Electrophysiological Characterization and Ionic Stoichiometry of the Rat Brain K⁺-dependent Na⁺/Ca²⁺ Exchanger, NCKX2*

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We have recently described a novel K⁺-dependent Na⁺/Ca²⁺ exchanger, NCKX2, that is abundantly expressed in brain neurons (Tsai, M., Rhee, K.-H., Bungard, D., Li, X.-F., Lee, S.-L., Auer, R. N., and Lytton, J. (1998) J. Biol. Chem. 273, 4115–4125). The precise role for NCKX2 in neuronal Ca²⁺ homeostasis is not yet clearly understood but will depend upon the functional properties of the molecule. Here, we have performed whole-cell patch clamp analysis to characterize cation dependences and ion stoichiometry for rat brain NCKX2, heterologously expressed in HEK293 cells. Outward currents generated by reverse NCKX2 exchange depended on external Ca²⁺ with a Kᵢ of 1.4 or 10 µM without or with 1 mM Mg²⁺, and on external K⁺ with a Kᵢ of about 12 or 36 mM with choline or Li⁺ as counter ion, respectively. Na⁺ inhibited outward currents with a Kᵢ of about 60 mM. Inward currents generated by forward NCKX2 exchange depended upon external Na⁺ with a Kᵢ of 30 mM and a Hill coefficient of 2.8. K⁺ inhibited the inward currents by a maximum of 40%, with a Kᵢ of 2 mM or less, depending upon the conditions. The transport stoichiometry of NCKX2 was determined by observing the change in reversal potential as individual ion gradients were altered. Our data support a stoichiometry for rat brain NCKX2 of 4 Na⁺:1 Ca²⁺:1 K⁺. These findings provide the first electrophysiological characterization of rat brain NCKX2, and the first evidence that a single recombinantly expressed NCKX polypeptide encodes a K⁺-transporting Na⁺/Ca²⁺ exchanger with a transport stoichiometry of 4 Na⁺:1 Ca²⁺:1 K⁺.

Cytoplasmic Ca²⁺ plays a key role in intracellular signaling in virtually all types of animal cells. Excitable cells in particular undergo frequent, dramatic, and transitory elevations in cytosolic free Ca²⁺ concentration ([Ca²⁺]ᵢ) (1, 2). The Ca²⁺ signal must then be removed rapidly from the cytosol, and Na⁺/Ca²⁺ exchange is a prominent component of this process, particularly in cardiac myocytes and brain neurons (3). Two families of Na⁺-Ca²⁺ exchange proteins have been described in mammalian tissues as follows: the cardiac-type Na⁺/Ca²⁺ exchanger (NCX), which has a generally accepted stoichiometry of 3 Na⁺:1 Ca²⁺ (Refs. 3 and 4, but see Ref. 5 for a recent reassessment of NCX stoichiometry); and a K⁺-dependent Na⁺/Ca²⁺ exchanger (NCKX), identified originally in retinal rod photoreceptors, which has a stoichiometry of 4 Na⁺:(1 Ca²⁺ + 1 K⁺) (6–8).

Three different genes (NCX1 or SLC8A1, NCX2 or SLC8A2, and NCX3 or SLC8A3) encoding proteins similar to the cardiac-type exchanger have been described (9). NCX1 is abundantly expressed in the heart, brain, and kidney and is also found in lower amounts in many other tissues (10–12). The expression of NCX2 and NCX3 is restricted mainly to brain and skeletal muscle (12–14). In contrast, the retinal rod exchanger (NCKX1 or SLC24A1) was originally thought to be expressed only in rod photoreceptors, and the unique properties of this molecule were thought to be an adaptation to the special ionic environment of this location (7, 15). Recently, however, both physiological (16, 17) and molecular (18–22) evidence has mounted for expression of NCKX1 and novel paralogs to this molecule, in a variety of tissues and organisms. The second member of the K⁺-dependent family of Na⁺/Ca²⁺ exchangers, NCKX2 (gene SLC24A2), was cloned first from rat brain (18) and subsequently from chick and human cone photoreceptors (22). NCKX2 is expressed broadly in brain neurons and has been shown to catalyze K⁺-dependent Na⁺/Ca²⁺ exchange when expressed in transfected cells (18, 22, 23).

NCX2 and NCX1 are co-expressed in many brain regions, and in some cases are probably present together within the same neurons (18, 24). The relative roles these two molecules play in neuronal Ca²⁺ homeostasis (1), however, is not yet clearly understood and will depend upon a detailed knowledge of their individual transport properties. Functional information for rat brain NCKX2 is quite limited, and many properties have been inferred largely from the similarity of this molecule to the retinal rod exchanger, NCX1. Although NCX1 function has been characterized extensively in situ (6, 8), the properties of the isolated protein molecule are not as well understood. Indeed, expression from cloned NCX1 has resulted in a number of reports of quite different properties, ranging from K⁺-independent exchange currents (25) to functional silencing of the bovine clone (26).

In this paper we describe experiments designed to characterize the transport and electrophysiological properties of rat brain NCKX2. Whole-cell patch clamp analysis was used on

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1 The abbreviations used are: NCX, Na⁺/Ca²⁺ exchanger; TMA, tetramethylammonium; NCKX, K⁺-dependent Na⁺/Ca²⁺ exchanger; TEA, tetraethylammonium; pF, picofarad.
transfected cells to characterize the cation dependences of NCKX2 and to measure its ionic stoichiometry. A preliminary account of these findings has been presented previously in abstract form (27).

