Functional characterization of the neuronal-specific K-Cl cotransporter: implications for \([K^+]_o\) regulation

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Payne, John A. Functional characterization of the neuronal-specific K-Cl cotransporter: implications for \([K^+]_o\) regulation. Am. J. Physiol. 273 (Cell Physiol. 42): C1516–C1525, 1997.—The neuronal K-Cl cotransporter isoform (KCC2) was functionally expressed in human embryonic kidney (HEK-293) cell lines. Two stably transfected HEK-293 cell lines were prepared: one expressing an epitope-tagged KCC2 (KCC2–22T) and another expressing the unaltered KCC2 (KCC2–9). The KCC2–22T cells produced a glycoprotein of \(~150\) kDa that was absent from HEK-293 control cells. The \(^{86}\)Rb influx in both cell lines was significantly greater than untransfected control HEK-293 cells. The KCC2–9 cells displayed a constitutively active \(^{86}\)Rb influx that could be increased further by \(1\) mM N-ethylmaleimide (NEM) but not by cell swelling. Both furosemide (Michaelis constant \((K_m)\) \(\sim 25\) \(\mu M\)) and bumetanide \((K_m \sim \pm 55\) \(\mu M\)) inhibited the NEM-stimulated \(^{86}\)Rb influx in the KCC2–9 cells. This diuretic-sensitive \(^{86}\)Rb influx in the KCC2–9 cells, operationally defined as KCC2 mediated, required external \(Cl^-\) but not external \(Na^+\) and exhibited a high apparent affinity for external \(Rb^+\) \((K_m) = 5.2 \pm 0.9 \text{ (SE) mM; } n = 5\) but a low apparent affinity for external \(Cl^-\) \((K_m > 50\) mM). On the basis of thermodynamic considerations as well as the unique kinetic properties of the KCC2 isoform, it is hypothesized that KCC2 may serve a dual function in neurons: 1) the maintenance of low intracellular \(Cl^-\) concentration so as to allow \(Cl^-\) influx via ligand-gated \(Cl^-\) channels and 2) the buffering of external \(K^+\) concentration \(([K^+]_o)\) in the brain.

furosemide; N-ethylmaleimide; external potassium homeostasis; postsynaptic inhibition

The potassium-chloride cotransporter is an integral membrane protein that mediates the obligatorily coupled, electroneutral movement of \(K^+\) and \(Cl^-\) across the plasma membranes of many animal cells. As an electroneutral transport mechanism, the direction of net movement of \(K^+\) and \(Cl^-\) by the cotransporter is determined solely by the sum of the chemical potential differences of the two ions. Under normal physiological conditions, the K-Cl cotransporter is an efflux pathway with the direction of driving force being dictated by the outwardly directed \(K^+\) chemical potential maintained by the Na\(^+\)-K\(^+\)-ATPase. The K-Cl cotransporter is characterized by its sensitivity to the sulfamoylbenzoic acid "loop" diuretics (e.g., furosemide and bumetanide) and by its activation by cell swelling or the application of the thiol alkylating reagent N-ethylmaleimide (NEM). The K-Cl cotransporter displays a slightly higher affinity for furosemide than bumetanide; however, its loop diuretic affinities are significantly lower than that of the Na-K-Cl cotransporter (4, 20). In red blood cells, activation of K-Cl cotransport by cell swelling and by NEM has been studied extensively, and both have been shown to depend on a dephosphorylation event (9, 18, 19).

In most cells where K-Cl cotransport has been described, its primary function appears to be the regulation of cell volume after swelling by promoting an efflux of \(K^+\) and \(Cl^-\) and osmotically obliged water. The K-Cl cotransporter also appears to be involved in the vectorial movement of salt and water across certain epithelia (e.g., Refs. 12, 31). A unique function of the K-Cl cotransporter has been proposed in neurons (e.g., Refs. 1, 3, 18a, 34). Postsynaptic inhibition involves the conductive movement of \(Cl^-\) through ligand-gated anion channels, i.e., \(\gamma\)-aminobutyric acidA (GABA\(_A\)) and glycine receptors. For these receptors to mediate postsynaptic inhibition, an inwardly directed \(Cl^-\) electrochemical gradient must be maintained so that an influx of \(Cl^-\) through the receptor-channel complex hyperpolarizes the postsynaptic membrane (i.e., inhibitory postsynaptic potential; IPSP) and stabilizes the membrane potential \((E_m)\) at or near the equilibrium potential for \(Cl^-\) \((E_{cl})\). A neuronal K-Cl cotransporter appears to function as the "active" \(Cl^-\) extrusion pathway that maintains \(E_{cl} < E_m\), as required for the IPSP (for reviews, see Refs. 3, 18a).

