The K⁺-Cl⁻ cotransporters (KCCs) are members of the cation-chloride cotransporter gene family and fall into two phylogenetic subgroups: KCC2 paired with KCC4 and KCC1 paired with KCC3. We report a functional comparison in Xenopus oocytes of KCC1 and KCC4, widely expressed representatives of these two subgroups. KCC1 and KCC4 exhibit differential sensitivity to transport inhibitors, such that KCC4 is much less sensitive to bumetanide and furosemide. The efficacy of other anion inhibitors is critically dependent on the concentration of extracellular K⁺, with much higher inhibition in 50 mM K⁺ versus 2 mM K⁺. KCC4 is also uniquely sensitive to 10 mM barium and to 2 mM trichloromethiazide. Kinetic characterization reveals divergent affinities for K⁺ (Kₘ values of 25.5 and 17.5 mM for KCC1 and KCC4, respectively), probably due to variation within the second transmembrane segment. Although the two isoforms have equivalent affinities for Cl⁻, they differ in the anion selectivity of K⁺ transport (Cl⁻ > SCN⁻ > Br⁻ > PO₄³⁻ > I⁻ for KCC1 and Cl⁻ > Br⁻ > PO₄³⁻ > I⁻ > SCN⁻ for KCC4). Both KCCs express minimal K⁺-Cl⁻ cotransport under isotonic conditions, with significant activation by cell swelling under hypotonic conditions. The cysteine-alkylating agent N-ethylmaleimide activates K⁺-Cl⁻ cotransport in isotonic conditions but abrogates hypotonic activation, an unexpected dissociation of N-ethylmaleimide sensitivity and volume sensitivity. Although KCC4 is consistently more volume-sensitive, the hypotonic activation of both isoforms is critically dependent on protein phosphatase 1. Overall, the functional comparison of these cloned K⁺-Cl⁻ cotransporters reveals important functional, pharmacological, and kinetic differences with both physiological and mechanistic implications.

In a great majority of cells, the plasma membrane is permeable to water. Movement of water across the cell membrane is largely dependent on the osmotic pressure gradient between the intracellular and extracellular space, such that water transport accompanies changes in the concentration of osmotically active molecules. Thus, when intracellular osmolality exceeds that of the extracellular milieu, cell volume increases due to the movement of water into the cell. To cope with the resultant cell swelling, cells have developed a series of complex mechanisms to achieve a regulatory volume decrease, primarily through the activation of efflux mechanisms for intracellular ions. In particular, net electroneutral release of K⁺-Cl⁻ is achieved by K⁺-Cl⁻ cotransport, the simultaneous operation of K⁺/H⁺ and Cl⁻/HCO₃⁻ exchangers, or through parallel, swelling-activated K⁺ and Cl⁻ channels (1).

K⁺-Cl⁻ cotransport was first described in red blood cells as a swelling- and NEM1-activated K⁺ efflux mechanism (2, 3), and red cells remain the primary model tissue for this class of ion transport. However, functional and physiological evidence has also been reported for the existence of K⁺-Cl⁻ cotransport in neurons (4), vascular smooth muscle (5), endothelium (6), epithelium (7, 8), heart (9), and skeletal muscle (10). Consequently, K⁺-Cl⁻ cotransport has been implicated not only in regulatory volume decrease, but also in transepithelial salt absorption (8), renal K⁺ secretion (11), myocardial K⁺ loss during ischemia (9), and regulation of neuronal Cl⁻ concentration (4). The physiological mechanisms invoked in cell volume regulation may also have broader roles in phenomena such as cell growth and apoptosis (1).