**EXPERIMENTAL PROCEDURES**

All chemicals were of analytical grade or better and were obtained from either Fisher, BDH, or Sigma, unless indicated otherwise. **Cell Culture and Transfection**—The rat brain NCKX2 cDNA tagged near the N terminus with the FLAG epitope in the pRC/CMV vector (Invitrogen) was as described previously (18). cDNA encoding the green fluorescent protein was from Life Technologies, Inc. (pGreen lab. 1). Rat heart NCX1.1 cDNA was in the pcDNA3.1 (+) vector (Invitrogen). Human embryonic kidney cells (the tsA201 variant of HEK293 cells expressing a temperature-sensitive mutant of the SV40 large T antigen) were grown in Dulbecco’s modified Eagles’s media supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 1% non-essential amino acids, and penicillin/streptomycin (Life Technologies, Inc.). Co-transfection of Giagn-purified FLAG-NCKX2 and green fluorescent protein cDNAs was performed using a standard calcium-phosphate precipitation protocol with BES buffer essentially as described previously (18). The FLAG-NCKX2 cDNA, cloned in the reverse orientation in the pMT2 vector, was used in control transfections. After co-transfection, the cells were cultured for 24 h, washed once to remove the precipitate, and provided with fresh medium, and the culture was continued for an additional 24 h to allow efficient protein expression from plasmid DNA. Finally, the cells were trypsinized, re-plated into 35-mm dishes, and used for electrophysiological recordings 2 h to 3 days later.

**Electrophysiology**—Electrophysiological measurements were carried out using the conventional whole-cell configuration of the patch clamp recording technique (28). Pipettes were prepared using a pipette puller (Sutter model P-87 Flaming/Brown micropipette puller) from borosilicate glass capillaries (Corning 8161 glass, outer diameter, 1.5 mm; inner diameter, 1.1 mm, from Warner Instrument Co.) and fire-polished to a resistance of 3–4 meqohms (Narishige MF-830 microforge) when filled with pipette solution (see below). A seal resistance of 2–10 gigaohms was formed by applying gentle negative pressure, and then a brief stronger suction, to the inside of the pipette to rupture the patch membrane and enter whole-cell mode.

Voltage clamp was conducted with a patch amplifier (Axopatch 200B; Axon Instruments, Inc.) and voltage clamp with a patch amplifier (Axopatch 200B; Axon Instruments, Inc.). Reverse and forward Na—/Ca2+ exchange currents were recorded under conditions where the exchanger does not function (see below). Glucose-containing, but not chloride-containing, pipette solutions (see below) resulted in a liquid junction potential of −10 mV that was unaffected by the nature of the bath perfusion solution and was corrected for in the applied potentials. Current traces were low-pass-filtered at 100 Hz and sampled at 1 kHz using pClamp software (version 8.0, Axon Instruments, Inc.). Changes in current were normalized with respect to either cell size as judged from cell capacitance (the 50% capacitance of 50 cells examined in this study was 32.5 ± 1.0 pF) or percentage of maximal current. Current-voltage (I-V) relationships were recorded from a holding potential of −20 mV with a ramp voltage protocol (dV/dt = 0.5 V/s) over the range −100 to +80 mV. Na—/Ca2+ + K+ exchange currents were isolated by digital subtraction, from currents elicited under conditions where the exchanger does not function (bath perfusion of LiCl and EGTA) from currents elicited under conditions compatible with exchanger function. All experiments were carried out at room temperature (22–24 °C).

**Solutions**—External solutions contained various combinations of NaCl, KCl, and LiCl (and in some cases, choline Cl) totaling 145 mM, either 0 or 1 mM MgCl2, 10 mM d-glucose, 10 mM HEPES/tetramethylammonium (TMA), and 0.5 mM EGTA, pH 7.4 (free [Ca2+]i < 0.1 nM). External [Ca2+]i was varied from 0 to 30 μM using 10 mM EGTA (osmotically balanced by reducing the LiCl concentration appropriately) and various amounts of CaCl2 calculated using the method described by Fabiato (29) or by unbuffered addition of CaCl2 above this range. For the experiments examining the influence of external K+ on inward currents and for reversal potential experiments, 20 mM tetraethylammonium chloride (TEA-Cl) was included in the external solution (replacing LiCl). TEA-Cl had no influence on the magnitude of Na—/Ca2+ + K+ exchange currents (data not shown). In all cases, the osmolality was measured and maintained at 280 mosm/kg (5100C Vapor Pressure Osmometer; Wescor, Inc.) by altering the precise concentration of LiCl used. Reverse exchange (outward) currents were recorded using a pipette solution containing 120 mM NaCl, 5 mM KCl, 2 mM MgCl2, 20 mM TEA-Cl, 1 mM Na2ATP, 8 mM d-glucose, 10 mM HEPES/TMA, and 5 mM EGTA, pH 7.2. Free [Ca2+]i under these conditions is less than 0.5 nM. In some experiments a free [Ca2+]i of 1 μM was generated by adding 4.28 mM CaCl2 to the above solution and readjusting the pH. Forward exchange (inward) currents were measured with a pipette solution containing 100 mM potassium gluconate, 20 mM KCl, 20 mM TEA-Cl, 1 mM Na2ATP, 10 mM EGTA, 9.4 mM CaCl2 ([Ca2+]i = 5 μM), 10 mM d-glucose, and 10 mM HEPES/TMA, pH 7.2. For studies on the influence of external K+ on inward currents using near-physiological conditions, the above pipette solution was supplemented with 3 mM NaCl and 1 mM MgCl2. Reversal potential experiments employed a pipette solution containing 18 mM NaCl, 100 mM potassium gluconate, 20 mM TEA-Cl, 1 mM Na2ATP, 10 mM EGTA, 6.40 mM CaCl2 ([Ca2+]i = 0.5 μM), 10 mM d-glucose, and 10 mM HEPES/TMA, pH 7.2.

