Cation transport by the neuronal $K^+\text{-Cl}^-$ cotransporter KCC2: thermodynamics and kinetics of alternate transport modes

Jeffery R. Williams and John A. Payne

Department of Physiology and Membrane Biology, School of Medicine, University of California, Davis, California 95616

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Williams, Jeffery R., and John A. Payne. Cation transport by the neuronal $K^+\text{-Cl}^-$ cotransporter KCC2: thermodynamics and kinetics of alternate transport modes. Am J Physiol Cell Physiol 287: C919–C931, 2004.—Both Cs$^+$ and NH$_4^+$ alter neuronal Cl$^-$ homeostasis, yet the mechanisms have not been clearly elucidated. We hypothesized that these two cations altered the operation of the neuronal K$^+\text{-Cl}^-$ cotransporter (KCC2). Using exogenously expressed KCC2 protein, we first examined the interaction of cations at the transport site of KCC2 by monitoring furosemide-sensitive $^{86}$Rb$^+$ influx as a function of external Rb$^+$ concentration at different fixed external cation concentrations (Na$^+$, Li$^+$, K$^+$, Cs$^+$, and NH$_4^+$). Neither Na$^+$ nor Li$^+$ affected furosemide-sensitive $^{86}$Rb$^+$ influx, indicating their inability to interact at the cation translocation site of KCC2. As expected for an enzyme that accepts Rb$^+$ and K$^+$ as alternate substrates, K$^+$ was a competitive inhibitor of Rb$^+$ transport by KCC2. Like K$^+$, both Cs$^+$ and NH$_4^+$ behaved as competitive inhibitors of Rb$^+$ transport by KCC2, indicating their potential as transport substrates. Using ion chromatography to measure unidirectional Rb$^+$ and Cs$^+$ influxes, we determined that although KCC2 was capable of transporting Cs$^+$, it did so with a lower apparent affinity and maximal velocity compared with Rb$^+$. To assess NH$_4^+$ transport by KCC2, we monitored intracellular pH (pHi) with a pH-sensitive fluorescent dye after an NH$_4^+$-induced alkaline load. Cells expressing KCC2 protein recovered pHi, much more rapidly than untransfected cells, indicating that KCC2 can mediate net NH$_4^+$ uptake. Consistent with KCC2-mediated NH$_4^+$ transport, pH recovery in KCC2-expressing cells could be inhibited by furosemide (200 μM) or removal of external [Cl$^-$]. Thermodynamic and kinetic considerations of KCC2 operating in alternate transport modes can explain altered neuronal Cl$^-$ homeostasis in the presence of Cs$^+$ and NH$_4^+$.

Fast hyperpolarizing inhibition mediated by ligand-gated anion channels (i.e., GABA$_A$ and glycine receptors) depends on an inwardly directed Cl$^-$ electrochemical gradient. Such a Cl$^-$ gradient can only be generated and maintained by active Cl$^-$ extrusion. There is now abundant evidence that the neuronal K$^+\text{-Cl}^-$ cotransporter (KCC2) functions as a significant Cl$^-$ extrusion mechanism and plays an important role in overall Cl$^-$ homeostasis of mature neurons (5, 19, 37, 39). Under normal conditions, KCC2 uses energy stored in the K$^+$ chemical gradient to drive Cl$^-$ out of the neuron. As a carrier protein, however, KCC2 is bidirectional and can mediate net ion efflux or influx, depending on the sum of the chemical potential differences across the plasma membrane for the transported ions. Because KCC2 is normally poised close to thermodynamic equilibrium, it can mediate net K$^+$ and Cl$^-$ uptake whenever there are subtle elevations of external [K$^+$] such as occurs with high neuronal activity (12, 21, 23).

The cation selectivity of K$^+\text{-Cl}^-$ cotransporter (KCC) systems represents an important issue from both physiological and experimental standpoints. Most studies examining the transport properties of the KCCs as well as other members of the cation-chloride cotransporter (CCC) family have used radio-isotopic Rb$^+$ ($^{86}$Rb$^+$) as a tracer for K$^+$. Such widespread use of this technique is not only due to the ready availability of $^{86}$Rb$^+$ and its convenient properties (i.e., high specific activity and convenient half-life) but also because it is generally accepted that Rb$^+$ is transported in a similar manner as K$^+$ by the CCCs. However, because Rb$^+$ is not the physiologically relevant cation, it is important to confirm that Rb$^+$ and K$^+$ are transported to the same degree and with similar kinetic properties. Such confirmation is particularly important for KCC2 because the operation of this transporter as an effective K$^+$ uptake system is dependent on it exhibiting an appropriately high transport affinity for external K$^+$. Although we and others have shown that KCC2 does indeed exhibit a high transport affinity for external Rb$^+$ ($K_m \approx 5$–9 mM; Refs. 35, 44), no study has yet confirmed that Rb$^+$ and K$^+$ are transported with similar kinetic parameters by this KCC isoform.

The transport of Cs$^+$ by KCC2 is important experimentally as it is often used to replace intracellular K$^+$ in patch-clamp studies because it can reduce K$^+$ channel activity in electrophysiological measurements. Unfortunately, Cs$^+$ has been shown to alter neuronal Cl$^-$ homeostasis. For example, Thompson and Gahwiler (46) demonstrated that the reversal potential for GABA ($E_{\text{GABA}}$) was more positive in hippocampal neurons recorded with Cs$^+$-filled microelectrodes than with K$^+$-filled microelectrodes, indicating that there was a net accumulation of intracellular Cl$^-$ when Cs$^+$ replaced intracellular K$^+$. The accumulation of intracellular Cl$^-$ when Cs$^+$ replaces K$^+$ in the intracellular compartment has recently been confirmed by studies using the gramicidin-perforated patch technique, a technique that maintains native intracellular [Cl$^-$] ([Cl$^-$]). With such Cs$^+$ replacement in the patch pipette, van Brederode et al. (47) reported a significantly more depolarized $E_{\text{GABA}}$ in the somata and dendrites of rat neocortical neurons. Moreover, using a gramicidin-perforated patch technique with rat dissociated lateral superior olive (LSO) neurons, which express robust KCC activity, Kakazu et al. (23) showed that replacement of intracellular K$^+$ with Cs$^+$, Li$^+$, or Na$^+$ caused [Cl$^-$] to increase. Kakazu et al. reasoned that if these latter cations were not substrates of KCC, then their replacement of K$^+$ in the intracellular compartment would force KCC to...
mediate net ion influx due to altered thermodynamics, resulting in net Cl\(^{-}\) uptake. Although both of these previous gramicidin-perforated patch-clamp studies could monitor net Cl\(^{-}\) movements, they could not provide detailed kinetic information about how the substitute cations interacted with the neuronal KCC, and therefore they could not define how Cs\(^{+}\) and the other substitute cations elicited their effects on the transporter.

Elevated serum ammonium can result either from inborn errors of the urea cycle enzymes or from liver failure. In extreme cases of acute liver failure, brain [NH\(_{4}\)]\(^{+}\) has been observed as high as 5 mM (45). Such hyperammonemic states are associated with significant effects on brain function, including altered synaptic transmission (for recent review, see Ref. 16). One well-established experimental effect of NH\(_{4}\)\(^{+}\) on neuronal transmission is depression of the hyperpolarizing inhibitory postsynaptic potential (IPSP; e.g., Refs. 1, 27–29, 33, 38). This effect of NH\(_{4}\)\(^{+}\) has been attributed to an apparent inhibition of active Cl\(^{-}\) extrusion, leading to elevated neuronal [Cl\(^{-}\)] and reduced driving force for the IPSP (28). This implies that there is either a direct or indirect effect of NH\(_{4}\)\(^{+}\) on KCC2.

