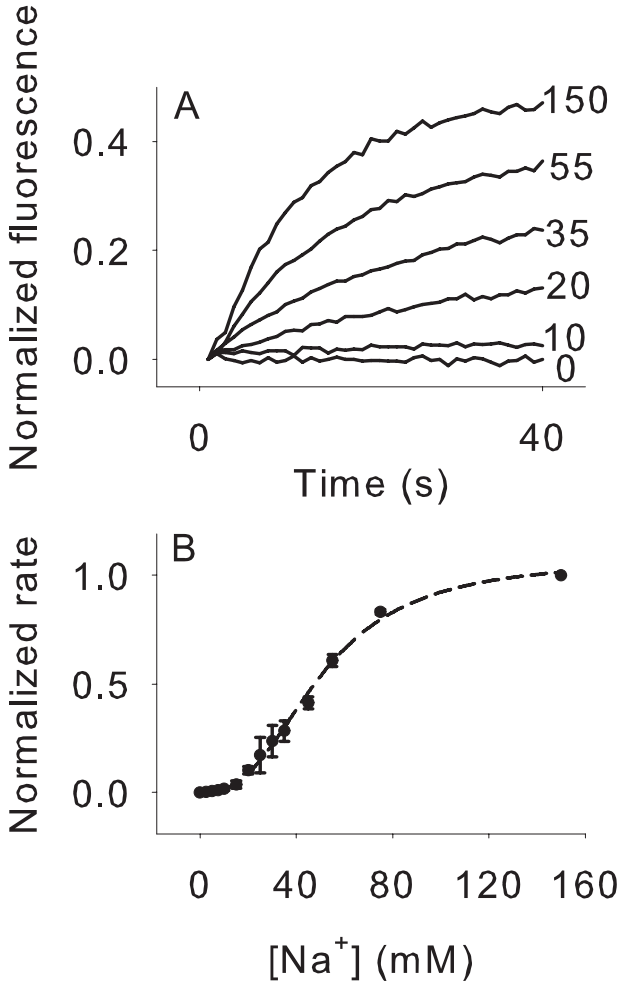

FIGURE 3. **Measurement of Na^+ affinity of NCKX2.** *A*, fluo-4FF-loaded NCKX2-transfected HEK293 cells were placed in cuvettes containing buffered 150 mM KCl and treated with 2 μM gramicidin, 0.4 μM Tg, and 2 μM FCCP. To initiate Ca^{2+} influx via reverse exchange, the indicated millimolar concentrations of NaCl were added at time 0 to separate cuvettes (in the presence of 250 μM external free Ca^{2+}). The traces shown are representative of results obtained in six other experiments. *B*, the initial rate of change in fluorescence attained after the addition of NaCl was used to derive a measure of the affinity of NCKX2 activity for internal Na^+ by normalizing the initial rate of change in fluorescence at each Na^+ concentration tested to the rate attained upon the addition of 150 mM NaCl. *Data points* represent averages, and *error bars* indicate S.D. ($n = 3$) fitted with a Hill function using SigmaPlot Version 7 software (K_d for $Na^+ = 50 \pm 1.6$ mM, Hill coefficient = 2.6).

cence increase at pH 8.8 was attributable to the decreased activity of PMCA *per se* because increased medium pH is also known to enhance the activity of the exchanger itself (31).

Fig. 2*B* compares the amplitude of the fluorescence increase in untreated NCKX2-transfected HEK293 cells with fluorescence amplitudes in NCKX2-transfected HEK293 cells pretreated with 0.4 μM Tg alone or with 2 μM FCCP. Although the fluorescence increase induced by the addition of 75 mM Na^+ in Tg-treated cells was somewhat higher compared with that in untreated control cells, the results were not statistically significant ($p > 0.05$, Student's *t* test, $n = 3$) (Fig. 2*B*). This suggested that SERCA alone did not contribute significantly to the magnitude of Ca^{2+} signals induced in HEK293 cells under our experimental conditions. On the other hand, the fluorescence amplitude more than doubled when cells were pretreated with Tg and FCCP in combination ($p < 0.05$, Student's *t* test, $n = 3$), suggesting that, in HEK293 cells, mitochondria are the major buffer of large NCKX2-mediated rises in cytosolic Ca^{2+}. Hence, to ensure in subsequent experiments that we were isolating the activity of the exchanger, all NCKX2-transfected HEK293 cells were pretreated with both 0.4 μM Tg and 2 μM FCCP prior to initiating reverse exchange (unless stated otherwise).

Measuring the Affinity of NCKX2 for Internal Na^+—To measure the affinity of NCKX2 for Na^+, we normalized the initial rate of change in fluo-4FF fluorescence attained upon the addition of various concentrations of Na^+ to the rate of fluorescence change obtained upon the addition of 150 mM NaCl (V_{max}) in the presence of 250 μM external free Ca^{2+} (Fig. 3*A*). In our experiments, Na^+ concentrations were simultaneously changed in both intracellular and external solutions, and this

was inevitable because of the use of gramicidin. The addition of 250 μM external free Ca^{2+} proved to be sufficient to suppress competition of Ca^{2+} influx by external Na^+ under our experimental conditions (data not shown). Fig. 3*B* shows the normalized rate of activity of NCKX2 in relation to the concentration of the substrate Na^+. The K_d of NCKX2 for internal Na^+ was 50 ± 1.6 mM ($n = 3$), and the fitted Hill plot gave a coefficient of 2.6, indicating that NCKX moves multiple Na^+ ions in each transport cycle. This is the first reported value of the K_d of NCKX for intracellular Na^+; it can be compared with values previously reported for the K_d for extracellular Na^+ of human and chicken NCKX2 expressed in the insect cell line High Five (38–48 mM Na^+, Hill coefficient of 1.9) (32), rat NCKX2 expressed in HEK293 cells (30 mM Na^+, Hill coefficient of 2.8) (33), and *in situ* bovine rod NCKX1 (35 mM Na^+, Hill coefficient of 2) (34, 35).