**Data Analysis**—Electrophysiological data were analyzed using pClamp software (Clampfit, version 8.0, Axon Instruments, Inc.). Currents were measured at the peak, and all data are presented as means ± S.E. Statistical comparisons were made using an unpaired Student’s t test. The ionic dependence of currents was fit using non-linear regression to various models with either Prism (GraphPad Software Inc, San Diego, CA) or MacCurveFit (Kevan Raner Software, Victoria, Australia). Equation 1 shows a cooperative activation model, where I is current (usually normalized); I max is the maximum current; K0 is the concentration of ion causing half-maximal activation of current; I0 is the concentration of the ion being varied, and nH is the Hill coefficient. In some cases, a simple activation model was used, in which I0 in Equation 1 was set to unity. Equation 2 gives a cooperative inhibition model, where the terms have the same meaning as in the activation model, except that K0H is the concentration giving half-maximal inhibition, and nH is the plateau current at infinite ion concentration.

The thermodynamic equilibrium of NCKX2, when the net work per transport cycle is 0, leads to a reversal potential (E_{Na,Cl-Ca}) equal to the weighted sum of the Nernstian membrane potentials for the transported ions according to the Equation 3 (Ref. 3) as follows, where E_{Na,Cl-Ca} and E_{k} are the Nernstian equilibrium potentials, and n_{Na}, n_{Cl}, and n_{k} are the number of ions that bind and are moved per unitary transport cycle for Na+, Ca2+, and K+, respectively.

Substituting for the individual Nernst potentials in Equation 3 gives Equation 4,

\[ E_{Na,Cl-Ca} = \frac{RT}{n_{Na}} \log(1 + \frac{I_{max}/I}{1 + [K_{0H}]^{n_{H}}}) \]

where B is an experimental constant, determined by the stoichiometry and the relevant set of fixed ionic concentrations. The denominator term in this equation (n_{Na} - 2n_{Cl} - n_{k}) is the net number of charges moved per transport cycle. Thus, a plot of E_{Na,Cl-Ca} against log(1/I) will have a slope that is a measure of n_{k}, the number of ions X, that move per unitary charge in each transport cycle.

**RESULTS**

Na+/Ca2+ + K+ Exchange Currents Generated by the Rat Brain NCKX2—Whole-cell patch clamp was used to record currents attributable to rat brain NCKX2 expressed in HEK293 cells. Large outward currents were generated when cells internally dialyzed with high Na+ concentrations were held at 0 mV and exposed to bath solutions containing both 1
mm Ca\(^{2+}\) and 40 mm K\(^+\) together but not with either alone (Fig. 1). The NCKX2 currents, which could be elicited repetitively, reached a peak magnitude of 51 ± 6 pA (n = 10) in about 1 s and then decayed over a few tens of seconds. NCKX2-induced currents were not significantly affected by external application of 20 mM TEA-Cl, 1 \(\mu\)M verapamil, or 1 mM BaCl\(_2\) (data not shown). When HEK293 cells were transfected with rat heart NCX1.1, Ca\(^{2+}\)-dependent outward currents recorded under the same conditions were independent of K\(^+\) (Fig. 1). The NCX1.1 currents usually had a larger peak magnitude than those of NCKX2 but also reached peak very quickly and decayed slowly with time. When either untransfected or control-transfected (NCKX2 cloned in the reverse orientation) HEK293 cells were analyzed under identical conditions, they did not produce any currents usually had a larger peak magnitude than those of NCKX2 but also reached peak very quickly and decayed slowly with time. When either untransfected or control-transfected (NCKX2 cloned in the reverse orientation) HEK293 cells were analyzed under identical conditions, they did not produce any obvious steady-state currents (Fig. 1), although sometimes a 1–2 pA solution change artifact was observed.

The voltage dependence of outward currents was examined as illustrated in Fig. 2. NCKX2 and control transfected cells held at 0 mV and perfused as described above were subjected to a voltage ramp protocol ranging from -80 to +80 mV. Under these conditions, which only permit outward Na\(^+\)/Ca\(^{2+}\) exchange currents, NCKX2-transfected cells displayed currents with an ohmic dependence on voltage, their magnitude increasing toward positive potentials. We did not observe any significant voltage-dependent currents in control or untransfected cells, as illustrated by the very shallow steady-state current-voltage (I-V) relationship with a reversal potential of 0 mV. Thus, the outward current observed in NCKX2-transfected cells appears to correspond to the electrogenic movement of Na\(^+\) through the molecule in exchange for Ca\(^{2+}\) and K\(^+\). Furthermore, under the conditions used to examine NCKX2 currents, control HEK293 cells had no significant potentially interfering membrane ion conductances.

To evaluate whether intracellular Ca\(^{2+}\) played a role in the regulation of Na\(^+\)/Ca\(^{2+}\) + K\(^+\) exchange currents generated by the rat brain NCKX2, as observed for NCX1 using giant excised patches (30), two different concentrations of free Ca\(^{2+}\) were included in the pipette solutions for outward current recordings. Fig. 3 shows the [Ca\(^{2+}\)]\(_i\) dependence of both rat heart NCX1.1 and rat brain NCKX2 outward currents elicited by a switch from Li\(^+\) to Ca/K. See “Experimental Procedures” and the legend to Fig. 1 for details of solution composition. A, representative current traces from a control- and an NCKX2-transfected cell are shown. B, I-V plots for control- and NCKX2-transfected cells, obtained by digital subtraction of the indicated voltage ramps labeled in A, illustrate the voltage dependence of Li-Li (c-a) and Ca/K-Li (b-a) currents. C, summary I-V plot from six experiments. Data are plotted as averaged normalized current ± S.E. for control- (○) or NCKX2-transfected (●) cells.
transfected cells regardless of the presence or absence of Ca\(^{2+}\) in the pipette. Nevertheless, the presence of Ca\(^{2+}\) in the pipette resulted in a small, but significant, increase in the magnitude of the observed NCKX2 currents. This pipette Ca\(^{2+}\)-dependent difference persisted using a variety of different extracellular K\(^{-}\) and Ca\(^{2+}\) concentrations (data not shown).