A major advance in the understanding of K⁺-Cl⁻ cotransport has been the recent molecular identification of mammalian genes that encode a total of four K⁺-Cl⁻ cotransporter (KCC) isoforms. These cotransporters were identified due to their similarity to other members of the electroneutral cation-chloride cotransporter gene family, the bumetanide-sensitive Na⁺-K⁺-Cl⁻ cotransporters and the thiazide-sensitive Na⁺-Cl⁻ cotransporter (12). The K⁺-Cl⁻ cotransporters have been designated KCC1 (13), KCC2 (14), KCC3 (15, 16), and KCC4 (16). KCC2 is a neuronal specific isoform, whereas the other three KCCs are widely distributed in multiple tissues. Phylogenetic and genomic analysis (16, 17) indicates that the four KCC

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1 The abbreviations used are: NEM, N-ethylmaleimide; KCC, K⁺-Cl⁻ cotransporter; KCC4, mouse KCC4 isoform; KCC1, rabbit KCC1 isoform; ⁸⁶Rb⁺, tracer rubidium; TM, transmembrane segment; DIDS, 4,4-dithiocyano-stilbene-2,2'-disulfonic acid; DIOA, (R(+)-(2S)-2-butyl-6,7-dichloro-2-cyclopenty1-2,3-dihydro-1-oxa-1-H-indenyl-5-yl)-oxygenetic acid; HEK 293, human embryonic kidney cell line; BSC2/NKCC2, bumetanide-sensitive Na⁺-K⁺-2Cl⁻ cotransporter 1 (renal specific); BSC2/NKCC1, bumetanide-sensitive Na⁺-K⁺-2Cl⁻ cotransporter 2.

2 In our previous work (16), we referred to the KCC on human chromosome 15q14 as KCC4 and the KCC on chromosome 5p15 as KCC3. However, in deference to the earlier publication of Hiki et al., we reversed the numbering of our GenBank™/EBI submissions to refer to the KCC on chromosome 15q14 as KCC3 and the KCC on chromosome 5p15 as KCC4 (see note added in proof in Ref. 16).
proteins form a separate subfamily of the cation-chloride co-
transporters. Furthermore, KCC2 and KCC4 form a closely
related subgroup, whereas KCC1 is more homologous to KCC3.
Alternative splicing and alternative promoter usage generate
further molecular heterogeneity. For example, there are at
least two alternative isoforms of KCC3, generated by transcrip-
tional initiation 5' of two separate first coding exons. The
longer isoform, KCC3a (16), utilizes exon 1a, whereas KCC3b
uses exon 1b, situated ~23 kilobases 3' within the human
KCC3 gene on chromosome 15q14.3 The predicted KCC3a and
KCC3b proteins, 1150 and 1099 amino acids, respectively, dif-
fer dramatically in the content and distribution of predicted
phosphorylation sites for protein kinases.

The extent of molecular heterogeneity in K'-'Cl' co-
transport was unexpected, even after the identification of KCC1 and
KCC2. In consequence, next to nothing is known about the
functional and pharmacological properties of the four major
KCC isoforms, or indeed of the physiological role of each iso-
form. One exception is the recent recognition that KCC2 en-
codes a developmentally regulated Cl' extrusion mechanism in
neurons, with crucial secondary effects on the response to γ-aminobutyric acid and other neurotransmitters that activate
neuronal chloride conductance (4). To begin to understand the
physiology and function of the individual KCCs, it is essential
to characterize the functional properties of each isoform. KCC1
cDNAs from human, mouse, rabbit, pig, and Caenorhabditis
elegans have been functionally expressed in human embryonic
kidney cells (HEK 293) and in Xenopus laevis oocytes (13, 17, 18). Rat KCC2 and human KCC3 cDNAs have also been ex-
pressed in HEK 293 cells (15, 19, 20). From these studies, it is
already quite clear that there are differences in the functional
and pharmacological properties of the K'-'Cl' co-
transporter isoforms. For example, kinetic analysis indicates that KCC2 exhibits significantly a higher affinity for potassium (14) than that of KCC1 (13) or KCC3 (20). In contrast, KCC2 is uniquely
volume-insensitive, exhibiting minimal if any activation by cell
swelling and considerable isosmotic transport activity (21). In
the present study, we extended the functional and pharmaco-
logical characterization of the recently cloned mouse KCC4
(16), using the Xenopus laevis oocyte expression system. The
functional comparison of shark and human BSC2/NKCC1 and
rabbit BSC1/NKCC2 has yielded important structure-function
information for the Na'-'K'-'2Cl' cotransporters (22-24). With this paradigm in mind, we simultaneously studied the func-
tional properties of KCC1 in our expression system and report
significant functional and pharmacological differences between
these representatives of the two molecular subgroups of the
KCCs.