Forward Exchange (Ca^{2+} Extrusion) Mode of NCKX2—To examine the forward exchange (Ca^{2+} extrusion) mode of NCKX2 operation, we loaded NCKX2-transfected HEK293 cells with Ca^{2+} via the reverse exchange mode of operation of NCKX2 by the addition of 75 mM NaCl in the presence of 250 μM external free Ca^{2+}, and after the fluorescence reached the steady-state plateau level, 1 mM EDTA was added to the cuvette to chelate external Ca^{2+}, thereby reversing the Ca^{2+} gradient across the plasma membrane. The fluorescence levels dropped precipitously upon the addition of EDTA presumably because of extrusion of Ca^{2+} from the cytosol via NCKX2 (Fig. 4*A*, *Ctrl trace*). We repeated this assay with Tg in the absence and presence of FCCP. Whereas the pattern of fluorescence change in Tg-treated cells mirrored that in untreated control NCKX2-transfected HEK293 cells, those cells treated with both Tg and FCCP had a greatly diminished rate of Ca^{2+} clearance. This suggested that the rapid decrease in fluorescence observed in the untreated cells was not mediated by the exchanger, at least not entirely, but in fact, mitochondria in that case effectively and very rapidly sequestered cytosolic Ca^{2+}. Hence, we proceeded to test the ability of NCKX2-transfected HEK293 cells pretreated with Tg and FCCP to extrude Ca^{2+} under a range of different Na^+ concentrations and found that the rate of Ca^{2+} extrusion diminished with increasing Na^+ concentrations (Fig. 4*B*). This finding was surprising, as we were expecting that Ca^{2+} extrusion rates would accelerate as the concentration of Na^+ was increased, according to kinetic considerations. In the example shown in Fig. 4*B*, the addition of 75 and 150 mM Na^+ promoted rapid Ca^{2+} influx through the reverse mode of operation of NCKX2; however, when the forward mode was engaged, the fluorescence decayed very slowly and did not reach the base line by the end of the data recording period (>2 min), signifying that the exchanger had somehow inactivated and was no longer able to effectively extrude internal Ca^{2+}.

Time Course of Inactivation of NCKX2—To exclude the possibility that elevating intracellular Na^+ in our assay resulted in the apparent inactivation of the forward mode of exchange because of competitive inhibitory interactions between Na^+ and Ca^{2+}-K^+ at the intracellular binding/transport site of the exchanger, we examined the time course of inactivation of NCKX2. In Fig. 5*A*, NCKX2-transfected HEK293 cells were loaded with Ca^{2+} via the exchanger by the addition of 75 mM