Cation Dependence of Outward Na\(^{+}\)/Ca\(^{2+}\) + K\(^{+}\) Exchange Currents—The kinetic cation dependence of NCKX2 at the extracellular face of the molecule was examined by subjecting NCKX2-transfected cells to a series of perfusion switches between solutions in which either K\(^{+}\) or Ca\(^{2+}\) was varied and recording outward currents carried by Na\(^{+}\) movement (reverse transport mode) using pipette solutions containing 1 \(\mu\)M [Ca\(^{2+}\)]\(_{free}\). Fig. 4 illustrates the external Ca\(^{2+}\) dependence of outward currents generated by NCKX2 in the presence of constant external K\(^{+}\) (40 mM) and either the presence or the absence of 1 mM MgCl\(_2\). Under these conditions, Ca\(^{2+}\)-activated outward currents in a dose-dependent manner that fitted very well with a model for a single Ca\(^{2+}\) activation site with an apparent dissociation constant of 1.4 or 101 \(\mu\)M [Ca\(^{2+}\)]\(_{free}\), in the absence or presence of Mg\(^{2+}\), respectively. Mg\(^{2+}\), alone (together with K\(^{+}\)) was unable to induce outward currents, indicating that it could not substitute for Ca\(^{2+}\) at the transport site. Furthermore, Mg\(^{2+}\) was without any significant effect on the maximum peak current (1.4 ± 0.2 pA/pF (n = 10) versus 1.2 ± 0.2 pA/pF (n = 9); p > 0.25) measured in 0 mM Mg\(^{2+}\) and 0.1 mM Ca\(^{2+}\) or in 1 mM Mg\(^{2+}\) and 5 mM Ca\(^{2+}\), respectively. This influence on apparent affinity and not on maximum current is consistent with a competitive interaction between Ca\(^{2+}\) and Mg\(^{2+}\), as reported previously for the bovine rod Na\(^{+}\)/Ca\(^{2+}\) + K\(^{+}\) exchanger (31).

Similar experiments were carried out to examine the external K\(^{+}\) dependence of NCKX2 function, recording outward currents under constant and saturating external Ca\(^{2+}\) (1 mM), using LiCl or choline Cl to maintain osmolarity, either with or without 1 mM MgCl\(_2\). As illustrated in Fig. 5, raising external [K\(^{+}\)] in the presence of LiCl increased the outward currents in a dose-dependent fashion. However, it appeared that the K\(^{+}\) effect did not reach saturation, even when external [K\(^{+}\)] was raised to 120 mM. The K\(^{+}\) dependence was not significantly altered by the presence or absence of 1 mM Mg\(^{2+}\) in the solution (data not shown). To evaluate the possibility that external Li\(^{+}\) might affect the K\(^{+}\) dependence of Na\(^{+}\)/Ca\(^{2+}\) + K\(^{+}\) exchange currents as suggested for rod NCKX1 (31), the concentration-response curve for K\(^{+}\) was repeated with LiCl replaced by choline Cl. As shown in Fig. 5, this substitution shifted the concentration-response curve for K\(^{+}\) to the left, reducing the apparent K\(_{app}\) about 3-fold. These data are consistent with a model where K\(^{+}\) interacts at a single site on the external face of NCKX2 with an apparent dissociation constant of 12 mM and that Li\(^{+}\) can compete for binding to this site, although only K\(^{+}\) is competent for transport.

We also investigated the ability of externally applied Na\(^{+}\) to inhibit outward NCKX2 currents recorded under constant external [Ca\(^{2+}\)] (1 mM) and [K\(^{+}\)] (40 mM). As illustrated in Fig. 6, raising external [Na\(^{+}\)] resulted in a concentration-dependent decrease in the outward current, with a K\(_{app}\) of about 60 mM that may reflect competition of Na\(^{+}\) at the Ca\(^{2+}\)- and/or K\(^{+}\)-binding sites, as anticipated from earlier studies on the rod NCKX (31, 32).

Cation Dependence of Inward Na\(^{+}\)/Ca\(^{2+}\) + K\(^{+}\) Exchange Currents—The kinetic external ionic dependence of inward NCKX2 currents (forward transport mode) was also investigated. Fig. 7 illustrates the external Na\(^{+}\) dependence of the inward currents generated by NCKX2. The data were fit to a...
cooperative activation model that resulted in a calculated $K_{12}$ for Na$^+$ of 30 mM and a Hill coefficient ($n_H$) of 2.8. Such Na$^+$-dependent inward currents were never observed with untransfected HEK293 cells or in recordings from NCKX2-transfected cells in which the patch pipette solution contained 5 mM EGTA. Thus, the currents we observed under these conditions must arise from the electrogenic inward movement of Na$^+$ through the forward mode of NCKX2 transport.

Local concentrations of extracellular K$^+$ can rise significantly above the resting level of about 3–5 mM, close to actively firing neurons (33). As extracellular K$^+$ has the potential to influence Ca$^{2+}$ homeostasis through a kinetic effect on NCKX2, we decided to examine the K$^+$ dependence of forward NCKX2 exchange (inward currents) using two different conditions. The first was chosen, based on the data of Fig. 7 above, to optimize stable inward currents. The second was chosen to mimic, as closely as practically possible, physiological levels of ions and membrane potential. As shown in Fig. 8, increasing [K$^+$] reduced inward currents by a maximum of 40%. The maximal effect of K$^+$ was already seen at 1 mM under close-to-physiological conditions and did not change upon further addition. Under conditions optimized for inward currents, the K$^+$ response was shifted to the right, but maximal effects were still seen by 10 mM. These data indicate that, in the physiological range of [K$^+$] likely to be experienced close to the surface of neurons (3–10 mM or higher), there is only a modest kinetic effect of K$^+$ on NCKX2 function.