MATERIALS AND METHODS

Xenopus laevis Oocyte Preparation—Adult female Xenopus laevis
frogs were purchased from Carolina Biological Supply Company (Bur-
lington, NC) and maintained at the Institution animal facility under
constant control of room temperature and humidity, 16 °C and 65%,
respectively. Frogs were fed with frog brittle dry food from Carolina
Biological Supply Company, and water was changed twice a week.
Oocytes were surgically collected from anesthetized animals under
constant control of room temperature and humidity, 16 °C and 65%,
under the strict supervision of Dr. Bliss Forbush III. To prepare cRNA, the KCC1 and KCC4 cDNAs were
full-length KCC4 and KCC1 cDNAs were previously subcloned into the high expression vector GEMHE (16); rabbit KCC1 was a gift of Dr. Bliss Forbush III. To prepare cRNA, the KCC1 and KCC4 cDNAs were
linearized at their 3'-ends with NheI and then transcribed in vitro
using the T7 RNA polymerase mMESSAGE kit (Ambion). Transcription
product integrity was confirmed on agarose gels, and concentration was
determined by absorbance reading at 260 nm (DU 640, Beckman, Ful-
erton, CA). cRNA was stored frozen in aliquots at −80 °C until used.
Transcription from mouse KCC4 (KCC4) or rabbit KCC1
mRNAs transcribed from mouse KCC4 (KCC4) or rabbit KCC1
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Transcription from mouse KCC4 (KCC4) or rabbit KCC1
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functional and pharmacological properties of the four major
KCC isoforms, or indeed of the physiological role of each iso-
form. One exception is the recent recognition that KCC2 en-
codes a developmentally regulated Cl' extrusion mechanism in
neurons, with crucial secondary effects on the response to γ-aminobutyric acid and other neurotransmitters that activate
neuronal chloride conductance (4). To begin to understand the
physiology and function of the individual KCCs, it is essential
to characterize the functional properties of each isoform. KCC1
cDNAs from human, mouse, rabbit, pig, and Caenorhabditis
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rabbit BSC1/NKCC2 has yielded important structure-function
information for the Na'-'K'-'2Cl' cotransporters (22-24). With this paradigm in mind, we simultaneously studied the func-
tional properties of KCC1 in our expression system and report
significant functional and pharmacological differences between
these representatives of the two molecular subgroups of the
KCCs.

Comparison of KCC1 and KCC4

Comparison of KCC1 and KCC4 in Xenopus Oo-
ocytes—In isotonic conditions, no differences were observed
among KCC4, KCC1, and water-injected oocytes (data not shown).
When uptakes were performed under hypotonic conditions,
microinjection of KCC4 and KCC1 cRNAs resulted in
heterologous expression of KCC4 and KCC1 in Xenopus Oo-
ocytes. In isotonic conditions, no differences were observed
among KCC4, KCC1, and water-injected oocytes (data not shown).
When uptakes were performed under hypotonic conditions,
microinjection of KCC4 and KCC1 cRNAs resulted in
cotransport activity, as compared with con-
trol oocytes injected with water. Fig. 1 summarizes
five experiments in which oocytes from different frogs were
injected with water or KCC4 or KCC1 cRNA, followed by 86Rb
uptake assay using a hypotonic uptake solution containing 10
and 50 mM of extracellular K' and Cl'. Sensitivity for several inhibitors was assessed by exposing groups of
oocytes to the inhibitors at concentrations varying from 20
and 147 μM of Cl'. Inhibitors were dissolved in 10% SDS, and tracer activity was determined for each
oocyte by β-scintillation counting.