Aickin et al. (1) investigated the indirect effect of intracellular pH (pHi) alkalinization induced by NH\(_{4}\)\(^{+}\) on Cl\(^{-}\) extrusion in crayfish stretch receptor neurons. By offsetting NH\(_{4}\)\(^{+}\)-induced intracellular alkalinization with coapplication of acetate, they demonstrated that the effect of NH\(_{4}\)\(^{+}\) on Cl\(^{-}\) extrusion was independent of neuronal pHi. Aickin et al. concluded that NH\(_{4}\)\(^{+}\) must have a direct effect on the neuronal Cl\(^{-}\) extrusion mechanism, i.e., KCC2. Recent studies have demonstrated that NH\(_{4}\)\(^{+}\) can substitute for K\(^{+}\) on the K\(^{-}\)-Cl\(^{-}\) cotransporters (7, 26); thus NH\(_{4}\)\(^{+}\) could directly affect KCC2 operation via transport kinetics and/or thermodynamics. Although Bergeron et al. (7) did not directly examine NH\(_{4}\)\(^{+}\) transport by KCC2, they did show that KCC1, KCC3, and KCC4 transported NH\(_{4}\)\(^{+}\) with nearly identical kinetics as Rb\(^{+}\). Liu et al. (26) used whole cell recordings to follow net changes in [Cl\(^{-}\)] of cultured neurons; thus they could not address the issue of altered transport kinetics. On thermodynamic grounds, however, Liu et al. concluded that the effect of NH\(_{4}\)\(^{+}\) on neuronal function was not due to Cl\(^{-}\) accumulation but rather to NH\(_{4}\)\(^{+}\) accumulation via KCC2, which places a continuous acid load on neurons. To date, the mechanism by which NH\(_{4}\)\(^{+}\) affects KCC2 operation and elicits its effect on neuronal function still has not been clearly elucidated.

In the present study, we tested the hypothesis that the effects of Cs\(^{+}\) and NH\(_{4}\)\(^{+}\) on neuronal Cl\(^{-}\) homeostasis could be explained by their action as cation substrates on KCC2, which in turn alters the thermodynamics and/or kinetics of KCC2 operation. In addition, we tested the hypothesis that KCC2 transports Rb\(^{+}\) and K\(^{+}\) with similar kinetic parameters. For our study, we used KCC2 protein exogenously expressed in a mutant Madin-Darby canine kidney (MDCK) cell line, LK-C1, that we have previously shown is a useful expression system for CCCs (36). To examine interaction at the cation translocation site of KCC2, we first monitored KCC2-mediated 86Rb\(^{+}\) influx as a function of external [Rb\(^{+}\)] at different fixed cation (i.e., Na\(^{+}\), Li\(^{+}\), K\(^{+}\), NH\(_{4}\)\(^{+}\), and Cs\(^{+}\)) concentrations. At concentrations as high as 100 mM, neither Na\(^{+}\) nor Li\(^{+}\) affected KCC2-mediated 86Rb\(^{+}\) influx, indicating that they do not interact at the cation translocation site of KCC2 and thus are not transport substrates. In contrast, K\(^{+}\), Cs\(^{+}\), and NH\(_{4}\)\(^{+}\) all inhibited KCC2-mediated 86Rb\(^{+}\) influx in a competitive manner consistent with the notion that each of these ions can interact with the cation translocation site of KCC2. First, by comparing nonradioisotopic Rb\(^{+}\) and K\(^{+}\) unidirectional influxes mediated by KCC2, we confirmed that Rb\(^{+}\) and K\(^{+}\) were transported with similar, but not identical, kinetic parameters. Second, by measuring nonradioisotopic unidirectional Cs\(^{+}\) influx, we showed that KCC2 was capable of operating in a Cs\(^{+}\)-Cl\(^{-}\)-cotransport mode. Last, by monitoring intracellular pH after an NH\(_{4}\)\(^{+}\)-induced alkaline load, we confirmed that KCC2 could operate as an NH\(_{4}\)\(^{+}\)-Cl\(^{-}\)-cotransporter. We conclude that thermodynamic and kinetic considerations of KCC2 operating in these alternative transport modes help explain the effects of Cs\(^{+}\) and NH\(_{4}\)\(^{+}\) on neuronal intracellular [Cl\(^{-}\)]-, and hence, on postsynaptic inhibition.

**METHODS**

Tissue culture and stable cell line production. Low K\(^{-}\)-resistant mutant MDCK (MDCK LK-C1) cells were maintained in growth medium containing DMEM, 10% fetal bovine serum, penicillin (50 U/ml), and streptomycin (50 µg/ml). The MDCK LK-C1 cell line, originally developed by McRoberts et al. (31), lacks endogenous Na\(^{+}\)-K\(^{-}\)-Cl\(^{-}\} cotransport activity and represents a useful expression system for the CCC proteins (36). Cells were maintained in a humidified incubator with 5% CO\(_{2}\) at 37°C.

Rat KCC2 (rtKCC2; Ref. 37) was stably expressed in MDCK LK-C1 cells using calcium phosphate precipitation and a previously described full-length rtKCC2 expression construct (35). After 3 wk of growth in 900 µg/ml Geneticin (GIBCO), single resistant colonies were amplified and screened by Western blot analysis (48) and by furosemide-sensitive 86Rb\(^{+}\) influx (see Radioisotopic 86Rb\(^{+}\) influx assay). The MDCK LK-C1 cells stably expressing rtKCC2 were maintained in growth medium containing 900 µg/ml Geneticin.

Equilibrium dialysis with nystatin. Intracellular ion composition of MDCK LK-C1 cells was altered using the nystatin technique (14). Cells were washed twice in ice-cold nystatin loading medium (solution a; see Table 1 for composition of all solutions). Nystatin prepared in DMSO (20 mg/ml stock) was added at a final concentration of 20 µg/ml to cells in ice-cold nystatin loading medium. Cells were incubated for 40 min on ice with gentle rocking. To remove nystatin after attainment of equilibrium dialysis and to restore normal low ion permeability, cells were warmed to 37°C and washed 10 times in nystatin loading medium containing 0.25% bovine serum albumin (fraction V) but no nystatin. After washing, cells were then used immediately for unidirectional cation influx assays. Nystatin-treated cells were used only in experiments presented in Fig. 5.

Radioisotopic 86Rb\(^{+}\) influx assay. Isotopic flux experiments were conducted at 24°C on preconfluent MDCK LK-C1 cells. Cells grown on 96-well plates were washed free of growth medium and incubated 10 min in preincubation medium-Rb (solution b) containing 0.1 mM ouabain, 1 mM N-ethylmaleimide (NEM), and ±2 mM furosemide. Cells were quickly washed twice and brought up in either of two flux media (solution d or e) containing 0.1 mM ouabain, 1 mM NEM, and ±2 mM furosemide. A trace amount of 86Rb\(^{+}\) was added to the flux media, and uptake was terminated at measured times by addition of Tris-buffered saline (TBS) plus 2 mM furosemide. Each well was washed seven times with TBS plus 2 mM furosemide to remove extracellular radioisotope. After washing, the cells were solubilized in 2% SDS and assayed for 86Rb\(^{+}\) by Cerenkov radiation and for protein by using the MicroBCA method (Pierce).

Nonradioisotopic cation influx assay. Nonradioisotopic cation influxes were performed on preconfluent stable MDCK LK-C1 KCC2 cells grown in 12-well plates. Cells were preincubated for 10 min in preincubation medium-K (solution c). After a brief wash, cells were incubated in a third flux medium (solution f) containing 0.1 mM ouabain, 1 mM NEM, and ±2 mM furosemide. Preliminary experi-
and 0.5 mM), and the change in pH i was measured for each change in range of pHi observed in our study (6.5 of MDCK LK-C1 cells expressing KCC2 was determined over the high-K/H11005 and cells were alkalinized with 10 mM NH4-MSA replacing an high-K/H11001 performed at the end of each experiment by using 10

10-min in

fl

40). Briefly, cells were perfused in a stepwise fashion with media containing decreasing concentrations of external NH4Cl (10, 4, 2, 1, and 0.5 mM), and the change in pHi was measured for each change in [NH4Cl]. To prevent transport of acid equivalents, we conducted experiments in nominally Na+- and HCO3−-free medium. Intracellular [NH4+]i was determined from the intracellular [NH3], pK_a, and pH by assuming that intracellular [NH3] was equivalent to external [NH3].