**NCKX2 Stoichiometry Determined from Reversal Potentials**—The overall operation of the rat brain NCKX2 exchanger depends not only on the kinetic properties examined above but also on thermodynamic properties. As we have demonstrated, NCKX2 function displayed absolute requirements for Na$^+$, in exchange for Ca$^{2+}$ and K$^+$. Thus, the reversal potential of Na$^+$/Ca$^{2+}$ K$^+$ exchange ($E_{Na-Ca-K}$, the thermodynamic equilibrium point, at which no net transport takes place) is dependent only on the transport stoichiometry of the molecule, as described by Equations 3–5 under “Experimental Procedures.” To determine the stoichiometry, $n_{eq}$, for an individual ion, X, the strategy is to measure the dependence of $E_{Na-Ca-K}$ on the bath concentration of X ($[X]_o$). Under these conditions a plot of $E_{Na-Ca-K}$ against log of $[X]_o$ will have a slope that provides a measure of the number of ions X that move per unitary charge in each transport cycle.

To measure the reversal potential of the [Na$^+$]$_o$-induced current, NCKX2-transfected cells were subjected to a series of perfusion switches from a bath solution containing LiCl and EGTA to one with fixed [Ca$^{2+}$] and [K$^+$] but varying [Na$^+$]. Voltage ramps were imposed before, during, and after each Na$^+$ superfusion condition. The currents obtained in LiCl and EGTA solutions were then subtracted from those elicited during subsequent perfusions, and the I-V relation was plotted, as illustrated in Fig. 9. From these data it is evident that different [Na$^+$] yielded I-V traces with different reversal potentials as anticipated and that the difference current in LiCl and EGTA solution before and after was essentially 0. As a further control,
were subjected to whole-cell patch clamp analysis using a holding potential of 0 mV and a bath perfusion solution containing 60 mM NaCl, 70 mM LiCl, 20 mM TEA-Cl, 1 mM MgCl$_2$, 10 mM D-glucose, 10 mM HEPES/TMA, pH 7.4. Bath perfusion solutions contained 1 mM MgCl$_2$, 10 mM D-glucose, and 10 mM HEPES/TMA, pH 7.2. Bath perfusion solutions contained 1 mM MgCl$_2$, 10 mM D-glucose, and 10 mM HEPES/TMA, pH 7.2. Bath perfusion solutions contained 1 mM MgCl$_2$, 10 mM D-glucose, and 10 mM HEPES/TMA, pH 7.2. Bath perfusion solutions contained 1 mM MgCl$_2$, 10 mM D-glucose, and 10 mM HEPES/TMA, pH 7.2.

Several members of the Na$^+$/Ca$^{2+}$ exchanger superfamily, including NCX1, NCX2, NCKX2, and NCKX3 (18, 24, 34, 35), have been reported to be highly expressed in a broad range of brain regions and cell types. Unraveling the unique physiological role for each exchanger sub-type will require, among other things, a comprehensive understanding of their functional properties. Rat brain NCKX2 was identified and cloned only recently and was found to be structurally similar to the well characterized NCX exchanger of retinal rod photoreceptors (18). Functional data on NCKX2, however, have been restricted largely to the demonstration of K$^+$-dependent Na$^+$/Ca$^{2+}$ exchange activity (18, 22, 23). In this study, we have examined kinetic and thermodynamic properties of ion transport for the rat brain K$^+$-dependent Na$^+$/Ca$^{2+}$ exchanger, NCKX2, providing the first detailed electrophysiological characterization for rat brain NCKX2, and the first stoichiometry determination for any cloned member of the K$^+$-dependent Na$^+$/Ca$^{2+}$ exchanger family.

To examine the properties of rat brain NCKX2, we have expressed the cDNA clone in HEK293 cells and measured membrane current generated by the exchange of Na$^+$ for Ca$^{2+}$ and K$^+$ using whole-cell patch clamp. Although there are no specific inhibitors of K$^+$-dependent Na$^+$/Ca$^{2+}$ exchangers, several features of the currents we have observed allow us to

Fig. 7. Na$^+$ dependence of inward NCKX2 currents. HEK293 cells transfected with vector alone (Control) or with rat brain NCKX2 were subjected to whole-cell patch clamp analysis using a holding potential of 0 mV and a pipette solution containing 100 mM potassium gluconate, 20 mM KCl, 20 mM TEA-Cl, 1 mM Na$_2$ATP, 10 mM EGTA, 9.68 mM CaCl$_2$ (Ca$^{2+}$)$_{pipet}$ = 5 µM), 10 mM D-glucose, and 10 mM HEPES/TMA, pH 7.2. Bath perfusion solutions contained 1 mM MgCl$_2$, 10 mM D-glucose, and 10 mM HEPES/TMA, pH 7.4, and either 145 mM NaCl and 1 mM MgCl$_2$. See “Experimental Procedures” and the legend to Fig. 7 for further details of solution composition. In either case, KCl additions replaced LiCl to maintain osmolarity. The average normalized current ± S.E. for 15 experiments under optimal conditions (●) or 12 experiments under near-physiological conditions (○) are shown. The curves represent the best fits of the data to a cooperative inhibition model (see Equation 2 under “Experimental Procedures”) with the indicated $K_{\text{Na}}$ and $n_{\text{H}}$ values; the plateau current was about 60% of the total. Note that the $K_{\text{Na}}$ and $n_{\text{H}}$ parameters for near-physiological conditions could not be calculated precisely from the fit of this data set.