To determine the ion transport kinetics of KCC4 and KCC1, we
performed experiments using varying concentrations of K' and Cl'.
The sensitivity for several inhibitors was assessed by exposing groups of
oocytes to the inhibitors at concentrations varying from 20 μM to 2 mM.
For these experiments, the desired concentration of the inhibitor was
present during both the incubation and uptake periods, except when noted.

Statistical Analysis—Statistical significance is defined as two-tailed
p < 0.05, and the results are presented as mean ± S.E. The significance
of the differences between groups was tested by one-way analysis of
variance with multiple comparison using Bonferroni correction or by the
Kruskal-Wallis one-way analysis of variance on ranks with the
Dunn's method for multiple comparison procedures, as needed.

RESULTS

Heterologous Expression of KCC4 and KCC1 in Xenopus Oo-
ocytes—In isotonic conditions, no differences were observed
among KCC4, KCC1, and water-injected oocytes (data not shown).
When uptakes were performed under hypotonic condi-
tions, microinjection of KCC4 and KCC1 cRNAs resulted in
significant K'-'Cl' cotransport activity, as compared with control
oocytes that were injected with water. Fig. 1 summarizes
five experiments in which oocytes from different frogs were
injected with water or KCC4 or KCC1 cRNA, followed by 86Rb
uptake assay using a hypotonic uptake solution containing 10
and 50 mM of extracellular K' and Cl', respectively. In control
oocytes, 86Rb uptake was 588 ± 91 pmol/oocyte·h⁻¹ in the
presence of Cl', indicating the presence of an endogenous K'-'Cl' co-
transporter. Microinjection of KCC4 cRNA resulted in an
increased 86Rb uptake to 24,457 ± 3,476 pmol/oocyte·h⁻¹. This
86Rb uptake was Cl'-dependent, in that uptake in KCC4
oocytes in the absence of extracellular Cl' was 1723 ± 402
pmol/oocyte·h⁻¹. In oocytes microinjected with KCC1, 86Rb
uptake increased to 12,632 ± 2205 pmol/oocyte·h⁻¹, and the

3 D. B. Mount and L. Song, unpublished data.
Comparison of KCC1 and KCC4

The uptake of $^{86}$Rb$^+$ was performed in hypotonic solutions with 120 mosmol/kg in the presence (open bars) or absence (black bars) of extracellular Cl$^-$. Each bar represents a mean of 90 oocytes extracted from five different frogs. $^{86}$Rb$^+$ uptake was performed during 60 min. * indicates a significant difference from uptake in KCC1 control group ($p < 0.01$).

Inhibitor Profile of KCC4 and KCC1—The effect of the loop diuretics furosemide and bumetanide was initially assessed using two different concentrations of extracellular K$^+$: 2 and 50 mM. In uptake medium with a K$^+$ concentration of 2 mM, relative KCC4 activity was 61 ± 3 and 90 ± 4% in the presence of 2 mM furosemide or bumetanide, respectively. Interestingly, the inhibition of KCC4 by loop diuretics was augmented when the uptake medium contained 50 mM K$^+$. Under these conditions, the KCC4 activity was 9 ± 4 or 17 ± 4% in the presence of furosemide or bumetanide, respectively. In contrast, for KCC1 this effect of extracellular K$^+$ was not observed for furosemide and was marginal for bumetanide. KCC1 function in the presence of furosemide was 9 ± 2% in 2 mM K$^+$ and 18 ± 8% in 50 mM K$^+$ ($p$ not significant), and in the presence of bumetanide it was 51 ± 12 versus 19 ± 7% in 2 and 50 mM K$^+$, respectively ($p = 0.05$; $t = 1.99$). To further define the differences in the K$^+$ effect on the sensitivity to loop diuretics between KCC4 and KCC1, we assessed the inhibitory effect of furosemide and bumetanide at several concentrations of extracellular K$^+$. The results of these series of experiments are shown in Fig. 2. The percentage inhibition of KCC4 by both furosemide and bumetanide was significantly affected by extracellular K$^+$ (Fig. 2, upper panels). The minimal and maximal inhibition by both loop diuretics was observed at 2 and 6 mM, respectively; no further effect was observed at higher K$^+$ concentrations. In contrast, the percentage of KCC1 inhibition by either furosemide or bumetanide did not vary as a function of extracellular K$^+$ concentration (Fig. 2, lower panels). Thus, to define differences between the two KCCs in sensitivity to loop diuretics, we used a 10 mM concentration of extracellular K$^+$ to assess the concentration curves for furosemide and bumetanide inhibition upon the Cl$^-$-dependent $^{86}$Rb$^+$ uptake induced by KCC4 or KCC1. As Fig. 3 illustrates, KCC4 exhibits apparent half-maximal inhibition ($K_{0.5}$) values of −900 μM for both furosemide and bumetanide. These are lower than the respective values for KCC1 (~180 μM for furosemide and bumetanide). Therefore, KCC4 clearly exhibits a lower affinity for loop diuretics than does KCC1. The inhibition of KCC1 by furosemide in Fig. 3 suggests the possibility of a second affinity site for the loop diuretic. However, this inhibition fitted well to a Michaelis-Menten kinetics pattern with one inhibitor-binding site. The data did not fit to an equation with two binding sites (data not shown).