Recently, we reported the molecular characterization of two distinct isoforms of the K-Cl cotransporter (KCC1, Ref. 10; KCC2, Ref. 28). These two K-Cl cotransporters exhibit \(~67\)% amino acid identity over their full length and display distinctly different tissue expression patterns. KCC1 was present in all tissues examined and appears to be a "housekeeping" isoform involved in cell volume regulation. Because KCC1 is highly expressed in tissues like kidney and lung, it is likely to be the isoform involved in epithelial salt transport as well. In contrast, KCC2 was found only in the brain where it is expressed in great abundance. Reverse transcriptase-polymerase chain reaction (PCR) and in situ hybridization studies indicate that within the central nervous system KCC2 is a neuronal-specific isoform (28). Based on its abundant neuronal expression, we proposed that KCC2 was likely the outwardly directed "Cl" pump of neurons (28). Although KCC1 has been clearly shown to mediate K-Cl cotransport, KCC2 has not yet been functionally characterized. In the present report, I functionally characterize KCC2 in stable human embryonic kidney (HEK-293) cell lines and demonstrate that it displays the basic transport features of a K-Cl cotransport system. The functional analysis revealed, however, that the expressed KCC2 protein exhibits a very high apparent affinity for external \(Rb^+\) \((K_m)\), Michaelis constant \((K_m) \sim 5\) mM. This high affinity for external \(Rb^+\) \((K_m)\) as well as thermodynamic considerations indicate that in addition to the maintenance of low...
neuronal Cl\(^{-}\), KCC2 may serve to help buffer external K\(^{+}\) concentration ([K\(^{+}\)]) in the brain.

A preliminary report of these results has been presented in abstract form (25).

MATERIALS AND METHODS

Preparation of expression constructs. Two KCC2 expression constructs were prepared from a full-length rat brain cDNA clone (5ERB14; see Ref. 28). This cDNA clone (rtKCC2: nucleotides \(-69\) to \(3635\)) lacks much of the 3'-untanslated region of the KCC2 sequence. One full-length expression construct was prepared by subcloning the 5ERB14 DNA into the mammalian expression vector, pBl20, at EcoRI I and Kpn I restriction sites of the polylinker. Another full-length expression construct was prepared by PCR mutagenesis to contain the c-myc epitope at the NH\(_2\) terminus of the KCC2 protein. The NH\(_2\) terminus is a poorly conserved region between the two K-Cl cotransporter isoforms as well as between all members of the cation-chloride cotransporter family. We have proposed that this region is not involved in ion translocation (29) and has limited functional significance (10). Therefore, it has proven to be a useful site for epitope addition on the cation-chloride cotransporters (10).

PCR mutagenesis was performed using 5ERB14 as template and synthetic oligonucleotide primers. The reverse primer (5'-GCAGAAGAGGATAAACCAGAGT-3') was used without purification. The 64-mer forward primer (adding an initiating Met and c-myc peptide epitope, EQKLISEEDL, directly to the original initiating Met of the KCC2 protein) was double high-performance liquid chromatography purified. The 399-base pair (bp) fragment was amplified in a reaction volume of 50 \(\mu\)l containing 10 ng cDNA template, 50 \(\mu\)M forward and reverse primer, 0.25 mM dNTP, 5 mM KCl, 10 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 9.0 at 25°C), 0.1% Triton X-100, 2.5 mM MgCl\(_2\), and 1.5 U Taq DNA polymerase (Promega). After the reaction contents were heated for 1 min at 94°C, 30 cycles of PCR were performed (each consisting of incubation of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C). The PCR product was then cloned into the plasmid pCR 1 (Invitrogen) and sequenced in both directions. After confirmation of the sequence, the PCR product and the 5ERB14 clone were ligated at a common Bsu36I site and subcloned into the mammalian expression vector, pBl20, at EcoRI I and Kpn I restriction sites of the polylinker.

Stable expression in HEK-293 cells. The human embryonic kidney cell line (HEK-293) was maintained in Dulbecco’s modified Eagle’s medium (Cellogro) supplemented with 10% fetal bovine serum, penicillin (50 U/ml), and streptomycin (50 \(\mu\)g/ml) in a humidified incubator (5% CO\(_2\) at 37°C). The two expression constructs were transfected into separate HEK-293 cells by calcium phosphate precipitation using previously described methods (29). After 3 wk of growth in 900 \(\mu\)g/ml Geneticin (GIBCO), single resistant colonies were amplified and screened by Western blot with a c-myc epitope monoclonal antibody or by \(^{86}\)Rb influx assay (see Functional \(^{86}\)Rb flux assay).