Intrinsic buffer capacity was calculated as the change in intracellular [NH3] divided by the change in pHi. (Δ[NH3]i/ΔpHi) was found to vary with pHi in a linear fashion. The buffer capacity (± SD) of 13 cells from two different cell preparations was 8.4 ± 2.0 mM/pHi unit at pH 7.0. The best-fit line to the data from each cell gave an average slope of −9.7 ± 3.5 mM/pHi unit2. Statistically, this buffer capacity was not significantly different from that found for the parent cell line, MDCK-LK-C1, which exhibited an average value of 9.9 ± 1.1 mM/pHi unit at pH 7.0 and an average slope of −12.3 ± 2.2 mM/pHi unit2 as determined from nine cells.

Rb+ and K+ competition with NH4+ uptake. MDCK-LK-C1 cells expressing KCC2 were initially perfused with imaging medium. Competition was then examined by switching the perfusing medium to one with varying [Rb+]i or [K+]i (0, 5, 10, 15 mM) in the presence of fixed 10 mM NH4+ (competition medium; see solution h in Table 1). pHi was monitored at 5-s intervals after addition of competition medium. The initial rate of acidification (ΔpHi/Δt) associated with NH4+ uptake was calculated from the linear portion of the pHi recovery, generally within the first 50 s after addition of competition medium. The rate of NH4+ uptake was calculated as the product of the rate of change in pHi (ΔpHi/Δt) and the intracellular buffer capacity. After 200 s, cells were switched back to imaging medium and the pHi was allowed to return to baseline. The return to baseline pHi was monitored at 15- to 20-s intervals and was variable in duration. A similar procedure was performed for each of the different [Rb+]i and [K+]i competition media in sequential fashion as shown in Fig. 8A. At the conclusion of each experiment, the cells were again perfused with 0 mM competing cation (Rb+ or K+) in the presence of 250 μM furosemide. The NH4+ uptake in this latter measurement was taken as the furosemide-insensitive NH4+ flux and was subtracted from each of the previous measurements to obtain the rate of furosemide-sensitive or KCC2-mediated NH4+ uptake. The KCC2-mediated NH4+ uptake rates in each of the different competing cation concentrations were normalized to the maximal rate in the absence of competing cation and presented as percent uninhibited initial velocity in Fig. 8B.

Protein analysis. Membranes were prepared from cultured cells by differential centrifugation as previously described (35, 36). Protein concentrations were determined using the MicroBCA protein kit (Pierce). Membrane proteins were resolved by SDS-PAGE using a 7.5% Tricine gel system. Gels were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon P; Millipore) in transfer buffer (192 mM glycine, 25 mM Tris-Cl, pH 8.3, and 15% methanol) for >3 h at 50 V by using a Bio-Rad Trans-Blot tank apparatus. The PVDF membrane with bound protein was visualized by staining with Coomassie brilliant blue R-250. The PVDF membrane was then blocked in PBS-milk (7% nonfat dry milk and 0.1%
Tween 20 in PBS, pH 7.4) for 1 h and then incubated in PBS-milk with affinity-purified KCC2 polyclonal antibodies (48) for 2 h at 24°C. After three 10-min washes in PBS-milk, the PVDF membrane was incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG; Amersham) for 2 h at 24°C in PBS-milk. After three washes in PBS-0.1% Tween 20, bound antibodies were detected using an enhanced chemiluminescence assay.

Data analysis. To determine Michaelis constants ($K_m$) and maximal velocities ($V_{max}$) of radioisotopic and nonradioisotopic cation fluxes, we used a nonlinear iterative procedure (DeltaGraph 4.5) to fit data points to a hyperbolic Michaelis-Menten equation. For cations acting as competitive inhibitors, the inhibitory constant ($K_i$) was determined by plotting the slope of the reciprocal plot as a function of inhibitor concentration and taking $K_i$ from the intercept on the inhibitor concentration axis (42). Data were analyzed statistically by use of a $t$-test in which experimental values were compared with control measurements. Error bars represent the standard error of the mean, and statistical significance was defined when $P < 0.05$.

RESULTS

Heterologous expression of KCC2 in a mutant MDCK cell line. We studied the operation of KCC2 using exogenously expressed protein in a mutant MDCK cell line, LK-C1 (31). This particular mutant MDCK cell line exhibits little measurable furosemide-sensitive $^{86}$Rb$^+$ influx and was originally described as a functional “knockout” for the Na$^+$/K$^+$/Cl$^-$ cotransporter (31). We recently characterized the MDCK LK-C1 cells as a useful expression system for the cation chloride cotransporters because these cells permit an unambiguous measurement of the activity of the stably transfected CCC protein (36). Figure 1A displays a Western blot using KCC2 antibodies (48) to detect the protein in membranes prepared from untransfected MDCK LK-C1 cells and MDCK LK-C1 cells expressing KCC2. It is clear that the stably transfected cells are producing abundant KCC2 protein, which is absent from untransfected control cells. The KCC2 protein was functional at the plasma membrane as we detected robust furosemide-sensitive $^{86}$Rb$^+$ influx in the transfected cells after treatment with NEM (Fig. 1B), a known activator of KCC2 (35). In contrast, the untransfected control cells exhibited no measurable furosemide-sensitive $^{86}$Rb$^+$ influx (Fig. 1B).

KCC2-mediated $^{86}$Rb$^+$ uptake was linear for 10 min in the KCC2 stable transfecants, and we routinely used 3- to 5-min uptakes to determine the initial $^{86}$Rb$^+$ influx rate in subsequent experiments.

Cation interactions at the external transport site of KCC2. To examine the interaction of various cations at the external transport site of KCC2, we measured the rate of furosemide-sensitive $^{86}$Rb$^+$ influx as a function of external [Rb$^+$] in the presence of different fixed concentrations of cations (0–100 mM Li$^+$, Na$^+$, K$^+$, and Cs$^+$; 0–10 mM NH$_4^+$). The data were fit to a Michaelis-Menten kinetic model (Fig. 2) to obtain kinetic constants ($K_m$ and $V_{max}$; summary data shown in Table 2). Because these data were not paired between the different cations, $V_{max}$ values cannot be directly compared between the different cations. Significant variation was observed in furosemide-sensitive $^{86}$Rb$^+$ influx rates because exogenous KCC2 protein expression levels tended to decrease as the stable cell line was propagated. Such a kinetic approach, however, permitted us to identify clearly those cations that were competitive inhibitors of furosemide-sensitive $^{86}$Rb$^+$ influx and, thus, likely KCC2 transport substrates. In general, competitive inhibitors increase the $K_m$ for the substrate without altering $V_{max}$. As shown in Fig. 2, neither Na$^+$ nor Li$^+$ significantly affected the furosemide-sensitive $^{86}$Rb$^+$ influx mediated by KCC2 (neither $K_m$ nor $V_{max}$ was altered; Table 2), indicating that they do not interact at the cation transport site of KCC2 and are not transport substrates. In contrast, K$^+$, which is the physiological transport substrate of KCC2, should exhibit competitive inhibition of the furosemide-sensitive $^{86}$Rb$^+$ influx in the MDCK LK-C1 cells expressing KCC2. This was indeed confirmed because at any given [K$^+$] the same $V_{max}$ could be attained if [Rb$^+$] was increased to high enough concentrations, a general characteristic of competitive inhibition (Fig. 2 and Table 2). Furthermore, the $K_m$ for external Rb$^+$ increased significantly from 4.27 ± 1.55 mM in the absence of [K$^+$] to 47.8 ± 15.7 mM with 100 mM K$^+$. Thus, as expected for competitive inhibition, transport affinity for external Rb$^+$ decreased as external [K$^+$] was elevated. We then examined furosemide-sensitive $^{86}$Rb$^+$ influx in the presence of varying external [Cs$^+$] or [NH$_4^+$]. Similar to what we observed with K$^+$, both Cs$^+$ and NH$_4^+$ exhibited characteristics of competitive inhibition of furosemide-sensitive $^{86}$Rb$^+$ influx, indicating that they also interact at the cation transport site of KCC2 (Fig. 2 and Table 2).