The sensitivity of the KCCs to other inhibitors of red cell K$^+$-Cl$^-$ cotransport was also assessed in oocytes injected with KCC4 or KCC1. Fig. 4 illustrates the effect of 100 μM DIDS and 100 μM DIOA on the $^{86}$Rb$^+$ uptake induced by the microinjection of each KCC cRNA. The effect of extracellular K$^+$ concentration on the inhibition of cotransport was very dramatic for DIDS. When the concentration of extracellular K$^+$ was 2 mM, the addition of DIDS to the extracellular medium resulted in reduction of KCC4 function to 65 ± 10% ($p < 0.003$) and of KCC1 to 65 ± 6% ($p = 0.113$, not significant). In contrast, when 50 mM of extracellular K$^+$ was used, DIDS resulted in significant decrease of KCC4 and KCC1 to 13 ± 4 and 13 ± 2%, respectively. The addition of 100 μM of DIOA to the extracellular medium also resulted in inhibition of the KCCs. However, inhibition of KCC4 was higher when extracellular K$^+$ was lower, although this was not the case for KCC1. DIOA is reportedly specific for K$^+$-Cl$^-$ cotransport over Na$^+$-K$^+$-2Cl$^-$ co-transport (27), and the same concentration of DIOA had no effect on the function of the Na$^+$-K$^+$-2Cl$^-$ co-transport activity of Xenopus oocytes (26) (data not shown).

We also tested the effect of a 2 mM concentration of the thiazide diuretic trichlormethiazide on the percentage of chlo-
Comparison of KCC1 and KCC4

**FIG. 3.** Concentration-response profiles for inhibition of KCC4 (circles) and KCC1 (squares) by furosemide (left panel) or bumetanide (right panel). Groups of 15 Xenopus oocytes microinjected with KCC4 or KCC1 were exposed to increased concentrations of furosemide or bumetanide in the preincubation and uptake mediums, from 20 to 2000 μM. Data were normalized as the percentage of influx in each KCC, taking 100% as the value observed in oocytes in which uptake was done in the absence of loop diuretics. Each point represents the mean ± S.E. of at least 15 oocytes.

**FIG. 4.** Effect of the inhibitors DIDS (upper panel) and DIOA (lower panel) upon $^{86}$Rb$^+$ uptake in KCC4- and KCC1-injected oocytes incubated in hypotonic conditions (120 mosmol/kg), in the presence of an extracellular K$^+$ concentration of 2 mM (open bars) or 50 mM (hatched bars). The Cl$^-$ concentration of the extracellular medium was 50 mM in both conditions. In all experiments, $^{86}$Rb$^+$ uptake was assessed in control groups of 2 and 50 mM K$^+$ concentration, in the absence of inhibitors, and experimental groups were exposed to a 100 μM of DIDS or DIOA during incubation and uptake periods. Each bar represents the mean ± S.E. of at least 15 oocytes.