Membrane protein identification and deglycosylation. Prestained molecular weight markers (Amersham or Bio-Rad) and protein samples from cell lysates or membrane preparations of stable cells were boiled in sample buffer \([3%\) sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH 8.5), 12% sucrose, 0.01% Serva Blue G, 0.002% phenol red, and 15.5 mg/ml diithiothreitol] and then separated by SDS-polyacrylamide gel electrophoresis using a 7.5% tricine gel system. Gels were electrophoretically transferred from unstained gels to polyvinylidene difluoride (PVDF) membranes (Immobilon P; Millipore) in transfer buffer [192 mM glycine, 25 mM Tris (pH 8.3), and 15% methanol] for 5 h at 20 V using a Bio-Rad Trans-Blot tank apparatus or 1 h at 25 V using a Bio-Rad Trans-Blot semi-dry apparatus. PVDF-bound protein was visualized by staining with Coomassie brilliant blue R-250. The PVDF membrane was blocked in phosphate-buffered saline (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 the mouse monoclonal c-myc peptide antibody either overnight at 4°C or 1 h at 24°C. After three 10-min washes in PBS-milk, the membrane was incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G; Zymed) for 2 h at 24°C in PBS-milk. After three washes in PBS-0.1% Tween 20, bound antibody was detected using an enhanced chemiluminescence assay (NEN Renaissance system).

To deglycosylate membrane proteins, membranes were isolated by differential centrifugation and denatured by boiling in 0.5% SDS. The deglycosylation of the c-myc-tagged KCC2 protein with n-glycosidase F required prior denaturation with SDS. Deglycosylation was carried out in a medium containing 0.5% n-actylglucoside, 20 mM sodium phosphate buffer (pH 8.0), 50 mM EDTA, protease inhibitors, and N-glycosidase F (20 U/ml; Boehringer Mannheim). The sample was incubated for 4 h at 37°C in a thermal cycler. Enzymatic treatment was terminated by addition of electrophoresis sample buffer supplemented with 6 M urea.

Functional \(^{86}\)Rb flux assay. Flux experiments were performed at 24°C on 1-day postconfluent cells grown in 96-well plates. Before flux measurement, cells were washed free of growth media with three successive washes in control flux media (135 mM NaCl, 5 mM RbCl, 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM NaHPO\(_4\), 2 mM glucose, 2.5 mM KCl, 1.5 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid titrated to pH 7.4 with N-methyl-2-glucamine base, and 0.1 mM ouabain; final osmolality, \(\approx\)290 mosmol/kg\(H_2O\)) in the presence or absence of 1 mM NEM. After being washed, the cells were then preincubated for 15 min in the presence or absence of 1 mM NEM. After preincubation, the cells were brought up in the same media as in the preincubation but containing 2 \(\mu\)Ci/ml \(^{86}\)RbCl. In transfected cell lines, the NEM-stimulated \(^{86}\)Rb uptake was linear for 7 min, and 3-min influx assays were routinely performed to obtain initial rates. In HEK-293 control cells, \(^{86}\)Rb influx was linear for 30 min, and 10-min influx assays were performed to obtain initial rates. The \(^{86}\)Rb influx was terminated by five washes in Tris-buffered saline (pH 7.4) containing 2 mM furosemide. Cells were solubilized in 2% SDS and assayed for \(^{86}\)Rb by Cerenkov radiation and for protein by the MicroBCA method (Pierce). In ion substitution experiments, N-methyl-D-glucamine replaced sodium, whereas glucuronate or methanesulfonate replaced chloride.

To assess the effect of swelling on the activity of KCC2, the cells were exposed to a medium rendered hypotonic (230 mosmol/kg\(H_2O\)) by the reduction of NaCl to 95 mM. The isotonic control media was prepared by the addition of sodium gluconate (final osmolality 290 mosmol/kg\(H_2O\)). Thus the isotonic and hypotonic media contained similar final Cl\(^{-}\) concentration ([Cl\(^{-}\)]) (104 mM). Cells were preincubated for 15 min in hypotonic or isotonic media before \(^{86}\)Rb flux analysis.

The ability of the loop diuretics furosemide and bumetanide as well as 4,4’-diisothiocyanostilbene-2,2’-disulfonic acid (DIDS) and [(dihydrodienyl)oxy]alkanoic acid (DIOA) to inhibit the KCC2-mediated \(^{86}\)Rb influx was examined. In these experiments, the cells were washed and preincubated for 15 min in control flux media containing 1
mM NEM and various concentrations of inhibitor. The initial rate of $^{86}$Rb influx was then monitored in fresh control flux media containing similar NEM and inhibitor concentrations as in the preincubation media.

Data analysis. A nonlinear iterative procedure (DeltaGraph, DeltaPoint, Monterey, CA) was used in Fig. 7 to calculate the inhibition constant ($K_i$) for furosemide and bumetanide inhibition. The data from each experiment were fit to the equation $J = J_{\text{max}} - (J_{\text{max}} - J_{\text{min}})/(1 + K_i/[\text{Inh}])$, where $J$ is the measured $^{86}$Rb influx, $J_{\text{max}}$ is the maximal $^{86}$Rb influx in the absence of diuretic, $J_{\text{min}}$ is the diuretic-insensitive $^{86}$Rb influx, and [Inh] is the diuretic concentration. The reported $K_i$ values are means ± SE of 4 separate experiments.

Results were analyzed statistically using either a t-test or single-factor analysis of variance in which experimental values were compared with control measurements. Error bars are ± SE. Statistical significance was defined as $P < 0.05$.