Fig. 8. K$^+$ inhibition of inward NCKX2 currents. HEK293 cells transfected with rat brain NCKX2 were analyzed by whole-cell patch clamp as described in Fig. 7, and the influence of increasing [K$^+$] on inward currents was tested under two conditions as follows: ●, optimal inward currents were recorded using a holding potential of 0 mV and a bath perfusion solution containing 60 mM NaCl, 70 mM LiCl, 20 mM TEA-Cl, 1 mM MgCl$_2$, 10 mM D-glucose, 10 mM HEPES/TMA, 0.5 mM EGTA, pH 7.4; ○, near-physiological conditions using a holding potential of ~50 mV, a bath solution containing 120 mM NaCl, 20 mM LiCl, 20 mM TEA-Cl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM D-glucose, and 10 mM HEPES/TMA, pH 7.4, and a pipette solution supplemented with 3 mM NaCl and 1 mM MgCl$_2$. See “Experimental Procedures” and the legend to Fig. 7 for further details of solution composition. In either case, KCl additions replaced LiCl to maintain osmolarity. The average normalized current ± S.E. for 15 experiments under optimal conditions (●) or 12 experiments under near-physiological conditions (○) are shown. The curves represent the best fits of the data to a cooperative inhibition model (see Equation 2 under “Experimental Procedures”) with the indicated $K_{\text{Li}}$ and $n_{\text{H}}$ values; the plateau current was about 60% of the total.
conclude they are derived exclusively from the operation of the NCKX2 protein. First, the relatively small size of the clamped cells (average capacitance 33 pF) allowed excellent control of intracellular ionic concentrations, whereas a high level of protein expression allowed precise measurements of currents with peak amplitudes as high as 51 pA. Second, no significant current could be elicited from control-transfected or untransfected cells under conditions appropriate for Na\(^+/\)Ca\(^{2+}\) or Na\(^+/\)Ca\(^{2+}\) + K\(^+\) exchange (either with, or without the presence of K\(^+\)). In fact, in the absence of cDNA encoding the exchanger, HEK293 cells demonstrated very low noise and only a very small membrane conductance (Figs. 2 and 9). Third, NCKX2-transfected cells displayed an obvious outward current that was dependent on the simultaneous presence of internal Na\(^+\), and both external Ca\(^{2+}\) and K\(^+\), and was not observed with either external Ca\(^{2+}\) or K\(^+\) alone. Fourth, NCKX2-transfected cells exhibited an inward current elicited by external Na\(^+\) perfusion only when both internal Ca\(^{2+}\) and K\(^+\) were present and not when internal Ca\(^{2+}\) was chelated with excess EGTA. Fifth, the observed currents were not significantly affected by the external application of verapamil, barium, or TEA.

The first set of experiments examined the external ionic dependence of outward currents, presumably carried by Na\(^+\) movement in what would be the “reverse” direction to the normal physiological operation of NCKX2 to extrude Ca\(^{2+}\) from the cell. Just as we had demonstrated previously by fura-2 imaging (18), Ca\(^{2+}\) entry through NCKX2 is strictly dependent upon external K\(^+\), in contrast to the operation of the heart-type NCX1.1 Na\(^+/\)Ca\(^{2+}\) exchanger (Fig. 1). Outward NCKX2 currents displayed a significant ohmic voltage dependence, becoming larger at more positive potentials, with a slight upward curvature visible (Fig. 2). These data are very similar to the voltage dependence of NCX1.1 currents measured under similar conditions, with the ion-binding sites close to saturation (5, 36). This observation suggests that, as for NCX1.1, there is only a small net effect of voltage on one or more of the ion binding reactions or on the conformational transitions associated with ion transport. A key distinction between NCX1.1 and NCKX2 outward currents was in their regulation by cytoplasmic Ca\(^{2+}\) (Fig. 3). While NCX1.1 was essentially inactive in the absence of cytoplasmic Ca\(^{2+}\), NCKX2 activity was reduced only modestly.

In this study we have characterized the kinetic properties of interaction at the external face of rat brain NCKX2 for all the transported ions. These data provide the first detailed and comprehensive characterization of the properties of the neuronal potassium-dependent Na\(^+/\)Ca\(^{2+}\) exchanger molecule, NCKX2. Furthermore, this study represents the first systematic examination of a variety of kinetic properties for any cloned member of the NCKX family, here examined using whole-cell patch clamp in a mammalian expression system. Table I presents a summary of the kinetic data obtained in this study compared with those for the retinal NCKX and mammalian NCX, which are derived from many different studies, using a variety of preparations and various methods. It is evident that in some cases there is a high degree of concordance between NCKX2 properties and those of the retinal NCX. For example Ca\(^{2+}\) activation of reverse exchange and Na\(^+\) activation of “forward” exchange in the absence of any competing ions are virtually identical. On the other hand, we report other properties for which no comparable data are available for NCKX proteins, for example the apparent Ca\(^{2+}\) affinity in the presence of close to physiological competing Mg\(^{2+}\), and the inhibitory influence of external Na\(^+\) on Ca\(^{2+}\) entry. Interestingly, where they can be compared, the values we obtain for NCKX2 are quite similar to those of mammalian NCX, with the exception of the K\(_{i}\) for activation of Ca\(^{2+}\) extrusion by Na\(^+\).