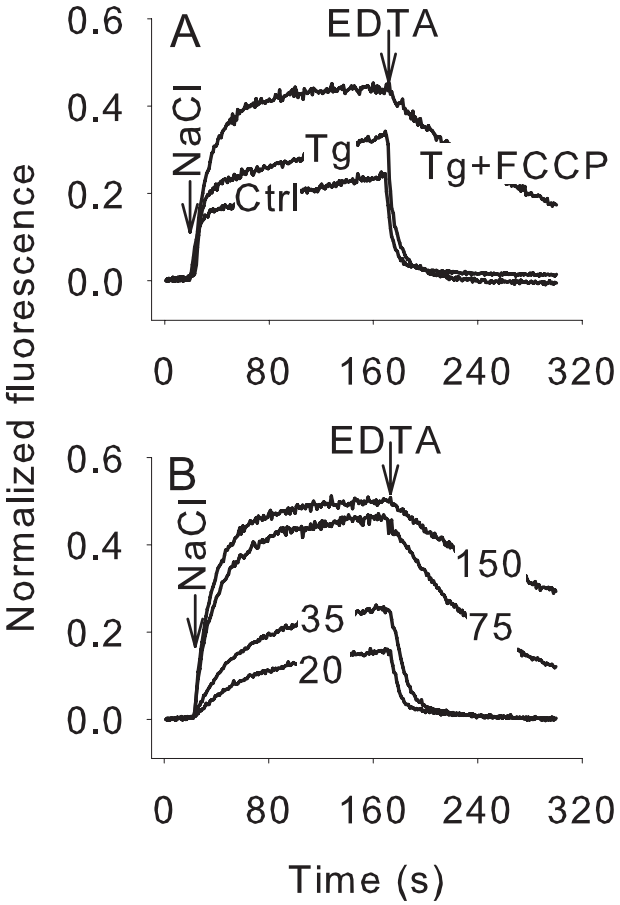

FIGURE 4. **Forward exchange (Ca^{2+} extrusion) mode of NCKX2.** *A*, fluo-4FF-loaded NCKX2-transfected HEK293 cells were placed in cuvettes with buffered 150 mM KCl and treated with 2 μM gramicidin. After loading the cells with Ca^{2+} using the reverse exchange mode of NCKX2 by the addition of 75 mM NaCl (in the presence of 250 μM external free Ca^{2+}), 1 mM EDTA was added to chelate external Ca^{2+}, thereby reversing the Ca^{2+} gradient and inducing Ca^{2+} extrusion via the exchanger. In separate cuvettes, cells were also treated with Tg alone to inhibit SERCA or with Tg and FCCP in combination to inhibit both SERCA and mitochondrial Ca^{2+} sequestration. Note that, in both untreated control (*Ctrl*) and Tg-treated cells, the fluorescence rapidly returned to base-line levels upon the addition of EDTA; however, when mitochondria were additionally disabled, the fluorescence decrease (internal Ca^{2+} clearance) rate was greatly diminished. The traces shown are representative of results obtained in six other experiments. *B*, fluo-4FF-loaded HEK293 cells were placed in separate cuvettes with buffered 150 mM KCl and treated with 2 μM gramicidin, 0.4 μM Tg, and 2 μM FCCP. At the *first arrow*, the indicated millimolar concentrations of NaCl were added to the cuvettes (in the presence of 250 μM external free Ca^{2+}). After 150 s, 1 mM EDTA was added to each cuvette to chelate external Ca^{2+} and to initiate forward exchange. Note that the rate of internal Ca^{2+} clearance diminished with increasing Na^+ concentrations. The traces shown are representative of results obtained in six other experiments.

Na^+ in the presence of 250 μM external free Ca^{2+}. At various time points thereafter, 1 mM EDTA was introduced to chelate extracellular Ca^{2+} and to initiate Ca^{2+} efflux from the cytosol via the exchanger. Fig. 5 shows the results from separate cuvettes using the same suspension of NCKX2-transfected HEK293 cells overlaid for illustrative purposes. The rate of Ca^{2+} clearance diminished with time, and it was clear that inactivation of the exchanger developed with a relatively slow time course; we estimated the $t_{1/2}$ of inactivation at 40 s. These results argue against competitive inhibition because the apparent inactivation process required significant time to develop and was not apparent when the cytosolic compartment was loaded with