RESULTS

The neuronal-specific K-Cl cotransporter was functionally characterized in stably transfected HEK-293 cell lines. Two full-length expression constructs were prepared in the mammalian expression vector, pJB20. The insert of one construct was prepared from unmodified KCC2 cDNA. The other construct was prepared by PCR mutagenesis to contain an epitope tag (c-myc peptide) at the NH$_2$ terminus of the KCC2 protein. The two KCC2 constructs were transfected into separate sets of HEK-293 cells via CaPO$_4$ coprecipitation. After 3 wk of growth in selection media, G418-resistant colonies were screened by Western blot (c-myc-tagged construct) or $^{86}$Rb influx (untagged control construct). In screening the cell lines for $^{86}$Rb influx, the cells were pretreated with NEM, an agent known to stimulate K-Cl cotransport in red blood cells as well as KCC1 expressed in HEK-293 cells (10). Seven cell lines expressing the unmutated control KCC2 construct and three cell lines expressing the c-myc-tagged KCC2 construct were successfully isolated. Due to their high levels of expression, I chose to characterize more fully the KCC2–9 (untagged control) and KCC2–22T (c-myc tagged) cell lines. Some of the cell lines functionally expressing KCC2 are shown in Fig. 1.

The KCC2–22T cell line was used to examine protein expression by Western blot analysis. This cell line expressed a membrane glycoprotein of 150 kDa that was completely absent from the untransfected control cells (Fig. 2). The KCC2 glycoprotein was similar in size to the KCC1 glycoprotein previously expressed in HEK-293 cells (10). Presumably, the 150-kDa KCC2 glycoprotein is the mature functional cotransporter delivered to the plasma membrane, since removal of the N-linked oligosaccharides with N-glycosidase F reduced the protein to a size predicted from the cDNA (~125 kDa).

In a number of preliminary experiments, both the KCC2–22T and KCC2–9 cell lines provided very similar functional data, substantiating the hypothesis that the NH$_2$-terminal epitope tag does not alter the functional expression of the KCC2 protein (data not shown). Under control conditions, the $^{86}$Rb influx in the KCC2–9 cell line was significantly greater than that of HEK-293 control cells and was completely inhibited by 2 mM furosemide (Fig. 3A). The furosemide-sensitive $^{86}$Rb influx was 6.2-fold higher in KCC2–9 cells (35.4 ± 1.8 nmol·mg protein$^{-1}$·min$^{-1}$) than untransfected control cells (5.7 ± 1.1 nmol·mg protein$^{-1}$·min$^{-1}$). Thus, as reported for KCC1, the KCC2 protein is capable of...
mediating a $^{86}\text{Rb}$ flux that is active without exogenous stimulation when transfected in HEK-293 cells.

The furosemide-sensitive $^{86}\text{Rb}$ influx in the KCC2–9 cells was stimulated ~1.6-fold by treatment with 1 mM NEM (Fig. 3A). In contrast, this flux was not significantly increased by cell swelling (Fig. 3B). In these latter experiments, external $[\text{Cl}^-]$ was held constant at 104 mM, and osmolarity was increased to isotonicity with the permeant anion methansulfonate; data not shown). This allowed me to directly compare initial flux rates in isotonic and hypotonic conditions, but because the affinity of the K-Cl cotransporter for external $[\text{Cl}^-]$ is low (see below), this resulted in a reduction in $^{86}\text{Rb}$ influx (compare control fluxes in Fig. 3, A and B). After treatment with NEM, the furosemide-sensitive $^{86}\text{Rb}$ influx was 20 times greater than that monitored in similarly treated untransfected HEK-293 cells. As previously reported, the NEM pretreatment significantly reduced the endogenous furosemide-sensitive $^{86}\text{Rb}$ influx of control HEK-293 cells, which is due predominately to an endogenous Na-K-Cl cotransporter (10). Because this improved the ability to monitor the expressed KCC2 protein in HEK-293 cells, NEM pretreatment was used in all subsequent studies to characterize the furosemide-sensitive $^{86}\text{Rb}$ influx in the KCC2–9 cell line. I operationally defined this NEM-stimulated $^{86}\text{Rb}$ influx in the KCC2–9 cells as KCC2 mediated.

Figure 4 presents the external ion dependency of the KCC2-mediated $^{86}\text{Rb}$ influx. In these experiments, the cells were first preincubated in control flux media containing 1 mM NEM for 15 min and then quickly washed three times in media lacking either external $\text{Na}^+$ (replaced by N-methyl-D-glucamine) or external $\text{Cl}^-$ (replaced by gluconate). The furosemide-sensitive $^{86}\text{Rb}$ influx was then monitored in these same $\text{Na}^+$- or $\text{Cl}^-$-free media. As expected for a K-Cl cotransport system, the KCC2-mediated $^{86}\text{Rb}$ influx required external $\text{Cl}^-$ but not external $\text{Na}^+$.