**FIG. 5.** Effect of 10 mM BaCl$_2$ upon the $^{86}$Rb$^+$ influx induced by microinjections of oocytes with KCC4 or KCC1 cRNA. Uptakes in the control groups were performed using a hypotonic uptake medium containing 40 mM NMDG chloride and 10 mM KCl, and uptakes in the BaCl$_2$ group were performed using a hypotonic medium containing 30 mM NMDG chloride, 10 mM BaCl$_2$, and 10 mM KCl. Each bar represents a mean of 20 oocytes. Open bars represent the normalized influx in control group, and black bars show normalized influx in BaCl$_2$ groups. *p < 0.01 versus uptake in control group.

The higher the inhibition by thiazides, since in 2 mM of extracellular K$^+$ $^{86}$Rb$^+$ uptake was reduced to 79 ± 3%, and at 50 mM it was reduced to 57 ± 9%. This difference was significant (p < 0.01). In KCC1-injected oocytes, trichlormethiazide reduced $^{86}$Rb$^+$ uptake by a statistically significant amount to 64 ± 4% in 2 mM K$^+$; this inhibitory effect was not statistically significant at 50 mM K$^+$ (74 ± 8% reduction in activity). Consistent sensitivity to trichlormethiazide is thus unique to KCC4.

Independent studies have suggested that barium can inhibit renal K$^+$-Cl$^-$ cotransporters (7, 8, 29). We thus assessed the effect of 10 mM extracellular barium on the function of KCC4 and KCC1. Fig. 5 shows that when 10 mM BaCl$_2$ was added to the uptake medium, KCC4-induced influx was reduced to 58 ± 4.3% of the uptake observed in KCC4-injected control oocytes. KCC1 function was only reduced to 79 ± 4.2%, hence the inhibitory effect of barium was significantly greater for KCC4 than for KCC1 (p < 0.01).

**Kinetic Properties of KCC4 and KCC1**—To determine and compare the kinetic properties of KCC4 and KCC1 in the same expression system, we measured $^{86}$Rb$^+$ uptake in KCC4- and KCC1-injected oocytes as a function of the concentration of each transported ion. The results of these series of experiments are depicted in Fig. 6. Uptakes were performed with K$^+$ or Cl$^-$ fixed at 50 mM, with changing concentrations of the counterion from 0 to 50 mM. Uptakes were also measured in water injected oocytes (data not shown), and the mean values for water groups were subtracted from corresponding KCC groups in order to assess only the $^{86}$Rb$^+$ uptake mediated by each heterologously expressed isoform. As shown in Fig. 1, $^{86}$Rb$^+$ uptake in water-injected oocytes was low, such that this correction was generally minor. In the case of KCC4, $^{86}$Rb$^+$ influx increased as the concentration of each transported ion was raised, until a plateau phase was reached at ion concentrations greater than 20–40 mM, compatible with Michaelis-Menten behavior. The calculated apparent $K_m$ and $V_{\text{max}}$ for extracellular K$^+$ concentration were 17.5 ± 2.7 mM and 32,370 ± 2115 pmol-oocyte$^{-1}$h$^{-1}$, respectively. The calculated apparent $K_m$ and $V_{\text{max}}$ values for extracellular Cl$^-$ concentration were 16.12 ± 4.2 mM and 41,440 ± 4174 pmol-oocyte$^{-1}$h$^{-1}$, respectively. The Hill coefficient for both ions remained close to unity.
1.08 ± 0.2 and 1.06 ± 0.3 for K⁺ and Cl⁻, respectively. KCC1 also exhibited a similar Michaelis-Menten behavior. The apparent $K_m$ and $V_{max}$ in KCC1 were 25.5 ± 3.2 mM and 39,540 ± 2199 pmol-oocyte⁻¹-h⁻¹ for extracellular K⁺ and 17.2 ± 8.3 mM and 14,930 ± 2822 pmol-oocyte⁻¹-h⁻¹ for Cl⁻. Hill coefficients for K⁺ (1.04 ± 0.13) and Cl⁻ (1.3 ± 0.5) in KCC1 also were close to unity.