These kinetic experiments permitted us to make a firm distinction between those cations that interact with the cation transport site of KCC2 (K$^+$, Cs$^+$, and NH$_4^+$) and those cations that do not (Li$^+$ and Na$^+$). However, such kinetic experiments, showing interaction with a transport site, do not prove that the...
competing cation is translocated across the membrane. That is, a competing cation could transiently occupy the cation binding site of KCC2 but not undergo translocation. In the following experiments, we examined in greater detail the translocation of Cs\(^{+}\)/H\(^{+}\), K\(^{+}\)/H\(^{+}\), and NH\(_{4}\)/H\(^{+}\) by KCC2.

Is Cs\(^{+}\) a transport substrate of KCC2? We investigated the possibility that Cs\(^{+}\)/H\(^{+}\) was a transport substrate of KCC2 by monitoring unidirectional Cs\(^{+}\)/H\(^{+}\) influx of MDCK LK-C1 cells. Because of the difficulty in acquiring radioisotopic Cs\(^{+}\)/H\(^{+}\) (\(^{137}\)Cs\(^{+}\)/H\(^{+}\)), we used ion chromatography to detect chemical Cs\(^{+}\)/H\(^{+}\) in influx. Gillen and colleagues (8, 11) recently showed the feasibility of using ion chromatography to monitor unidirectional cation influx in animal cells and tissues. In preliminary experiments, we determined that the appearance of Rb\(^{+}\)/H\(^{+}\) or Cs\(^{+}\)/H\(^{+}\) was easily detected in MDCK LK-C1 cells stably expressing KCC2 after 5–10 min and that in influx was linear for 15 min for Rb\(^{+}\)/H\(^{+}\) and 45 min for Cs\(^{+}\)/H\(^{+}\) (data not shown). We routinely used 10-min influxes to determine initial rates for Rb\(^{+}\)/H\(^{+}\) and Cs\(^{+}\)/H\(^{+}\) influx. As shown in Fig. 3 for paired experiments, both Rb\(^{+}\)/H\(^{+}\) and Cs\(^{+}\)/H\(^{+}\) influxes exhibited a significant ouabain-sensitive component in control and KCC2-transfected cells, indicating the translocation of both cations by the Na\(^{+}\)/H\(^{+}\)-K\(^{+}\)/H\(^{+}\)-ATPase. With NEM treatment, there was a significantly elevated furosemide-sensitive component in control and KCC2-transfected cells, indicating the translocation of both cations by the Na\(^{+}\)/H\(^{+}\)-K\(^{+}\)/H\(^{+}\)-ATPase.

Table 2. Summary of kinetic constants for furosemide-sensitive \(^{86}\)Rb\(^{+}\) influx in the presence of various cations

<table>
<thead>
<tr>
<th>Cation</th>
<th>(K_m), mM</th>
<th>(V_{max}), nmol/mg protein/(\text{min})</th>
<th>(n)</th>
<th>(K_i), mM</th>
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<tr>
<td>Na(^{+})</td>
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<td>0 mM</td>
<td>4.25±0.69</td>
<td>5.2±0.69</td>
<td>3</td>
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<tr>
<td>50 mM</td>
<td>4.82±0.94</td>
<td>6.4±1.03</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>100 mM</td>
<td>4.83±0.99</td>
<td>6.9±1.82</td>
<td>3</td>
<td></td>
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<tr>
<td>Li(^{+})</td>
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<tr>
<td>0 mM</td>
<td>3.33±0.31</td>
<td>12.1±4.4</td>
<td>3</td>
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<tr>
<td>50 mM</td>
<td>3.46±0.45</td>
<td>15.1±6.0</td>
<td>3</td>
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</tr>
<tr>
<td>100 mM</td>
<td>3.48±0.16</td>
<td>15.3±6.7</td>
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<tr>
<td>K(^{+})</td>
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<td></td>
<td></td>
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<tr>
<td>0 mM</td>
<td>4.27±1.55</td>
<td>17.5±7.0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>50 mM</td>
<td>21.4±3.4</td>
<td>21.4±7.7</td>
<td>3</td>
<td></td>
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<tr>
<td>100 mM</td>
<td>47.8±15.7</td>
<td>25.9±15.2</td>
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<td></td>
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<tr>
<td>Cs(^{+})</td>
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<td></td>
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<tr>
<td>0 mM</td>
<td>4.14±0.29</td>
<td>19.6±7.4</td>
<td>3</td>
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<tr>
<td>50 mM</td>
<td>33.7±4.7</td>
<td>17.2±6.3</td>
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<tr>
<td>100 mM</td>
<td>86.5±15.3</td>
<td>26.1±10.3</td>
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<tr>
<td>NH(_{4})(^{+})</td>
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<tr>
<td>0 mM</td>
<td>5.96±0.37</td>
<td>36.8±1.3</td>
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<tr>
<td>5 mM</td>
<td>7.18±0.68</td>
<td>30.6±1.5</td>
<td>4</td>
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</tr>
<tr>
<td>10 mM</td>
<td>8.93±1.35</td>
<td>29.7±1.1</td>
<td>4</td>
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Values are means ± SE. \(K_m\), Michaelis constant; \(V_{max}\), maximal velocity; \(K_i\), inhibitory constant; NA, not applicable.

Fig. 2. Effect of various cations on furosemide-sensitive \(^{86}\)Rb\(^{+}\) influx of MDCK LK-C1 cells expressing KCC2 plotted as a function of \([\text{Rb}^{+}]\). Unpaired representative experiments are shown for each cation. Values are means ± SE of 5 replicates. Summary data from at least 3 experiments are presented in Table 2. Furosemide-sensitive \(^{86}\)Rb\(^{+}\) influxes were measured in the presence of 0 (\(F\)), 50 (\(E\)), or 100 mM (\(R\)) external [Na\(^{+}\)] and [Li\(^{+}\)] (A), 0 (\(F\)), 50 (\(E\)), or 100 mM (\(R\)) external [K\(^{+}\)] and [Cs\(^{+}\)] (B), and 0 (\(F\)), 5 (\(E\)), or 10 mM (\(R\)) external [NH\(_{4}\)\(^{+}\)] (C).
sensitive component of the Rb$^+$ and Cs$^+$ influxes in the KCC2-expressing cells compared with control cells. These data clearly demonstrated that Cs$^+$, like Rb$^+$, is translocated by KCC2. It is evident from the data presented in Fig. 3 that the Cs$^+$ influxes were much reduced from those exhibited by Rb$^+$. To understand the transport of Cs$^+$ by KCC2 in greater detail, we examined in paired experiments the kinetics of both KCC2-mediated Cs$^+$ and Rb$^+$ influx in the stably transfected MDCK LK-C1 cells. In these paired experiments, we monitored furosemide-sensitive Cs$^+$ and Rb$^+$ influxes as a function of external [Cs$^+$] and [Rb$^+$], respectively (Fig. 4 and Table 3). As we noted above in Fig. 3, the furosemide-sensitive Cs$^+$ influx was significantly less than that of Rb$^+$, because the $V_{\text{max}}$ of Cs$^+$ influx by KCC2 was $\sim 20\%$ of that for Rb$^+$. Notably, the $K_m$ of KCC2 for external Rb$^+$ that we measured with chemical Rb$^+$ influx (6.2 $\pm$ 1.3 mM) was similar to that previously determined with radioisotopic $^{86}\text{Rb}^+$ (5.2 $\pm$ 0.9 mM) in stable HEK-293 cells (35). This finding confirms the validity and accuracy of the chemical method. The $K_m$ of KCC2 for external Cs$^+$ (14.0 $\pm$ 1.1 mM) was significantly higher than for external Rb$^+$ (Table 3). These data clearly indicate that although Cs$^+$ is transported by KCC2, it represents a poor substrate as exemplified by its low $V_{\text{max}}$-$K_m$ ratio.