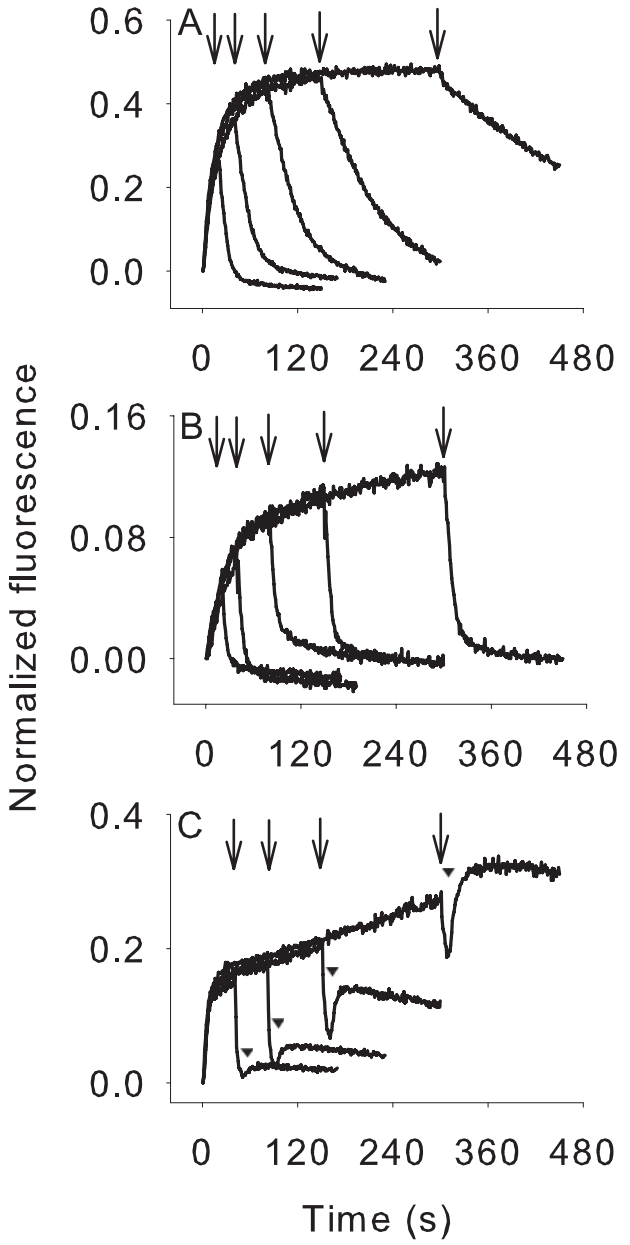

FIGURE 5. **Time course of inactivation of NCKX2 and accumulation of the mitochondrial Ca^{2+} pool.** *A*, fluo-4FF-loaded NCKX2-transfected HEK293 cells were placed in cuvettes with buffered 150 mM KCl and treated with 2 μM gramicidin, 0.4 μM Tg, and 2 μM FCCP. At time 0, 75 mM NaCl was added to each cuvette in the presence of 250 μM external free Ca^{2+}. At various time points thereafter, 1 mM EDTA was added to the cuvette as indicated by the *arrows*. The data shown are from separate cuvettes overlaid to illustrate the difference in kinetics of Ca^{2+} clearance. The traces shown are representative of results obtained in four other experiments. *B*, the same experimental conditions as described for *A* were used, except that Ca^{2+} influx was induced by the addition of 20 mM NaCl at time 0 (concentration not sufficient to produce strong inactivation; see Fig. 4*B*). The traces shown are representative of results obtained in two other experiments. *C*, fluo-4FF-loaded NCKX2-transfected HEK293 cells were placed in cuvettes with buffered 150 mM KCl and treated with 2 μM gramicidin and 0.4 μM Tg. In separate cuvettes, at time 0, 75 mM NaCl was added, and at the *arrows*, 1 mM EDTA was added to the cuvette to chelate external Ca^{2+} and to initiate Ca^{2+} efflux. Following the addition of EDTA at 10 s, 2 μM FCCP was added to release accumulated Ca^{2+} in mitochondria (indicated by the *arrowheads*). The data shown are from four separate cuvettes overlaid for illustrative purposes. The traces shown are representative of results obtained in three other experiments.

high free Ca^{2+} for periods of up to 40 s while still exposed to high levels of internal Na^+ (75 mM). If competitive inhibition by Na^+ was prominent, then the apparent inactivation process would have been expected to occur instantaneously upon the addition of EDTA. As a control, we examined the time course of the Ca^{2+} efflux component of NCKX2-transfected HEK293 cells loaded with 20 mM Na^+ in the presence of 250 μM external free Ca^{2+}; this concentration of Na^+ was not sufficient to produce inactivation, as illustrated in Fig. 4*B*. As shown in Fig. 5*B*, in the presence of 20 mM Na^+, NCKX2 was competent in rapidly clearing cytosolic Ca^{2+} loads regardless of the incubation time with Na^+. Because of the results shown in Fig. 4*A*, we were interested in examining the time course of development of mitochondrial Ca^{2+} accumulation in NCKX2-transfected HEK293 cells. To this end, gramicidin- and Tg-treated NCKX2-transfected HEK293 cells were loaded with Ca^{2+} by the addition of 75 mM Na^+ in the presence of 250 μM external free Ca^{2+}, and at various time points thereafter, 1 mM EDTA was added to chelate external Ca^{2+}. After 10 s, 2 μM FCCP was applied to release accumulated Ca^{2+} in the mitochondria. Fig. 5*C* shows the results from four separate cuvettes displaying different Ca^{2+} loading periods and overlaid to illustrate the development of Ca^{2+} accumulation in mitochondria. At the earliest time point (20 s), very little Ca^{2+} had accumulated in the mitochondria in comparison with the Ca^{2+} released by application of FCCP after 300 s of Ca^{2+} loading via NCKX2. These experiments also suggested that, under our experimental conditions, there was a dynamic equilibrium established between NCKX2 mediating Ca^{2+} influx into the cytosol and mitochondria accumulating Ca^{2+} from the cytosol. The experiments also suggested that the reverse mode of operation of NCKX2 seemed to operate continuously, as the magnitude of the Ca^{2+} pool accumulated by mitochondria grew larger with time presumably because of the continued influx of external Ca^{2+} through the exchanger.

NCKX2 Inactivation Is Dependent on External Ca^{2+}—To further address whether the apparent inactivation of the exchanger may have been due to application of high Na^+ at the intracellular surface of the exchanger, thereby effectively competing with Ca^{2+} at the Ca^{2+}-K^+-binding pocket, we examined the process of inactivation of NCKX2 in HEK293 cells loaded with different starting values of Ca^{2+}. We had predicted that, if competitive inhibition was a major contributing factor to the apparent inactivation, then lowering the starting external Ca^{2+} levels should enhance the apparent inactivation (because less Ca^{2+} would enter the cytosolic compartment via the exchanger, and hence, Na^+ would more effectively compete with Ca^{2+} for the intracellular binding pocket). However, we observed the opposite effect; as the starting concentration of Ca^{2+} was decreased, inactivation did not develop. Fig. 6 shows a typical example of NCKX2-transfected HEK293 cells assayed with either 250 or 10 μM external free Ca^{2+}. In both experiments, the cells were loaded with Ca^{2+} via the reverse exchange mode of NCKX2 by the addition of 75 mM Na^+. The cells that had been exposed to 10 μM external free Ca^{2+} were able to rapidly clear the cytosolic load of Ca^{2+}, whereas those exposed to 250 μM external free Ca^{2+} (the concentration used in previous experiments) displayed a diminished rate of Ca^{2+} clearance