Figure 5 shows the dependence of KCC2-mediated $^{86}\text{Rb}$ influx on external $\text{Rb}^+$ and $\text{Cl}^-$ concentrations. We have previously reported (10) that KCC1 expressed in HEK-293 cells has low apparent affinities for external $\text{Rb}^+ (K_m > 25 \text{ mM})$ and external $\text{Cl}^- (K_m > 50 \text{ mM})$. Although the furosemide-sensitive $^{86}\text{Rb}$ uptake of the KCC2–9 cells also displayed a low apparent affinity for external $\text{Cl}^- (K_m > 50 \text{ mM})$, it exhibited a relatively high apparent affinity for...
This represents at least a fivefold higher affinity for external Rb\(^+\) (K\(_m\) = 5.2 ± 0.9 mM; n = 5) than that reported for the KCC1 isoform expressed in the same cell line (10). This high apparent affinity for external Rb\(^+\) exhibited by KCC2 is an important functional difference between the two K-Cl cotransporter isoforms.

Furosemide and bumetanide inhibited the \(^{86}\)Rb influx in the KCC2–9 cells with K\(_i\) values of 25 ± 3 µM (n = 4) and 55 ± 13 µM (n = 4), respectively (Fig. 6). Similar furosemide and bumetanide K\(_i\) values were obtained for inhibition of the \(^{86}\)Rb uptake mediated by KCC1 expressed in HEK-293 cells (furosemide, 40 ± 4 µM; bumetanide, 59 ± 9 µM; Ref. 10). In addition to the loop diuretics, a number of other compounds are known to inhibit K-Cl cotransport in red blood cells (6, 35). These include the stilbene disulfonic acid DIDS and the alkanoic acid DIOA. As shown in Fig. 7, both of these drugs significantly inhibited the \(^{86}\)Rb uptake of KCC2–9 cells; however, the inhibition by either drug at 100 µM was not complete when compared with the furosemide inhibition (~80% of the furosemide-sensitive influx).

**DISCUSSION**

In a previous study, a K-Cl cotransporter (KCC1), displaying a ubiquitous tissue distribution, was functionally characterized in stable HEK-293 cells (10). Concurrent with the isolation of KCC1, we identified a closely related but distinct gene product (KCC2) that was specifically expressed in neurons throughout the central nervous system (28). On the basis of its high identity to KCC1 (67%) and unique tissue distribution, we proposed that this second gene product represented a neuronal-specific isoform of the K-Cl cotransporter. In the present study, I have functionally characterized KCC2 as a novel K-Cl cotransporter and demonstrated that it exhibits unique functional properties. These unique properties provide insight into potential roles of KCC2 in ion homeostasis within the central nervous system.

Functional characterization of KCC2. With the use of an epitope-tagged construct expressed in a stable HEK-293 cell line, the biochemical characteristics of the KCC2 protein were first examined. Like KCC1, KCC2 is a glycoprotein with a molecular mass of ~150 kDa when expressed in HEK-293 cells. Treatment of membranes prepared from the stable cell line with N-glycosidase F reduced the KCC2 protein to a mass of ~125 kDa, which is the size of the core KCC2 protein predicted from the cDNA. These data demonstrate that KCC2, although an apparent neuronal-specific protein, is properly processed and delivered to the plasma membrane of HEK-293 cells. As with all other members of the cation-chloride cotransporter gene family, both K-Cl cotransporter isoforms are glycoproteins. The
region of the primary sequence that likely harbors the oligosaccharides is the large predicted extracellular loop between putative transmembrane segment (TM) 5 and TM6 where both KCC isoforms contain four putative glycosylation sites.

Confirmation that KCC2 is expressed at the cell surface and encodes a K-Cl cotransporter protein was obtained from \(^{86}\)Rb flux analysis of the stable HEK-293 cell lines. Cells expressing the unmutated KCC2 protein (KCC2–9) exhibited a constitutively active furosemide-sensitive \(^{86}\)Rb influx that was approximately six-fold greater than untransfected HEK-293 cells. K-Cl cotransport has been well studied in vertebrate red blood cells where both cell swelling and the alkylating reagent NEM have been shown to stimulate this cation-chloride cotransporter (for review see Ref. 21). Hypotonic swelling and NEM are also known to activate the KCC1 isoform when stably expressed in HEK-293 cells (10). Much less is known about the regulation of the KCC2 isoform. Unlike KCC1, the KCC2 isoform expressed in HEK-293 cells was not stimulated by osmotic swelling. Like KCC1, however, KCC2 activity was significantly increased by NEM treatment. It is interesting to note that in the presence of NEM, the furosemide-sensitive \(^{86}\)Rb influx mediated by the KCC2–9 cells (56 ± 3 nmol·mg protein\(^{-1}\)·min\(^{-1}\)) was substantially larger than that reported by Gillen et al. (10) for KCC1-expressing cells (~1 nmol·mg protein\(^{-1}\)·min\(^{-1}\)). This large difference could be explained by differences in protein expression and/or transport kinetics. Because both studies used 5 mM Rb\(^{+}\) in the flux medium, it is likely that the high external Rb\(^{+}\) affinity exhibited by KCC2 contributed significantly to its larger \(^{86}\)Rb influx.