Examination of the ionic dependence of NCKX2 outward

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**Fig. 9. Reversal potential determinations for NCKX2.** HEK293 cells transfected with either vector alone (Control) or rat brain NCKX2 were analyzed by whole-cell patch clamp using holding potential of 0 mV and a pipette solution containing 18 mM NaCl, 100 mM potassium gluconate, 20 mM TEA-Cl, 1 mM MgCl\(_2\), 10 mM glucose, 10 mM HEPES/TMA, pH 7.2. Bath perfusion solutions contained 1 mM MgCl\(_2\), 10 mM glucose, 20 mM TEA-Cl, 10 mM HEPES/TMA, pH 7.4, and either 125 mM LiCl, 0.5 mM EGTA (buffered with 10 mM EGTA in the range of 0.3–30 μM or unbuffered above this range). Each ion was varied individually with the others held constant at 50 mM Na\(^+\), 10 mM K\(^+\), or 0.5 mM Ca\(^{2+}\). Voltage ramps from −100 to +80 mV were applied during each perfusion, and in Li at the start and the end of the experiment. A, representative current traces for Control- or NCKX2-transfected cells subjected to perfusion switches of varying [Na\(^+\)]. B, I-V curves obtained from the indicated ramps during the perfusions illustrated in A, with the I-V curve obtained in Li at the start of the experiment (marked a) digitally subtracted in some cases, as shown. Arrows in B indicate the reversal potentials recorded for NCKX2 under the three different [Na\(^+\)] perfusions.
expected to compete for the K\(^+\)-binding site, further lowering the effective apparent K\(^+\) affinity. Thus, it seems likely that under normal physiological conditions, even if K\(^+\) were to rise close to the extracellular surface of a neuron, K\(^+\) occupancy of its external binding site would not limit NCKX2 function. The experimentally determined apparent K\(^+\) affinity for NCKX2 is dramatically different from the value of about 1 m\(M\) reported for retinal rod NCKX (8, 38). The retinal experiments, however, were conducted under dramatically different conditions from those used here. More recent determinations of the apparent K\(^+\) affinity for recombinantly expressed NCKX molecules under conditions more closely matching those used in our study also revealed a non-saturating K\(^+\) dependence with an apparent affinity of about 10–20 m\(M\) or higher (21, 22).

Raising extracellular [Na\(^+\)] inhibited outward currents, in a manner consistent with expectations from experiments on retinal rod NCKX (31). As we have not performed a systematic comparison of the interaction between Na\(^+\) and either K\(^+\) or Ca\(^{2+}\), it is not possible to be certain that the inhibition is purely competitive. Whatever kinetic model is used to fit the Na\(^+\) inhibition data, it is clear that as [Na\(^+\)] approaches physiological levels, operation of NCKX2 in the reverse direction would be significantly reduced (Fig. 6), especially considering that internal [Na\(^+\)] and [K\(^+\)] were chosen to favor reverse exchange in this experiment.

The Na\(^+\) dependence of rat brain NCKX2 operating in the forward mode was also determined using inward currents. The data fit well to a cooperative model of activation by Na\(^+\) with a \(K_{1/2}\) of 30 m\(M\). This value matches very closely that determined for the retinal rod exchanger, although the cooperativity for Na\(^+\) interacting with NCKX2 is apparently greater (Hill coefficient of 2.8) than observed for retinal rod NCKX (Hill coefficient of 1.8–2 (23, 32, 39, 40)). Consequently, one would expect that the externally oriented Na\(^+\)-binding sites of NCKX2 would be saturated under physiological conditions, hence driving the Ca\(^{2+}\)-efflux, or forward, direction of the rat brain exchanger. K\(^+\) has been shown to compete for Na\(^+\) binding to the retinal rod exchanger (32, 37), which would be expected to reduce the rate of Ca\(^{2+}\)-efflux. Since [K\(^+\)] is thought to vary over a broad range at the extracellular surface of actively firing neurons (33), one might suggest that this competitive interaction serves an important regulatory influence on NCKX2 function. As illustrated in Fig. 8, we tested the effect of increasing [K\(^+\)] on inward NCKX2 current, and we found that K\(^+\)-inhibited current magnitude by about 40%. As this effect saturated at rather low [K\(^+\)], especially under near-physiological conditions, in fact it seems unlikely that acute changes in [K\(^+\)] would have a regulatory effect on rat brain NCKX2 function. The large effect of K\(^+\) at low concentrations, which remained incomplete even at much higher [K\(^+\)], suggests an influence not related to competition at the ion-binding sites but may instead reflect the significant changes in thermodynamic driving force that occur in this concentration range. As a consequence, it appears that under physiological conditions, NCKX2 is probably operating at only about 60% of its intrinsic maximal rate.

In addition to kinetic considerations of the rate of NCKX2-mediated Ca\(^{2+}\) transport, thermodynamic driving forces that stem from the stoichiometric coupling of ions during transport have a dramatic influence on the direction of transport. To determine the stoichiometry of rat brain NCKX2 we have taken the approach of measuring reversal potentials at different external ion concentrations. This is therefore a thermodynamic approach to measure an inherently thermodynamic problem and, consequently, rests on fewer assumptions than other approaches, which use kinetics methods to address the question. To calculate stoichiometry from the reversal potential meas-

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**Fig. 10. Stoichiometry determinations for NCKX2.** Plots of reversal potential against log of the ion concentration for Na\(^+\) (A), Ca\(^{2+}\) (B), or K\(^+\) (C), determined as illustrated in Fig. 9. Averages ± S.E. for between 5 and 8 determinations at each data point are shown; in some cases the excursion of the error is smaller than the symbol size. Solid lines show the fit of the data to Equation 5 under “Experimental Procedures.” The stoichiometry (\(n_{Na}\), the number of ions \(X\) moved per net charge) extracted from the slope of these fits using a value of 58.5 for the term 2.303 \((RT/F)\) is shown in each panel. For comparison, dashed lines show the theoretical relationship described by Equation 5 under “Experimental Procedures” with the indicated number \((n)\) of ions moved per net charge. See “Experimental Procedures” for further details.
measurements, however, there are two necessary conditions as follows: (i) that the current being measured is a pure NCKX2-exchanger, and (ii) that we have good control over intracellular ion concentrations (or, at the very least, that these do not change during the course of the experiment or from one external condition to another). The question of the origin of the currents was considered near the beginning of “Discussion,” and it seems clear that we were able to record NCKX2 currents not contaminated with other conductances. The second assumption merits further examination. We have conducted several experiments to establish that control over the ionic environment close to the internal face of the membrane is very good.