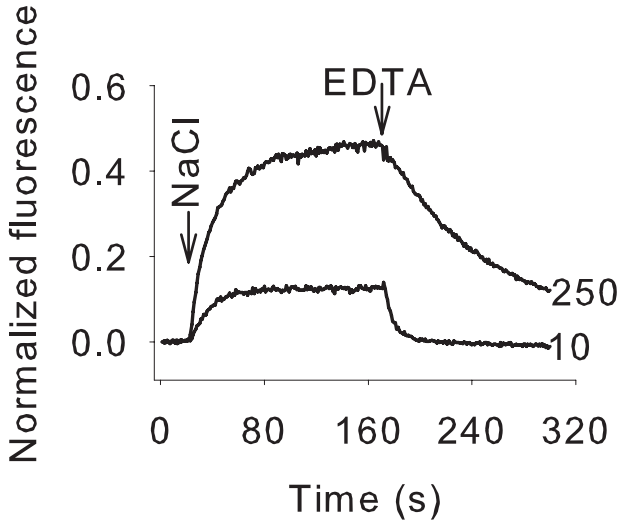

FIGURE 6. **NCKX2 inactivation does not develop with low extracellular Ca^{2+}.** Fluo-4FF-loaded NCKX2-transfected HEK293 cells were placed in cuvettes with buffered 150 mM KCl and treated with 2 μM gramicidin, 0.4 μM Tg, and 2 μM FCCP. At the *first arrow*, 75 mM NaCl was added in the presence of 250 or 10 μM external free Ca^{2+}, achieved by the addition of 1 mM HEDTA and 8 mM Ca^{2+}/HEDTA (see "Experimental Procedures"). At the *second arrow*, 5 mM EDTA was added to chelate external Ca^{2+} and to initiate Ca^{2+} extrusion via the exchanger. Note that the rate of Ca^{2+} efflux was markedly slower in the cells incubated with 250 μM external free Ca^{2+}, signifying inactivation of NCKX2. The traces shown are representative of results obtained in three other experiments.

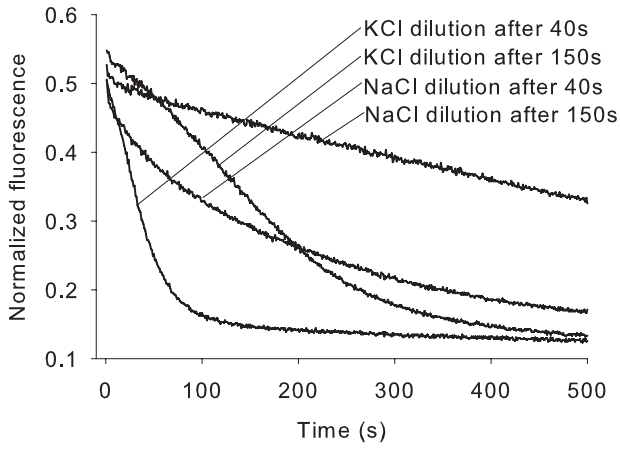

FIGURE 7. **Relief of inactivation of NCKX2.** Fluo-4FF-loaded NCKX2-transfected HEK293 cells were placed in cuvettes with 500 μl of buffered 100 mM NaCl and 50 mM KCl, and treated with 2 μM gramicidin, 0.4 μM Tg, and 2 μM FCCP. The cells were then loaded by the addition of 250 μM external free Ca^{2+} to the cuvette. After either 40 or 150 s, the medium in the cuvette was diluted 4-fold by the addition of 1500 μl of buffered 150 mM KCl (supplemented with 1 mM EDTA) or 1500 μl of buffered NaCl (supplemented with 1 mM EDTA). The traces represent dilution with either buffered KCl or NaCl and, after a period of 40 or 150 s, incubation with Na^+; dilution was at time 0 in each case. The traces shown are representative of results obtained in three other experiments.

from the cytosol upon initiation of Ca^{2+} extrusion. This suggested that inactivation of NCKX2 required high intracellular Na^+ and high extracellular Ca^{2+}; the results also argued against competitive inhibitory interactions between Na^+ and Ca^{2+}. In addition, we considered the possibility of abrupt changes in pH mediating the observed inactivation process. To this end, we loaded NCKX2-transfected HEK293 cells with the proton indicator cell-permeant form of the dye 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (Molecular Probes) and carried out the assays described herein, but under no conditions did we detect any large or abrupt changes in intracellular pH, even upon the addition of FCCP and the various solutes (data not shown).

Relief of Inactivation of NCKX2—Further evidence that the inactivation of the exchanger was both time- and Na^+-dependent was that it could be relieved by reducing the Na^+ concentration in the cuvette. This was achieved by loading the cells with Ca^{2+} using the reverse exchange mode of NCKX2 in 500 μl of buffered 100 mM NaCl and 50 mM KCl, after a passage of 150 s, the medium was diluted 4-fold with 150 mM buffered KCl (containing 1 mM EDTA) to decrease the Na^+ concentration from 100 to 25 mM. This caused a time-dependent relief of inactivation (Fig. 7). In contrast, upon dilution with 150 mM buffered NaCl (containing 1 mM EDTA), the inactivated state of forward exchange was maintained despite the fact that the high Na^+ concentration was expected to saturate the external binding sites and to maximize Ca^{2+} efflux. When the assay medium was diluted with buffered 150 mM KCl (containing 1 mM EDTA) 40 s following initiation of reverse exchange, the fluorescence levels dropped precipitously, consistent with results of the time dependence plots in Fig. 5*A*, suggesting that exposure to high intracellular Na^+ alone was not sufficient to completely inactivate the exchanger and that the full inactivation required >40 s to become apparent. Note that dilution with 150 mM buffered NaCl (containing 1 mM EDTA) 40 s following initiation of reverse exchange resulted initially in rapid clearing of cytosolic Ca^{2+}, with the rate then subsiding due to progressive inactivation. As a result, the final cytosolic Ca^{2+} concentration at the end of our recording in 150 mM buffered NaCl was higher than the level achieved by dilution of the assay medium with buffered 150 mM KCl.