The KCC2-mediated \(^{86}\)Rb influx displayed many functional characteristics of a K-Cl cotransporter protein. For example, it exhibited complete dependence on the presence of external Cl\(^{-}\) but not external Na\(^{+}\). Additionally, the NEM-stimulated \(^{86}\)Rb influx was inhibited by the loop diuretics with a greater sensitivity to furosemide than bumetanide. Other known inhibitors of K-Cl cotransport such as the stilbene disulfonic acid DIDS (6) and the alkanolic acid DIOA (35) significantly reduced the KCC2-mediated \(^{86}\)Rb influx. Furthermore, the KCC2-mediated \(^{86}\)Rb influx displayed simple Michaelis-Menten kinetics with respect to external [Rb\(^{+}\)] and [Cl\(^{-}\)], and such data are consistent with the electroneutral cotransport of 1Rb\(^{+}\)(K\(^{+}\)):1Cl\(^{-}\).

Involvement of putative TM2 in ion binding. The molecular and functional characterization of numerous members of the cation-chloride cotransporter gene family has provided important information concerning structure-function relationships of these transporters. One region that has received particular attention is putative TM2 of the Na-K-Cl cotransporter (NKCC). This region has been implicated in forming part of the cation binding site on the Na-K-Cl cotransporter (17), and it is reasonable to suspect that structural differences in TM2 help explain the large measured differences in ion affinities between the shark rectal gland and human colonic Na-K-Cl cotransporters (29). Furthermore, this region is encoded by an alternatively spliced 96-bp cassette exon in the absorptive isoform of the mammalian kidney, leading to three distinct splice variants with differing TM2 segments (NKCC2a-b-f; see Refs. 15, 26). Remarkably, these NKCC2 splice variants are restricted to the thick ascending limb of the loop of Henle (TALH) where they display a distinct axial heterogeneity (15). The presence of variant Na-K-Cl cotransporters possessing different ion affinities within the TALH has important functional implications for NaCl reabsorption in the kidney (see Ref. 27).

In comparing the primary structures of KCC1 and KCC2, not surprisingly TM2 is the most divergent predicted transmembrane segment, sharing only 60% amino acid identity. This divergence is almost entirely at the postulated extracellular side of TM2 (Fig. 8).

Seven of the remaining eleven TMs display >90% amino acid identity, including TM1, -3, -6, -8, -10, -11, and -12. The large difference in apparent affinity for external Rb\(^{+}\)(K\(^{+}\)) and the low TM2 identity are consistent with the hypothesis that among the cation-chloride cotransporters TM2 is involved in cation binding. However, as the KCC proteins do not transport Na\(^{+}\), TM2 of the K-Cl cotransporters cannot be involved in Na\(^{+}\) binding. One residue in KCC2 of particular interest is the glutamic acid predicted to be at the extracellular side of TM2 (Fig. 8). This residue is replaced by glutamine in KCC1. Thus it is interesting to speculate that the negative charge of the glutamic acid side chain might be involved in providing a K\(^{+}\) binding site in KCC2 with a significantly higher affinity than that in KCC1.

Physiological function of K-Cl cotransport. The existence of two different K-Cl cotransporters begs the obvious question concerning functional differences. KCC1 shares many functional characteristics of the vertebrate red cell K-Cl cotransporter that has been the subject of much study. For example, it is activated by cell swelling and by NEM, and it has a low apparent
affinity for external Rb\(^+\) (K\(^+\)) and external Cl\(^-\) (10). These characteristics, along with its ubiquitous tissue distribution, provide strong circumstantial evidence that KCC1 is the housekeeping isoform involved in cell volume regulation (10). There is also evidence that KCC1 may participate in net transepithelial salt movement, since it is highly expressed in tissues such as lung and kidney (10). KCC2, on the other hand, displays some very different functional characteristics, including the lack of stimulation by cell swelling and a very high apparent affinity for external Rb\(^+\) (K\(^+\)). The unique characteristics of KCC2 as well as its neuronal specificity raise the possibility that the physiological functions of KCC2 differ from those of KCC1. Furthermore, there are significant differences in the primary sequence between the two KCC isoforms that may confer isoform-specific differences in regulation. For example, KCC2 has a large 44-amino acid insertion within the large COOH-terminal hydrophilic region that contains numerous negatively charged amino acids as well as a unique potential protein kinase C phosphorylation site. This region also contains a potential tyrosine kinase phosphorylation site that is not present in KCC1.