**Kinetics of K$^+$ transport by KCC2.** As with our analysis of Cs$^+$ transport by KCC2, we examined the kinetics of KCC2-mediated K$^+$ transport using ion chromatography to detect K$^+$ uptake in KCC2 expressing cells. To accurately determine unidirectional K$^+$ influx using this technique, however, it was necessary to replace intracellular K$^+$ with an alternate cation by using an equilibrium dialysis method that employs the polyene antibiotic nystatin to induce high membrane conductance for monovalent ions (10). In preliminary experiments, we determined that normal cell volume could be maintained in the presence of nystatin by using 40 mM sucrose in the loading solution (data not shown). As shown in Fig. 5A, we could replace intracellular K$^+$ as the major intracellular cation with Rb$^+$ using this technique. In these experiments, we prepared both high-Rb$^+$ and high-K$^+$ cells to compare K$^+$ and Rb$^+$ uptake in nystatin-treated cells. It should be pointed out that nystatin-treated cells were also loaded with elevated [Cl$^-$] given that we used 130 mM KCl or RbCl in the loading solutions. After equilibrium dialysis with the loading solutions, we removed nystatin from the plasma membrane by repeated washing of cells in loading solution lacking nystatin but containing 0.25% bovine serum albumin. Figure 5B shows the time course of $^{86}\text{Rb}^+$ influx in untreated cells and in cells that underwent equilibrium dialysis with subsequent removal of

| Table 3. Summary of kinetics constants for furosemide-sensitive cation influx in intact and nystatin-treated cells |
|-----------------|--------------------|-----------------|-----|
|                  | $K_m$, mM          | $V_{\text{max}}$, nmol/mg protein/min$^{-1}$ | $n$ |
| Intact cells     |                    |                              |     |
| Rb$^+$           | 6.19$\pm$1.28      | 52.8$\pm$4.7               | 4   |
| Cs$^+$           | 14.0$\pm$1.1       | 11.7$\pm$3.2               | 4   |
| Nystatin-treated |                    |                              |     |
| Rb$^+$           | 7.94$\pm$2.03      | 71.5$\pm$5.6               | 5   |
| K$^+$            | 9.84$\pm$0.83      | 87.1$\pm$1.3               | 5   |

Values are means $\pm$ SE.

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Fig. 3. Paired Rb$^+$ and Cs$^+$ influxes of untransfected MDCK LK-C1 cells (A and C) and of MDCK LK-C1 cells stably expressing KCC2 (B and D). After 10 min of treatment with (NEM) or without (control) 1 mM N-ethylmaleimide, Rb$^+$ and Cs$^+$ influxes were measured by ion chromatography (see methods). Total (open bars), ouabain-sensitive (shaded bars), and furosemide-sensitive (solid bars) Rb$^+$ and Cs$^+$ influxes are shown. Values are means $\pm$ SE of $>3$ separate experiments. **Significantly different from total influx of MDCK LK-C1 cells; ***significantly different from total influx of control; #significantly different from furosemide-sensitive influx of MDCK LK-C1 cells; ##significantly different from furosemide-sensitive influx of control ($P < 0.05$ paired t-test).

Fig. 4. Kinetics of furosemide-sensitive Rb$^+$ (●) or Cs$^+$ (○) influx of MDCK LK-C1 cells expressing KCC2 after treatment with 1 mM NEM. Values are means $\pm$ SE from 4 paired experiments. Curves represent best fits of data to a model of activation at single sites. The Michaelis constant ($K_m$) and maximal velocity ($V_{\text{max}}$) for summary data are presented in Table 3 (intact cells).
nystatin from the plasma membrane. The fact that the furosemide-insensitive component of $^{86}\text{Rb}^+$ influx is similar to that of untreated cells confirms removal of nystatin from the plasma membrane and restoration of normal low ion permeability. Interestingly, we noted that nystatin-treated cells consistently exhibited much greater rates of furosemide-sensitive $^{86}\text{Rb}^+$ influx than did untreated control cells (Fig. 5A).

Using ion chromatography, we determined the kinetics of furosemide-sensitive $\text{Rb}^+$ and $K^+$ influx in paired experiments on nystatin-treated cells. As shown in Table 3, the transport affinity $K_m$ exhibited by KCC2 for external $\text{Rb}^+$ was not significantly different between intact cells and nystatin-treated cells, indicating that nystatin treatment does not alter this kinetic parameter. In nystatin-treated cells, in which paired KCC2-mediated $K^+$ and $\text{Rb}^+$ influxes were measured, we found that KCC2 exhibits similar transport affinities for external $K^+$ and external $\text{Rb}^+$ (Fig. 5C and Table 3). In contrast, the $V_{\text{max}}$ of furosemide-sensitive $K^+$ influx was \( \sim 20\% \) greater than that of furosemide-sensitive $\text{Rb}^+$ influx.

Is ammonium a transport substrate of KCC2? We tested the hypothesis that $\text{NH}_4^+$ was a transport substrate of KCC2 by monitoring $\text{pH}_i$ acidification associated with net $\text{NH}_4^+$ uptake after an acute $\text{NH}_4^+$-induced alkaline load. The MDCK LK-C1 cells expressing KCC2 were loaded with the fluorescent $\text{pH}$-sensitive dye BCECF, and changes in $\text{pH}_i$ of single cells were monitored by fluorescence microscopy. As shown in Fig. 6A, applying $30\,\text{mM} \text{NH}_4^+$ to the medium of KCC2 expressing cells caused a rapid $\text{pH}_i$ increase (from $a$ to $b$, due to entry of the weak base $\text{NH}_3$) followed by a slower $\text{pH}_i$ recovery (from $b$ to $c$, due to entry of acidic $\text{NH}_4^+$ via transport mechanisms). The initial slope from $b$ to $c$ can be used to determine the rate of $\text{NH}_4^+$ uptake ($\text{d}\text{pH}/\text{dt}$). We observed varying levels of $\text{NH}_4^+$ transport in the KCC2 stable cells, and the initial rates of $\text{pH}_i$ recovery from the population were well fit to a single Gaussian distribution (Fig. 6B). If KCC2 transports $\text{NH}_4^+$, we hypothesized that the varying levels of $\text{NH}_4^+$ transport were due to differences in KCC2 protein expression among the population of cells. To correlate the level of $\text{NH}_4^+$ transport with KCC2 protein expression in the same set of cells, we first measured the initial rates of $\text{pH}_i$ recovery and then performed immunocytochemistry on the cells in situ within the perfusion chamber using our KCC2 antibodies (48). After photobleaching the BCECF fluorescence, we applied fixation and immu-
nontaining solutions directly through the perfusion system while maintaining the original visual field. In Fig. 6C, we compare the BCECF fluorescence and the KCC2 immunofluorescence of the same two cells used to obtain the pHr tracings presented in Fig. 6A. We quantified KCC2 protein expression as pixel intensity (arbitrary units) within the same region of interest chosen for the pHr measurements. As shown in Fig. 6D for a population of 29 cells, we observed a good correlation between the initial rate of pHr recovery and KCC2 protein expression. Figure 6D also shows the initial rate of pHr recovery of untransfected MDCK LK-C1 cells and of the two cells imaged in Fig. 6, A and C. Those cells from the KCC2 stable population that showed little KCC2 protein expression also exhibited low initial rates of pHr recovery similar to those observed for the untransfected cells. These data clearly demonstrate that KCC2 can mediate NH4+ transport. Some of the variability we observed in the correlation between KCC2 function and protein expression in Fig. 6D is no doubt due to the fact that we are not quantifying surface KCC2 protein expression but rather total cellular protein expression. Nonetheless, the correlation is remarkably strong. Because there are cells from the KCC2 stable population that exhibited low pHr recovery rates similar to those of untransfected cells, we used these “low expressers” as internal controls in subsequent experiments.