NCKX2 Inactivation Is an Inherent Property of the Exchanger Related to Its Affinity for Internal Na^+—The NCKX2 inactivation described above appeared to be related to occupancy of the internal binding sites of NCKX2 with Na^+. To test this notion, it would be desirable to have NCKX2 mutants with altered Na^+ affinity. We are currently in the process of screening all the NCKX2 mutants reported previously (21) for changes in Na^+ affinity using the experimental protocol illustrated in Fig. 3. Two single residue substitutions were found to be useful for this study, as they resulted in a marked increase in affinity for internal Na^+. Previously, we had reported that a key acidic residue, Asp^{548}, which lies within the midplane of transmembrane-spanning domain H8 of NCKX2, is important for the binding and transport of both Ca^{2+} and K^+ (22). Substitution of Asp^{548} with glutamate (D548E) caused dramatic decreases in the affinity of the exchanger for K^+ and Ca^{2+} by 10- and 100-fold, respectively. In this study, we assayed mutant D548E for internal Na^+ affinity at the same concentrations tested with wild-type NCKX2. As shown in Fig. 8*A*, the apparent affinity for intracellular Na^+ was increased with the D548E substitution; the K_d value was 19.3 ± 1.6 mM ($n = 3$) as opposed to 50 mM for wild-type NCKX2 (Fig. 3*B*). The Hill coefficient was slightly increased to a value of 3.0 as opposed to 2.6 for wild-type NCKX2. Another residue whose substitution we also found to increase the apparent affinity of the exchanger for Na^+ was Asn^{572}; its substitution to cysteine (N572C) also resulted in

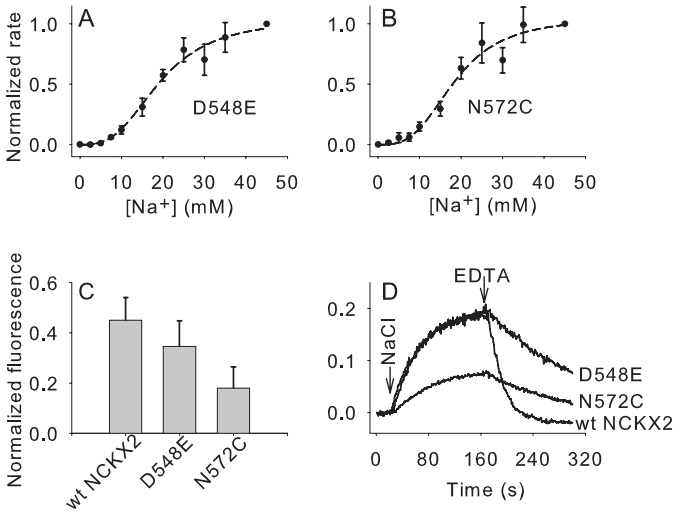

FIGURE 8. **Enhanced inactivation of NCKX2 mutants with increased Na^+ affinity.** *A*, fluo-4FF-loaded HEK293 cells transfected with the NCKX2 mutant D548E were assayed for Na^+ affinity as described in the legend to Fig. 3. *Data points* represent averages, and *error bars* indicate S.D. The K_d for Na^+ was 19.3 ± 0.6 mM, and the fitted Hill plot gave a Hill coefficient of 3.0. *B*, fluo-4FF-loaded HEK293 cells transfected with the NCKX2 mutant N572C were assayed for Na^+ affinity as described in the legend to Fig. 3. The K_d for Na^+ was 18.5 ± 0.3 mM, and the fitted Hill plot gave a Hill coefficient of 3.1. *C*, shown is a comparison of the V_{max} values for wild-type (*wt*) NCKX2, D548E, and N572C based on the maximal plateau amplitude of fluorescence attained. *D*, fluo-4FF-loaded HEK293 cells transfected with wild-type NCKX2, D548E, or N572C were placed in cuvettes with buffered 150 mM KCl and treated with 2 μM gramicidin, 0.4 μM Tg, and 2 μM FCCP. At the *first arrow*, 35 mM NaCl was added to the cuvettes in the presence of 250 μM external free Ca^{2+}, and 1 mM EDTA was subsequently added at the *second arrow* 150 s following the addition of NaCl. Note that, although wild-type NCKX2-transfected HEK293 cells were able to rapidly clear the cytosolic load of Ca^{2+}, both D548E- and N572C-transfected HEK293 cells had a diminished rate of Ca^{2+} clearance, signifying inactivation of these exchangers at lower Na^+ concentration.