Regulation of neuronal intracellular [Cl\(^-\)]. One potential function of KCC2 might be to maintain the low neuronal [Cl\(^-\)] and the favorable inwardly directed Cl\(^-\) electrochemical gradient required for the proper function of ligand-gated Cl\(^-\) channels in postsynaptic inhibition (28). The hyperpolarization that occurs in postsynaptic neurons after activation of GABA\(_A\) receptors is due to an influx of Cl\(^-\) through the ligand-gated channel. This represents the underlying cellular mechanism for the IPSP. For GABA\(_A\) receptors to mediate such a hyperpolarization, the electrochemical potential difference for Cl\(^-\) must be kept more negative than the resting membrane potential. This requires the presence of a transport mechanism that can move Cl\(^-\) out of the neuron against an electrochemical gradient, i.e., an active Cl\(^-\) extrusion mechanism. Under normal physiological conditions, there is enough energy stored in the K\(^+\) chemical gradient to move Cl\(^-\) against its chemical gradient out of the neuron via electroneutral K-Cl cotransport, and this can help maintain the low neuronal [Cl\(^-\)] and external Cl\(^-\) concentrations.

KCC2 functions of KCC2 differ from those of KCC1. Furthermore, the K\(^+\) released during activity must eventually be returned to the neuron to prevent the depletion of neuronal K\(^+\). I propose that the neuronal K-Cl cotransporter can participate in the process of K\(^+\) uptake after neuronal activity.

As an electroneutral transporter, the direction and magnitude of net K-Cl cotransport will be dictated by the sum of the K\(^+\) and Cl\(^-\) chemical potential differences. In most cells, the K-Cl cotransporter has only been considered as a net efflux pathway because plasma K\(^+\) concentration is tightly regulated and the K\(^+\) and Cl\(^-\) chemical gradients almost always favor net efflux. However, in many neurons where intracellular [Cl\(^-\)] ([Cl\(^-\)]\(_o\)) is quite low (7–9 mM if E\(_{Cl}\) < E\(_m\)), the driving force for net K-Cl cotransport is very close to thermodynamic equilibrium. Using typical values reported for neuronal [K\(^+\)] (100 mM; Ref. 11) and external [Cl\(^-\)] (145 mM; Ref. 7), Fig. 9 presents the driving force for net K-Cl cotransport as a function of [K\(^+\)]\(_o\) at different neuronal [Cl\(^-\)]\(_o\). In neurons like pyramidal cells of the hippocampus where [Cl\(^-\)]\(_o\) has been estimated to be ~7 mM (16), [K\(^+\)]\(_o\) needs only to increase above ~5 mM to reverse the calculated driving force for net K-Cl cotransport, thus allowing KCC2 to operate as a net influx pathway.

For a K-Cl cotransporter to operate efficiently as a [K\(^+\)]\(_o\) buffer, it should exhibit a number of important features. 1) Its direction of net transport should be sensitive to subtle changes in [K\(^+\)]\(_o\). Because the driving force for net K-Cl cotransport is situated very near thermodynamic equilibrium in the brain and KCC2 appears to be constitutively active (at least as expressed in HEK-293 cells), it can respond rapidly to any increase in [K\(^+\)]\(_o\). 2) The affinity of the cotransporter for external K\(^+\) should be compatible with the range of the observed [K\(^+\)]\(_o\) (3–10 mM), allowing a high rate of cotransport activity. This requirement appears to be a unique feature of the KCC2 isoform, as it exhibits a rather high affinity for external Rb\(^+\) (K\(_m\) = 5.2 mM), whereas the KCC1 isoform displays a lower affinity for external Rb\(^+\) (K\(_m\) > 25 mM; Ref. 10). Because KCC2 exhibits an affinity for external Rb\(^+\) (K\(_m\)) that is near the normal [K\(^+\)]\(_o\), it is an excellent candidate for [K\(^+\)]\(_o\) buffering. In other words, the rate of K-Cl cotransport will approach maximum velocity as [K\(^+\)]\(_o\) increases to its ceiling level of 10–12 mM. 3) To be an effective buffer, responses should be rapid. This is an issue of transport capacity, requiring high levels of protein expression. Although KCC2 protein has not yet been

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examined in the brain, RNA analysis, including both Northern blot and in situ hybridization studies, have demonstrated that the KCC2 transcript is expressed at very high levels in neurons throughout the central nervous system (28). Thus, on the basis of thermodynamic considerations as well as unique properties of the KCC2 protein, including its transport kinetics, distribution, and density, KCC2 is well suited to function as an influx pathway and an effective \([K^+]_o\) buffer under conditions of elevated \([K^+]_o\). Although the K-Cl cotransporter cannot function alone in \([K^+]_o\) homeostasis, it can complement other neuronal \(K^+\) uptake systems (e.g., Na\(^+-\)K\(^+\)-ATPase) and glial spatial buffering and contribute significantly to the overall process of rapidly returning \(K^+\) to the neuron.