Confirmation that KCC2 was indeed mediating much of the NH4+ uptake in the KCC2-expressing cells was obtained by showing that NH4+ uptake after an acute alkaline load was significantly inhibited by furosemide (250 μM), by bumetanide (200 μM; data not shown), and by removal of external [Cl−] (Fig. 7). Significantly, the inhibition of KCC2-mediated NH4+ uptake by furosemide was fully reversible, consistent with the action of furosemide as a high Kd diuretic that rapidly dissociates from CCCs in less than a minute (Fig. 7A).

We examined the degree of inhibition exhibited by Rb+ and K+ at a fixed [NH4+] of 10 mM. As expected for competitive inhibition, the rate of NH4+ influx decreased as the concentration of competing cation increased (Fig. 8). In the case of K+, the pHr recovery rate reached half maximal at ~15 mM, whereas for Rb+, the half-maximal rate was attained at ~9 mM. The data for K+ inhibition of KCC2-mediated NH4+ transport are remarkably similar to our earlier findings in Table 2, from which we calculated the Ki for the effect of external K+ on Rb+ transport to be 12.7 mM. Thus it appears that K+ has a weaker inhibitory effect on KCC2 transport than either Rb+ or NH4+, implying that Rb+ and NH4+ are more similar in their interaction with KCC2 than K+. The similarity between Rb+ and NH4+ as cation substrates for KCC2 is further supported by the fact that both of these cations are nearly equivalent in their ability to inhibit each others transport by KCC2 (Table 2 and Figs. 2C and 8B).

**DISCUSSION**

In this study, we examined cation transport by KCC2 using an heterologous expression system in which transport kinetics could be accurately determined. We found that Na+ and Li+ did not affect KCC2-mediated 86Rb+ influx, indicating that neither of these ions interacts with the cation transport site of KCC2. In contrast, K+, Cs+, and NH4+ all behaved as competitive inhibitors of KCC2-mediated 86Rb+ influx. We confirmed with transport measurements that K+, Cs+, and NH4+ are indeed translocated by KCC2, and we determined their transport kinetics. KCC2 transports Rb+, K+, and NH4+ with roughly similar kinetic parameters, but Cs+ is transported with significantly increased Km and reduced Vmax by KCC2.

K+ transport by KCC2. In our initial kinetic studies of KCC2 expressed in HEK-293 cells using 86Rb+ isotopic in-

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Fig. 6. Correlation of net NH4+ uptake and KCC2 protein expression in MDCK LK-C1 cells expressing KCC2. A: 2 representative traces showing rapid alkalinization (from a to b) and subsequent intracellular pH (pHi) recovery (from b to c) after addition of 30 mM NH4+. B: histogram of the initial rates of pHi recovery (~dpH/dt) after NH4+-induced alkaline load from 57 cells from 5 experiments. Data were fit to a single Gaussian distribution. C: 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) fluorescence and KCC2 immunocytochemistry of the same visual field. Cells labeled 1 and 2 were used to obtain the pHr tracings shown in A. D: scatterplot of a population of 29 cells from 3 experiments in which both ~dpH/dt and KCC2 protein expression (mean pixel intensity from immunocytochemistry) were measured within the same region of interest. The mean pHi recovery observed for untransfected MDCK LK-C1 cells is also shown (□; bars = SE). Cells 1 and 2 shown in A and C are represented in D as ▼ or ▲, respectively.
fluxes, we determined the apparent affinity $K_m$ for external $Rb^+$ to be $5.2 \pm 0.9$ mM (35). More recently, Song et al. (44), using $^{86}$Rb$^+$ influx measurements of KCC2 expressed in the Xenopus oocyte, reported the $K_m$ of KCC2 for external Rb$^+$ to be $9.3 \pm 1.8$ mM. Both of these previously published values using $^{86}$Rb$^+$ are similar to what we obtained in the present study using chemical Rb$^+$ influxes for both intact ($6.19 \pm 1.28$ mM) and nystatin-treated cells ($7.94 \pm 2.03$ mM). It is commonly assumed that Rb$^+$ and K$^+$ are transported by the K$^+-\text{Cl}^-$ cotransporters as equivalent substrates, but only rarely have the kinetic parameters of KCC-mediated Rb$^+$ and K$^+$ transport been measured together in the same study. One recent report examined the use of Rb$^+$ as a tracer for K$^+$ transport by the K$^+-\text{Cl}^-$ cotransporter of rabbit red blood cells and determined that the $V_{\text{max}}$ values of NEM-stimulated effluxes were $\sim 30\%$ greater when measured with $^{86}$Rb$^+$ than with K$^+$ (22). This finding is in general agreement with that reported for K$^+-\text{Cl}^-$ cotransport in other mammalian red blood cells (15, 25). Our kinetic analysis using nystatin-treated cells to measure KCC2-mediated K$^+$ and Rb$^+$ influx in paired experiments revealed only minor kinetic differences between these two cations. In contrast to what was observed for KCC in red blood cells, we observed a $\sim 20\%$ greater $V_{\text{max}}$ for K$^+$ transport than for Rb$^+$ transport by KCC2 (Table 2 and Fig. 5). The reason for this difference is unknown but may be related to functional differences between KCC isoforms because the KCC present in mammalian red blood cells is likely KCC1 and/or KCC3. Although the $K_m$ for external K$^+$ ($9.84 \pm 0.83$ mM; $n = 5$) was slightly greater than that observed for external Rb$^+$ ($7.94 \pm 2.03$ mM; $n = 5$), these values were not statistically significantly different. Thus we concur with Jennings and Adame (22) that although the available data indicate that Rb$^+$ and K$^+$ transport by the KCCs are not kinetically identical, the differences for the most part are relatively minor, and Rb$^+$ can be used with confidence as an appropriate indicator of K$^+$ transport on the K$^+-\text{Cl}^-$ cotransporters.

We found that the nystatin-treated cells exhibited a significantly greater rate of furosemide-sensitive $^{86}$Rb$^+$ influx than that observed for untreated control cells (Fig. 5B). This comparison is valid because the two treatment groups were paired. The main difference between these two groups is the fact that the nystatin-treated cells likely had greatly elevated [Cl$^-$]$_i$. We have determined [Cl$^-$]$_o$ in untreated MDCK LK-C1 cells expressing KCC2 to be $32 \pm 1.4$ mM (mean $\pm$ SE, $n = 7$; unpublished results), which is much less than the $\sim 90$ mM [Cl$^-$]$_i$ that we calculate would be attained in the nystatin-treated cells, assuming a Cl$^-$ Donnan ratio of 0.7 with 130 mM [Cl$^-$]$_i$ in the loading solution. There is strong evidence that [Cl$^-$]$_i$ plays a key role in modulating activity of certain members of the CCC gene family. We hypothesize that [Cl$^-$]$_i$ is a key factor in modulating KCC activity, where elevated [Cl$^-$]$_i$ stimulates KCC activity. Such a hypothesis is consistent with our finding that KCC2 activity was much greater in nystatin-treated cells, which likely had elevated intracellular [Cl$^-$]. Furthermore, it is consistent with the operation of KCC2 as an important regulator of neuronal [Cl$^-$]. That is, if KCC2

![Fig. 7. Furosemide inhibition (A) and Cl$^-$ dependence (B) of pH recovery of MDCK LK-C1 cells expressing KCC2 after an NH$_4^+$-induced alkaline load. Representative traces from high (KCC2; shaded) and low expressing (control; solid) cells are shown.](http://ajpcell.physiology.org/)

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functions predominantly as a neuronal “Cl− extruder” to maintain low [Cl−], then activation of KCC2 by elevated [Cl−], provides a simple feedback system to aid in neuronal Cl− homeostasis. Studies on duck red blood cells by Lytle and McManus (30) have shown that [Cl−]i is a critical modulator of KCC activity; as [Cl−]i increases, the common volume set point at which Na+/K+/Cl− cotransporter inactivates and KCC activates is shifted to lower cell volumes.