**Anion Dependence of KCC4 and KCC1**—It has been shown that some extracellular anions other than Cl⁻ can support ion translocation through the K⁺-Cl⁻ cotransporter of both sheep and human erythrocytes (30). It was thus of interest to measure $^{86}$Rb⁺ transport by KCC4 and KCC1 in the presence of different anions. The $^{86}$Rb⁺ influx of KCC4- and KCC1-injected oocytes using an uptake solution containing 40 mM potassium gluconate and 10 mM KCl served as the reference activity for these experiments, as compared with uptake activity in oocytes exposed to medium containing 40 mM potassium gluconate and 10 mM of KBr, KH₂PO₄, KI, potassium gluconate, or KSCN. Fig. 7 shows the percentage of KCC4 (upper panel) and KCC1 (lower panel) function when uptakes were performed using these different anion substitutions. KCC4 shows the higher $^{86}$Rb⁺ influx in the presence of 10 mM KCl. $^{86}$Rb⁺ influx was still observed in the presence of other anions: 58 ± 9% with 10 mM KBr, 22 ± 5.9% with 10 mM KH₂PO₄, and 17 ± 3.8% with KI, whereas potassium gluconate and KSCN did not support transport. These results are in contrast to those observed in KCC1-injected oocytes, for which the order of anion-supported transport was Cl⁻ > SCN⁻ > Br⁻ > PO₄⁻³ > I > gluconate.

**Regulation of KCC4 and KCC1**—One of the most distinctive characteristics of K⁺-Cl⁻ cotransport in several cells and species is activation by the alkylating agent NEM (2). We therefore analyzed the effect of NEM on $^{86}$Rb⁺ influx in groups of oocytes under isotonic or hypotonic conditions. Again, in all of the experiments in which we assessed $^{86}$Rb⁺ influx in oocytes that were incubated in isotonic medium (210 mosmol/kg), the uptake observed in KCC4- or KCC1-injected oocytes was not different from the uptake in water-injected oocytes. However, the addition of 1 mM NEM in isotonic conditions resulted in a 5-fold activation of KCC4 (214 ± 12 pmol/oocyte⁻¹-h⁻¹) in the KCC4 control group versus 1062 ± 70 pmol/oocyte⁻¹-h⁻¹ in the NEM-treated group, p < 0.001) and a 2.6-fold activation of KCC1 (120 ± 27 versus 319 ± 76 pmol/oocyte⁻¹-h⁻¹, p < 0.05) (Fig. 8, A and B). Of note, when uptake were performed in hypotonic medium, the addition of NEM resulted in a dramatic inhibition of both isoforms (Fig. 8, C and D), such that $^{86}$Rb⁺
uptakes induced by KCC4 and KCC1 were reduced by 68 and 55%, respectively. In the same experiments, $^{86}\text{Rb}^+$ uptake due to the endogenous oocyte K\(^{-}\)-Cl\(^{-}\) cotransporter (H\(_2\)O-injected oocytes) was significantly increased when uptakes were done under both isotonic and hypotonic conditions (data not shown).

It has been known for some time that the inhibition of protein phosphatases prevents the activation of red cell K\(^{-}\)-Cl\(^{-}\) cotransport by either cell swelling or NEM. Since the role of phosphatases in the control of the cloned KCCs is unclear, we studied the effect of three inhibitors of protein phosphatases. We used 100 nM calyculin A, which inhibits the function of protein phosphatases 1 and 2A. The relative role of specific phosphatases was assessed using okadaic acid at 1 nM, a concentration that only affects protein phosphatase 2A, and cypermethrin at 100 pm, a concentration in which this compound inhibits the function of protein phosphatase 2B. As Fig. 9 shows, the addition of calyculin A completely prevents the activation of KCC4 and KCC1 by cell swelling. In contrast, neither okadaic acid, nor cypermethrin prevented this activation. These results indicate that protein phosphatase 1 is required for the activation of both KCC4 and KCC1 by cell swelling.