In addition to the K-Cl cotransporter, the Na-K-Cl cotransporter also exhibits some of the features required for an efficient \([K^+]_o\) buffer discussed above; that is, it has a strong net inwardly directed driving force and a high affinity for external \(K^+\) (29). The Na-K-Cl cotransporter has been well characterized by Russell and co-workers (5) in the squid giant axon, and recently, NKCC1 was localized in neurons of the rat brain (30). In the rat brain, NKCC1 appears to be much more restricted in its neuronal distribution and density than KCC2. For example, Plotkin et al. (30) found NKCC1 at high levels only in sensory neurons and certain neurons of the brain stem, and this may limit its role in \([K^+]_o\) buffering within the neuronal microenvironment. On the other hand, NKCC1 does exhibit very high expression within the epithelial cells of the choroid plexus, which are responsible for the production of the cerebrospinal fluid. Plotkin et al. (30) have suggested that NKCC1 plays an important role in the regulation of \([K^+]_o\) in this secreted fluid as well as the regulation of \([Cl^-]_o\) in certain neurons. Therefore, both the K-Cl and Na-K-Cl cotransporters appear to serve important roles in the regulation of neuronal \([Cl^-]_i\) and \([K^+]_o\) in the brain.

Functional consequences of KCC2-dependent \([K^+]_o\) buffering. The involvement of KCC2 in the regulation of neuronal \([Cl^-]_i\) and \([K^+]_o\), in the brain is mutually exclusive. In other words, it can function in the regulation of only one of these parameters at any given time. However, as touched on above, because the driving force for K-Cl cotransport is situated very near thermodynamic equilibrium, KCC2 becomes sensitive to subtle changes in either \([Cl^-]_i\) or \([K^+]_o\), and, therefore, it can function as a “dynamic buffer,” responding to and reducing changes in either parameter. However, an important consequence of the neuronal K-Cl cotransporter operating in “reverse mode” as a net influx pathway is that it will contribute to an increase in neuronal \([Cl^-]_i\). This will alter the driving force for \(Cl^-\) movement through conductive pathways (\(E_m-E_{Cl^-}\)) like GABA\(_A\) and glycine receptors, and may contribute to the neuronal \(Cl^-\) accumulation that is largely responsible for depression of the GABA\(_A\) inhibitory response after repetitive stimulation (22, 34). A well-characterized consequence of repetitive stimulation even at low frequency is a significant and transient increase in \([K^+]_o\) that results from the release of neuronal \(K^+\) via conductive pathways (e.g., Ref. 14). Although it has been suggested that the elevated \([K^+]_o\) would lead to \(Cl^-\) accumulation through inhibition of outward K-Cl cotransport (34), this hypothesis does not properly account for the thermodynamics of K-Cl cotransport. I hypothesize that the \(Cl^-\) accumulation that occurs after repetitive stimulation is largely a result of the K-Cl cotransporter operating as a net influx pathway because of the elevated \([K^+]_o\) and subsequent reversal of K-Cl cotransport driving force. The possible involvement of KCC2 in determining the direction and magnitude of the driving force for \(Cl^-\) movement through GABA\(_A\) receptors indicates this cotransporter may contribute significantly to the genesis of epileptiform activity in the brain. Significantly, furosemide application
(24, 33, 34) as well as elevation of $[K^+]_o$ (typically 8.5 mM; see Ref. 23), both of which are expected to result in the accumulation of neuronal $[Cl^-]$ through effects on the K-Cl cotransporter, have been shown to cause epileptiform activity in vitro preparations.

In summary, the KCC2 protein has been functionally characterized in stably transfected HEK-293 cell lines, demonstrating that it exhibits many of the basic transport features of a K-Cl cotransport system. The KCC2 protein, however, displays some unique functional characteristics that were distinct from the KCC1 isoform, including the lack of swelling activation and relatively high apparent affinity for $[K^+]_o$. The unique properties of the KCC2 protein along with the thermodynamics of K-Cl cotransport support the hypothesis that KCC2 can function efficiently as a net influx pathway under conditions of elevated $[K^+]_o$.

Because the driving force for K-Cl cotransport in many neurons is situated very near thermodynamic equilibrium and relatively high apparent affinity for $[K^+]_o$, the direction of net movement of K and Cl through the cotransporter will be very sensitive to changes in $[Cl^-]$ and $[K^+]_o$. Thus KCC2 may play an important role in the homeostasis of both neuronal $[Cl^-]$ and $[K^+]_o$ in the brain.

NOTE ADDED IN PROOF

A recent article (K. Kaila, K. Lamsa, S. Smirnov, T. Taira, and J. Volpo. J. Neurosci. 17: 7662–7672, 1997) has addressed the cellular mechanisms that account for the large positive shift in the GABA_A reversal potential ($E_{GABA_A}$) in pyramidal neurons following repetitive stimuli. Data from this study are fully consistent with a K-Cl cotransporter mediating a significant increase in $[Cl^-]$ under conditions of elevated $[K^+]_o$ and contributing to a large positive shift in $E_{GABA_A}$ in the postsynaptic neuron.

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