Cs+ transport by KCC2. Our finding that KCC2 transports Cs+ bears importantly on the interpretation of several previous patch-clamp studies that have examined neuronal Cl− homeostasis under circumstances in which intracellular K+ was replaced by Cs+ (46, 47). This maneuver has become common practice because it inhibits K+ channel activity and reduces background noise. Because KCC2 transports Cs+ much less rapidly than it does K+ (at comparably high concentrations), the replacement of intracellular K+ with Cs+ could substantially alter the steady-state concentration of Cl− in the neuron and thereby affect GABA A or glycine receptor function. Indeed, previous studies have shown that EGABA was more depolarized when intracellular Cs+ replaced K+ (46, 47).

Significantly, Kakazu et al. (23) examined the cation selectivity of KCC in LSO neurons in rats using the gramicidin-perforated patch technique. They found that replacement of intracellular K+ with Cs+, Na+, or Li+ resulted in an increase of [Cl−], above that predicted from a passive Cl− distribution. To explain these results, Kakazu et al. concluded that these cations were not substrates of the cotransporter and that replacement of intracellular K+ with Cs+, Na+, or Li+ created a significant gradient for net Cl− flux via KCC, leading to elevated [Cl−]i above that of a passive distribution. Unfortunately, the measurement of net Cl− fluxes by KCC using the gramicidin-perforated patch technique does not permit one to make firm conclusions about how these cations interact with the transporter. The advantage of the kinetic analyses we performed in the present study is that one can more closely examine the interaction of cations with the cotransporter and make firmer conclusions about the nature of those interactions. In the case of Na+ and Li+, it is clear that neither ion interacts with the cation transport site of KCC2 as shown by the overlapping Michaelis-Menten plots (Fig. 2A). In contrast to the findings of Kakazu et al., however, we found that Cs+ was indeed a
transport substrate of KCC2 but that it was not transported in an equivalent fashion as Rb⁺. We determined that the V_{max} of Cs⁺ transport by KCC2 was ~20% of that for Rb⁺ in paired measurements. This dramatically reduced V_{max} value would make it difficult to distinguish Cs⁺ as a transport substrate from nontransported species such as Na⁺ and Li⁺ when using net Cl⁻ measurements as performed by Kakazu et al. Because Kakazu et al. reported an increase in intracellular [Cl⁻] above that of a passive distribution, another active Cl⁻ transport system must exist in LSO neurons to explain their data. To explain the reduced V_{max} of KCC2 when transporting Cs⁺, we speculate that Cs⁺ may easily interact with the cation site of KCC2 as demonstrated by its low inhibitory constant (Kᵢ = 9.7 mM); however, the Cs⁺ ion may be too large to permit easy passage through the subsequent conformational steps needed for ion translocation (ionic radii: Cs⁺ = 1.69 Å). In contrast, the smaller size of the K⁺ (r = 1.33 Å) and Rb⁺ (r = 1.48 Å) ions is less likely to impede the conformational steps needed for ion translocation. The slower translocation of Cs⁺ by KCC2 has important implications for those experiments in which Cs⁺ is used as a replacement for intracellular K⁺ because the rate of Cl⁻ extrusion by KCC2 will be significantly impaired, leading to intracellular Cl⁻ accumulation.

**NH₄⁺ transport by KCC2.** In addition to the alkali cations, we also examined the effect of NH₄⁺ on KCC2-mediated ⁸⁶Rb⁺ uptake and noted that it acted as a competitive inhibitor, indicating that NH₄⁺ might also be a transport substrate of KCC2. Indeed, recent studies have demonstrated that the K⁺-Cl⁻ cotransporters are capable of transporting NH₄⁺ at the K⁺ site (7, 26), and our data corroborate these findings for KCC2. NH₄⁺ substitutes for K⁺ on both isoforms of the Na-K⁺-Cl⁻ cotransporter (3, 17, 24, 34), thus NH₄⁺ transport is a general feature of the K⁺-transporting CCCs. This has important implications for cellular H⁺ and Cl⁻ transport, especially that occurring in epithelia and in neurons.

The application of NH₄⁺ salts or the excess production of endogenous NH₄⁺ as a result of a metabolic error or liver failure has long been associated with seizure activity (2, 6, 43). Although numerous studies have attempted to explain this proconvulsant effect of NH₄⁺, Lux et al. (29) were the first to report the most plausible explanation. In cat spinal motoneurons, they demonstrated that NH₄⁺ salts dramatically and reversibly reduced synaptic inhibition by diminishing the driving force for the IPSP. Similar observations have since been reported for other neuronal preparations (1, 20, 27, 32, 33, 38). Synthetic inhibition is critical to the normal operation of the brain, and any condition that reduces its effectiveness will promote seizure activity (e.g., epilepsy). Lux (28) suggested that the effect of NH₄⁺ on the IPSP was the direct result of elevated neuronal [Cl⁻] due to the reversible inhibition of an “active Cl⁻ extrusion mechanism.” Indeed, numerous studies have shown that neuronal [Cl⁻] was elevated in the presence of NH₄⁺ salts (4, 13, 41). It is now well accepted that the major Cl⁻ extrusion mechanism of neurons is KCC2 (5, 19, 37, 39), but no study has yet clarified the issue of how NH₄⁺ salts might elicit their effect on this important Cl⁻ transporter. One possibility that has been investigated is inhibition of Cl⁻ extrusion by the intracellular alkalinization caused by NH₄⁺. This issue was addressed in an early study using crayfish neurons (1). Aickin et al. (1) showed that the decline in the IPSP caused by NH₄⁺ was still observed even when the pHₗ alkalization induced by NH₄⁺ was offset by coapplication of acetate. Thus the effect of NH₄⁺ on the IPSP driving force could not be the result of KCC2 inhibition by an NH₄⁺-induced alkalinization. This finding has been confirmed by a recent study showing that KCC2 activity actually increased as pHₗ was elevated from 7 to 8 (7). Aickin et al. concluded that the NH₄⁺-induced decline in IPSP driving force must be interfering in some direct manner with the neuronal Cl⁻ extrusion mechanism.

Recently, Liu et al. (26) investigated the transport of NH₄⁺ by neuronal KCC, likely KCC2. They reported a positive shift in GABA-induced currents on application of NH₄⁺ to cultured neurons, indicating that, as others have shown, NH₄⁺ caused an elevation of [Cl⁻]. Interestingly, however, Liu et al. suggested that the deleterious effects of NH₄⁺ on neuronal function were not the result of alterations in neuronal [Cl⁻], but rather the result of an acid load placed on neurons by KCC2-mediated NH₄⁺ uptake. Unfortunately, this conclusion of Liu et al. is based largely on a misinterpretation of the thermodynamic driving force of a carrier protein transporting two competing cation substrates, NH₄⁺ and K⁺. Furthermore, the idea that the deleterious effects of NH₄⁺ on neuronal function would be due to a maintained acid load seems to ignore the presence of robust H⁺ extrusion mechanisms (i.e., Na⁺/H⁺ exchange) in neurons that are present to provide pHₗ regulation. We agree with Lux et al. (29) that the effects of NH₄⁺ on neuronal function can be explained predominantly by an elevation of neuronal [Cl⁻], and subsequent reduced driving force for IPSPs. Furthermore, we propose that the NH₄⁺-induced increase in neuronal [Cl⁻] can be explained by a proper consideration of the thermodynamics and kinetics of KCC2 operating simultaneously in two transport modes, i.e., a K⁺-Cl⁻ and an NH₄⁺-Cl⁻ cotransport mode.