increased Na^+ affinity, with K_d = 18.5 ± 1.9 (n = 3) (Fig. 8*B*) and a Hill coefficient of 3.1. Fig. 8*C* compares the V_{max} values of the three NCKX2 constructs as judged by the maximal amplitude of the Na^+-induced changes in fluo-4FF fluorescence changes. Wild-type NCKX2 and the D548E mutant showed comparable values, whereas the V_{max} of the N572C mutant was lower. Because we had observed that NCKX2 inactivated when exposed to high intracellular Na^+, we predicted that those mutants with increased affinity for intracellular Na^+ would display inactivation of the forward mode of exchange at lower Na^+ concentrations than would wild-type NCKX2. Fig. 8*D* shows that this was indeed the case; although we observed inactivation of wild-type NCKX2 only when intracellular Na^+ was elevated to 75 mM or higher (see Fig. 4*B*), both the D548E and N572C mutants displayed greatly decreased Ca^{2+} clearance when intracellular Na^+ was elevated to 35 mM. Note that with the level of Ca^{2+} loading induced by the addition of 35 mM Na^+, D548E-transfected HEK293 cells were loaded with the same level of Ca^{2+} as cells transfected with wild-type NCKX2; however, those transfected with N572C had about half the level of intracellular Ca^{2+} increase upon the addition of Na^+. When looking at the forward exchange mode, only the mutants (with increased Na^+ affinity) were inactivated and did not rapidly lower intracellular Ca^{2+} back to the base line upon the addition of EDTA, whereas wild-type NCKX2-transfected HEK293 cells rapidly cleared the cytosolic load of Ca^{2+} when exposed to 35 mM Ca^{2+} (see also Fig. 4*B*). Although in our previous work we had shown that D548E displays a decreased affinity for Ca^{2+} and K^+ (22), this does not account for this inability of this mutant protein to clear cytosolic Ca^{2+} (as shown in Fig. 8) because, when forward exchange was engaged at an earlier time point, D548E rapidly cleared the rise in intracellular Ca^{2+} (as did N572C) (data not shown).

DISCUSSION

The *SLC24* gene family of NCKX Na^+/Ca^{2+}-K^+ exchangers comprises bidirectional and electrogenic Ca^{2+} transporters. The transport of Ca^{2+} through these carriers is dependent on the relative gradients of Na^+, Ca^{2+}, and K^+ as well as membrane potential. NCKX displays a high turnover rate and hence a large capacity to change intracellular free Ca^{2+} over a large range of free Ca^{2+} concentrations and even at low intracellular free Ca^{2+} in the nanomolar range (36). Here, we have described a method for measuring the affinity of the human retinal cone/brain exchanger NCKX2 for Na^+ and how to examine the physiologically relevant mode of Ca^{2+} extrusion from the cytosol; in the process, we discovered a novel feature of Na^+-dependent inactivation of Ca^{2+} transport. We suggest that this kinetic transition between an active and inactive mode of the exchanger serves as a regulatory mechanism to prevent lowering of intracellular Ca^{2+} to unfavorable levels.

Controlling Alkali Cation Concentrations Using Gramicidin—The method of using the gramicidin clamp to control alkali cation concentrations across the plasma membrane has proven very useful and indeed necessary for accurately and quantitatively assaying the affinity of the exchanger for Na^+. The resting Na^+ concentration of cells is typically ~10 mM, which is the concentration of Na^+ at the initial sigmoidal ramp of exchanger activity (Fig. 3*B*); hence, relying on the low resting intracellular Na^+ concentration to drive Ca^{2+} import can be very variable because of the sensitivity of the exchanger to small changes in Na^+ concentrations in this range. In the presence of gramicidin and high Na^+, NCKX2 was able to drive intracellular free Ca^{2+} to high levels of 15–20 μM, necessitating the use of the low affinity Ca^{2+} indicator fluo-4FF to avoid saturation of the dye. The dynamic Ca^{2+} fluxes observed here for HEK293 cells expressing NCKX2 are not a consequence of overexpression of the NCKX2 protein in this system, as they are similar to those observed *in situ* for Ca^{2+} fluxes and NCKX currents in the outer segments of retinal rod (28, 36) and cone (12) photoreceptors.

Interplay between NCKX2 and Other Cellular Ca^{2+}-handling Mechanisms—As our results have clearly shown, whereas NCKX can mediate large increases in intracellular Ca^{2+}, when the Na^+ gradient is reversed so as to favor Ca^{2+} efflux, only a portion of the Ca^{2+} efflux signal component is actually mediated by the exchanger (when intracellular Na^+ is high). Specifically, it appears that a dynamic equilibrium of Ca^{2+} influx via the exchanger and Ca^{2+} sequestration by mitochondria is established once intracellular Ca^{2+} levels reach a certain threshold. On the basis of experiments we carried out with the two dyes fluo-3 (with a reported K_d for Ca^{2+} of ~0.4 μM; data not shown) and fluo-4FF (with K_d for Ca^{2+} of ~10 μM), we estimated that the level at which mitochondria began accumu-

lating Ca^{2+} was ~2–3 μM. Kim *et al.* (37) found a similar pattern when investigating Ca^{2+} clearance at synaptic terminals, where Na^+/Ca^{2+} exchange activity (including a significant K^+-dependent component) dominates in Ca^{2+} clearance, with mitochondria participating only when Ca^{2+} reaches levels >2.5 μM or the Ca^{2+} load is prolonged. Likewise, in adrenal chromaffin neuroendocrine cells, mitochondria do not participate in clearance of Ca^{2+} loads (induced by depolarization) up to 0.5 μM; however, mitochondria are the major mechanism for Ca^{2+} clearance when intracellular Ca^{2+} reaches 2–3 μM (38). Coincidentally, the level of Ca^{2+} at which mitochondria started to accumulate Ca^{2+} in our cells was the same as that required to observe inactivation of the exchanger, raising the possibility of an underlying interaction between mitochondria and the exchanger at the plasma membrane. Such an interaction between the plasma membrane Na^+/Ca^{2+} exchanger and mitochondria has been suggested previously to mediate inactivation of exchange of NCX (39). Although our results cannot conclusively rule out this intriguing possibility, we do not think that such is the case for NCKX; however, this issue deserves further investigation.