First, the data presented in this work were obtained using ramp protocols that ascended from −100 to +80 mV. The extremes of voltage during this protocol will drive significant exchange current and hence have the potential to alter the ionic conditions under the membrane with time (41). We also performed the same experiment using instead voltage ramps that descended from +80 to −100 mV. Under these conditions, any changes in ionic composition with time would be opposite to those generated by the ascending ramp. Consequently, poor ionic control would result in different reversal potentials depending upon the ramp direction. In fact, we observed under several different extracellular conditions that the reversal potential was not dependent upon the direction of the ramp. Furthermore, we measured the reversal potential using an initial holding potential of either 0 or −20 mV, and we obtained identical results, further confirming precise control over intracellular ionic conditions.

Second, if there were inadequate intracellular dialysis via the patch pipette, then ionic control would be compromised, and voltage ramps performed at different times following membrane break-through into the whole-cell patch clamp configuration would give different reversal potentials. In experiments where identical ramps were administered, separated by 2 min, we always observed identical reversal potentials.

Third, in many cases, particularly when amplitudes were high, we observed a slow time-dependent decrease in NCKX2 current magnitude. Such an effect might have been due to the build up of ions under the membrane, which would then influence the reversal potential measurements. Therefore, voltage ramps were performed twice, at approximately 2 and 7 s following the perfusion switch to initiate current. Reversal potentials measured from these two time points were not significantly different.

Fourth, we used a “calcium-jump” experiment to test for sub-membrane changes in [Ca2+]i, using two conditions that differed only in the bath [Ca2+]i as follows: (i) when bathed with solution containing 3 mM Ca2+, NCKX2-transfected cells displayed large outward currents associated with Ca2+ movement through the exchanger into the cell; (ii) when the bath solution contained only 30 μM Ca2+, a small inward current was observed, associated with Ca2+ extrusion from the cell. If Ca2+ movement through the exchanger resulted in poor ionic control under the membrane, then a rapid switch between 3 mM Ca2+ and 30 μM Ca2+ should have resulted in a large peak of inward current (due to the larger local concentration of Ca2+ carried in by the exchanger) that decayed with time. This was never observed, indicating that under our experimental conditions we had good control over sub-membrane ion concentrations.

Fifth, if control over ionic concentration was poor in these experiments, we would not have expected the reversal potential measurements to fall close to the theoretical predicted values.
In fact, as illustrated in Fig. 10, our data sets fall extremely close to the theoretical values of 4 Na\(^+\):(1 Ca\(^{2+}\) + 1 K\(^+\)) in all cases.

Thus we conclude that control over ionic concentration was very good in our experiments and that we were able to determine a precise and reliable stoichiometry from our reversal potential measurements for rat brain NCKX2 of 4 Na\(^+\):(1 Ca\(^{2+}\) + 1 K\(^+\)) (Fig. 10). Our stoichiometry determination is identical to the measurements made on retinal rods, which express NCKX1 (6, 8). This result is important for several reasons. It is the first clear, direct evidence that a cloned and heterologously expressed member of the K\(^-\)dependent family of Na\(^+\)/Ca\(^{2+}\) exchangers actually transports K\(^+\) (as opposed simply to being regulated in an allosteric manner, as observed for NCX1 by alkali cations (42)). Thus, all ion-binding sites required for full function of NCKX2, and likely other members of the family, must be contained within a single polypeptide chain. These binding sites, including that for K\(^-\), are likely to be composed of amino acids contained within the regions highly conserved between retinal rod NCKX1 and brain NCKX2 and predicted to lie in the hydrophobic portion of the membrane (18). Such a result is consistent with the now established notion that the phylogenetically conserved and Na\(^+\)/Ca\(^{2+}\) exchanger superfamily defining \(\alpha\)-repeat elements form the ion transport binding pocket (9, 43). On the other hand, it is in contradiction to two reports published recently that suggested that either another subunit was required for K\(^-\)-dependent operation of bovine NCKX1 (25) or that a segment of the proposed intracellular loop of NCKX1 was an essential part of the K\(^-\)/Ca\(^{2+}\) exchanger gating pocket (9, 43).

The determined stoichiometry for rat brain NCKX2 of 4 Na\(^+\):(1 Ca\(^{2+}\) + 1 K\(^+\)) confirms that this exchanger will function to extrude Ca\(^{2+}\) from cells over a far wider range of conditions than would be possible for an exchanger that did not couple the movement of K\(^-\) or that only transported 3 Na\(^+\) in exchange for 1 Ca\(^{2+}\), such as NCX1. Combined with the kinetic properties of the ion-binding sites, it seems likely that under conditions experienced in normal cells NCKX2 operates exclusively in Ca\(^{2+}\) efflux mode. These newly defined properties of rat brain NCKX2 will be critical in considering the role this molecule plays in the integrated control of neuronal Ca\(^{2+}\) homeostasis.

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Electrophysiological Characterization and Ionic Stoichiometry of the Rat Brain K\(^+\) -dependent Na\(^+\)/Ca\(^{2+}\) Exchanger, NCKX2

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