The present study shows that the kinetic parameters of KCC2 transporting either K⁺ or NH₄⁺ are quite similar. These findings are not too surprising given the fact that NH₄⁺ exhibits physical properties that are quite similar to those of K⁺ (i.e., it has a diffusion coefficient and an ionic radius that are similar to those of K⁺; Ref. 18). In stark contrast to the similarity of the kinetics of K⁺ and NH₄⁺ transport by KCC2, the thermodynamics of KCC2 operating in the K⁺-Cl⁻ and the NH₄⁺-Cl⁻ cotransport modes are quite different (Fig. 9).
cotransport modes are quite different. As with any electroneutral ion transporter, the thermodynamic driving force of KCC2 is determined by the sum of the chemical potential differences of the transported ions. In the case in which two substrate cations compete for the same transport site, each transport mode must be considered as a separate transport event subject to its own thermodynamic driving force. In other words, once a cation and an anion are bound to the transporter, only their chemical potential differences need be considered to derive the thermodynamic force driving that transport event. It is inappropriate to combine the two chemical potential differences for the cations into a single term as performed by Liu et al. (26) (see their Eqs. 2 and 3). Hence, the thermodynamic driving forces for the K\(^{+}\)-Cl\(^{-}\) (\(\Delta\mu_{\text{KCC}}\)) cotransport mode and the NH\(_{4}\)^{+}-Cl\(^{-}\) (\(\Delta\mu_{\text{ACC}}\)) cotransport mode are

\[
\Delta\mu_{\text{KCC}} = RT \ln \left( \frac{[K^{+}]}{[K^{+}]_o} \right) + RT \ln \left( \frac{[Cl^{-}]}{[Cl^{-}]_o} \right) \tag{1}
\]

\[
\Delta\mu_{\text{ACC}} = RT \ln \left( \frac{[NH_{4}^{+}]}{[NH_{4}^{+}]_o} \right) + RT \ln \left( \frac{[Cl^{-}]}{[Cl^{-}]_o} \right) \tag{2}
\]

where the subscripts o and i denote extracellular and intracellular, respectively, \(R\) is the gas constant, and \(T\) is absolute temperature. Because NH\(_{4}\)^{+} in aqueous solution is in equilibrium with NH\(_3\), and NH\(_3\) is permeable across most cell membranes, it follows that at equilibrium, which is attained within milliseconds, log \([NH_{4}^{+}]_i/[NH_{4}^{+}]_o = pH_{i} - pH_{o}\) (derived from the Henderson-Hasselbalch equation). Therefore, Eq. 2 can be rewritten as

\[
\Delta\mu_{\text{ACC}} = 2.303 RT (pH_{i} - pH_{o}) + 2.303 RT \log \left( \frac{[Cl^{-}]}{[Cl^{-}]_o} \right) \tag{3}
\]

From Eq. 3, it becomes immediately apparent that the driving force for the ACC cotransport mode does not strictly depend on the concentration of NH\(_{4}\)^{+} in the system but rather on the difference between the pH\(_i\) and pH\(_o\). Using normal physiological extracellular and intracellular ion concentrations for a mature neuron, one can easily demonstrate that the thermodynamic driving forces of the two cotransport modes exhibit opposite polarity, i.e., the KCC mode is a net Cl\(^{-}\) extruder, whereas the ACC cotransport mode is a net Cl\(^{-}\) accumulator. In Fig. 9, we show how the equilibrium level of Cl\(^{-}\) changes as a function of [K\(^{+}\)]\(_i\) when KCC2 operates solely in the KCC mode or as a function of pH\(_i\) when KCC2 operates solely in the ACC mode (given normal physiological [K\(^{+}\)]\(_i\) = 100 mM, [Cl\(^{-}\)]\(_o\) = 135 mM, and pH\(_o\) = 7.4) using the following equations:

\[
\Delta\mu_{\text{KCC}} = 0: \left[ Cl^{-} \right]_i = \left[ Cl^{-} \right]_o \times \left[ K^{+} \right]_o / \left[ K^{+} \right]_i \tag{4}
\]

\[
\Delta\mu_{\text{ACC}} = 0: \left[ Cl^{-} \right]_i = \left[ Cl^{-} \right]_o / \left( T/RT \right)^{pH_{i} - pH_{o}} \tag{5}
\]

When KCC2 is operating in the KCC mode, Eq. 4 predicts that an equilibrium [Cl\(^{-}\)]\(_i\) of \(~5\) mM will be obtained when [K\(^{+}\)]\(_i\) is 4 mM. In contrast, when KCC2 is operating in the ACC mode with pH\(_i\) of 7.1, Eq. 5 predicts an equilibrium [Cl\(^{-}\)]\(_i\) of nearly \(~68\) mM. If K\(^{+}\) and NH\(_{4}\)^{+} are present together in the system, the [Cl\(^{-}\)]\(_i\) that ultimately will be attained at steady state will fall between these two extremes and will depend on the kinetics of each transport mode of KCC2, i.e., on the concentrations of K\(^{+}\) and NH\(_{4}\)^{+} on both sides of the cell membrane. Our data indicate that KCC2 exhibits remarkably similar kinetic properties for transport of K\(^{+}\) and NH\(_{4}\)^{+} with \(K_m\) values between 5 and 10 mM. Thus we predict that Cl\(^{-}\) loading in neurons would begin to be observed as external [NH\(_4\)^{+}] approached near millimolar concentrations (>0.5 mM). Remarkably, this is within the range (0.5–3 mM) in which Lux and colleagues (28, 29) began to observe effects of NH\(_4\)^{+} on IPSPs in cat spinal neurons. We conclude that NH\(_4\)^{+} does not inhibit Cl\(^{-}\) extrusion by KCC2, as originally proposed by Lux et al. (29), but rather its addition to the extracellular compartment alters the transport mode of KCC2 to favor Cl\(^{-}\) accumulation via NH\(_4\)^{+}-Cl\(^{-}\) cotransport. The resulting increase in neuronal [Cl\(^{-}\)] then diminishes the driving force for the IPSP. Obviously, such an effect of NH\(_4\)^{+} on KCC2 is completely reversible, as originally shown by Lux et al. (29). Once NH\(_4\)^{+} is removed from the system, KCC2 will extrude the accumulated Cl\(^{-}\) by operating in the KCC mode.

In summary, our findings indicate that Rh\(^{+}\), K\(^{+}\), Cs\(^{+}\), and NH\(_{4}\)^{+} are all transport substrates of KCC2, whereas Na\(^{+}\) and Li\(^{+}\) are not. The kinetic constants of KCC2 transport are roughly similar for external Rh\(^{+}\), K\(^{+}\), and NH\(_{4}\)^{+}. In contrast, Cs\(^{+}\) supports only a fraction of the \(V_{\text{max}}\) observed in the presence of the other transportable cations. Thus replacement of intracellular K\(^{+}\) with Cs\(^{+}\) will cause transporters to be recruited into the more slowly translocated Cs\(^{+}\) bound state, resulting in reduced rate of Cl\(^{-}\) extrusion. The addition of NH\(_{4}\)^{+} to the extracellular compartment permits KCC2 to operate in an alternate transport mode that can mediate significant Cl\(^{-}\) accumulation as external [NH\(_4\)^{+}] reaches levels near its \(K_m\). Thus replacement of intracellular K\(^{+}\) with Cs\(^{+}\) or the addition of even low millimolar concentrations of extracellular NH\(_{4}\)^{+} will compromise Cl\(^{-}\) extrusion by KCC2, resulting in significant cellular Cl\(^{-}\) loading. Such a mechanism can explain the positive shifts in \(E_{\text{Cl}}^{\text{ABA}}\) and \(E_{\text{Cl}}^{\text{glycine}}\) that are observed in the presence of these two monovalent cations.

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