Distinguishing Inactivation from Competitive Inhibition of NCKX2 Activity—Arguably, the use of gramicidin introduced the caveat that Na^+ was present on both sides of the membrane (likewise with K^+), and hence, exchange activity in the reverse (or forward) mode may have been slowed because of competition of Na^+ on the external (or internal) side of the membrane. We do not believe this to be the case because, by carrying out the assay in a medium of high KCl, we were able to effectively minimize inhibitory interactions between Na^+ and Ca^{2+} by favoring the binding of Ca^{2+}, as was shown previously in studies of Na^+/Ca^{2+}-K^+ exchange *in situ* in rod outer segments (36). Moreover, several of our findings herein argue against competitive inhibition. First, the inactivation process was time-dependent as illustrated in Fig. 5, although if competitive inhibition was prominent, it would have been observed at all time points examined. In line with this finding, we have also shown time-dependent relief of inactivation by lowering intracellular Na^+ from 100 to 25 mM (Fig. 7). Second, inactivation did not develop when extracellular Ca^{2+} was lowered to 10 μM (Fig. 6). This result was intriguing because it would be expected that lowering the external Ca^{2+} and ultimately the Ca^{2+} availability in the cytoplasm would promote competitive inhibition in the presence of high internal Na^+. Interestingly, the process of Na^+-dependent inactivation described herein for NCKX2 is similar in characteristics to Na^+-dependent inactivation described for the cardiac Na^+/Ca^{2+} exchanger (40), for which it was proposed that the inactive state occurs when the exchanger transport sites are exposed to high Na^+ only on the cytoplasmic side and that it requires high extracellular Ca^{2+} as well. Hilgemann *et al.* (40) suggested that inactivation of the cardiac exchanger does not develop with low extracellular Ca^{2+} because, under those conditions, the exchanger binding sites would favor the exoplasmic face, which prevents them from exposure to high internal Na^+ on the cytoplasmic face, the only conformation that will inactivate the exchanger. Finally, our results with the NCKX2 point mutants D548E and N572C suggest that inactivation of NCKX2 is dependent on its affinity for Na^+, as both these mutants showed enhanced inactivation at lower intracellular Na^+, concomitant with their increased affinity for this ion (Fig. 8).

Residues Important for Na^+ Binding—No studies have reported previously on residues important for Na^+ transport of NCKX transporters or on residues in the α2 repeat of NCX transporters important for Na^+ transport. Asp^{548} and Asn^{572} are located and conserved in the α2 repeats of all NCX and NCKX sequences currently in the data base, and our observation of altered Na^+ affinities suggests that these two residues may form part of the cation-binding pocket of all NCKX and NCX transporters. We reported previously that Asp^{548} is one of the two most important residues (together with Glu^{188}) that determine the Ca^{2+} affinity of NCKX transporters (22).

Physiological Implications—Previous work from our laboratory on retinal rod outer segments that carry the related NCKX1 exchanger suggest that the NCKX1 exchanger operates in "bursts" of activity, which then drastically diminish (41, 42). Our results here suggest the presence of different kinetic states of the NCKX2 exchanger and reveal some of the ionic conditions necessary for the process of inactivation to occur: high extracellular Ca^{2+} (≥250 μM) and high intracellular Na^+ (>35 mM). Little is known about the molecular mechanism of the above regulatory features of NCKX1 and NCKX2 and to what degree they are related or distinct. The experimental paradigms described in this study and the capability of HEK293 cells to express (mutant) NCKX proteins at levels comparable with those found *in* situ in rod and cone photoreceptors will be useful to further elucidate the regulatory mechanisms and kinetic states of NCKX exchangers.

Inactivation of NCKX2 may be a useful regulatory mechanism that operates under physiological conditions. Mounting evidence points to NCKX2 being an important Ca^{2+} clearance mechanism in neuronal synaptic terminals (13–15), which are specialized compartments that experience frequent and dynamic Ca^{2+} fluxes and depolarized membrane potential resulting from frequent invasions of action potential spikes. Persistent presynaptic activity leads to persistent elevations in presynaptic terminal Ca^{2+} concentrations (43, 44), associated with an increase in intraterminal Na^+ levels (up to peaks of 80 mM) (45). This accumulation of intraterminal Na^+ has been suggested to induce Ca^{2+} influx into the neuronal terminal through plasma membrane Na^+/Ca^{2+} exchangers operating in the reverse mode as well as from delayed release of Ca^{2+} from mitochondria (46, 47). Although NCX operating at an exchange stoichiometry of three Na^+ ions for one Ca^{2+} ion may indeed reverse under such conditions, NCKX with a stoichiometry of four Na^+ ions for one Ca^{2+} ion + one K^+ ion is less likely to reverse and begin Ca^{2+} import. However, a significant component of the persistent rise in intraterminal Ca^{2+} may be explained by the diminished rate of Ca^{2+} clearance by NCKX2 as it becomes inactivated by the prolonged high levels of intracellular Na^+ until presynaptic activity subsides, at which point ionoregulatory mechanisms can restore resting ionic conditions, and the exchanger may resume in clearance of the presynaptic terminal of the high load of Ca^{2+}.

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