**DISCUSSION**

We have recently shown (16) that heterologous expression of the mouse KCC4 cDNA induced the expression of a $^{86}\text{Rb}^+$ influx pathway that is activated by cell swelling, dependent on the presence of extracellular Cl\(^{-}\) and inhibited by 2 mM of the loop diuretic furosemide. These data established that KCC4 functions as a K\(^{-}\)-Cl\(^{-}\) loop diuretic furosemide. These data established that KCC4

**FIG. 8.** Effect of 1 mM NEM on the $^{86}\text{Rb}^+$ uptake in KCC4-injected (A and C) and KCC1-injected (B and D) oocytes, under both isotonic (A and B) and hypotonic (C and D) conditions. Each bar represents the mean of 20 oocytes. Uptakes in the control conditions are shown in open bars, in the absence of extracellular Cl\(^{-}\) in black bars, and in the presence of NEM in hatched bars. *, p < 0.0001 versus control bars.

Fig. 9. Effect of the protein phosphatase inhibitors calyculin A (100 nM) (hatched bar), okadaic acid (1 nM) (black bar), and cypermethrin (100 nM) (gray bar) upon the swelling-induced activation of KCC4 or KCC1. In both panels, the white bar represents the control group, $^{86}\text{Rb}^+$ influx in hypotonic medium in the absence of inhibitor. Each bar represents the mean ± S.E. of at least 15 oocytes.

ners were markedly activated, albeit with a different magnitude (KCC4 > KCC1). These findings differ from previous observations (13, 15, 17, 19, 20) that indicate minimal hypotonic activation of KCC1, KCC2, and KCC3 when these cotransporters were expressed in HEK 293 cells, but agree with the cell swelling-induced activation of KCC1 found by Su et al. (18) using *Xenopus* oocytes as an expression system. Thus, when expressed in *Xenopus* oocytes, KCC4 and KCC1 cotransporters can be activated by cell swelling, suggesting that HEK 293 cells may not possess the appropriate signaling pathways for the activation of the cotransporters by swelling. In comparison, volume-regulated transport pathways recapitulate their *in vivo* physiology when expressed in *Xenopus* oocytes. Thus, shrinkage-activated transport pathways such as the Na\(^{+}\)-K\(^{-}\)-2Cl\(^{-}\) cotransporter (26) or the epithelial sodium channel ENaC (31) and swelling-activated pathways such as the calcium-activated intermediate K\(^{-}\) channel mIK1 (32) are regulated appropriately in this expression system. Of the four KCCs, KCC4 seems to be the isoform that exhibits the highest activation by hypotonicity. Influx mediated by KCC4 in this study was consistently higher than KCC1, although the amount of injected cRNA was equivalent. There are, however, several possible explanations for the greater hypotonic activation of KCC4. For example, KCC4 cRNA may be intrinsically more stable or better translated than KCC1 in oocytes. Of note, however, rat
KCC2 reportedly encodes a K⁺-Cl⁻ cotransporter with significant activity under isonicotic conditions when expressed in Xenopus oocytes and only minimal activation by cell swelling (21). Therefore, it is likely that much of the observed differences in volume sensitivity is due to variation in the structure of the four KCC proteins.

The two major loop diuretics inhibit KCC4, with an inhibitor sensitivity that is lower than that observed for KCC1. The reported effect of external potassium ([K⁺]e) on the inhibition of K⁺-Cl⁻ cotransport by loop diuretics (33) was observed for KCC4 but not KCC1. We observed a significantly different effect of furosemide and bumetanide on KCC4 at variable [K⁺]e, with the minimal and maximal effect at [K⁺]e of 2 and 6 mM, respectively. In contrast, no effect of [K⁺]e was observed for the inhibition of KCC1 by loop diuretics. This finding suggests that, as the isoform with the lower inhibitor affinity, the inhibition of KCC4 is more dependent on the positive effect of [K⁺]e on the interaction between the transporter protein and loop diuretics. Similar to our loop diuretic experiments, the anion transport inhibitor DIDS inhibited the function of KCC4 and KCC1, with an apparent Kᵢ that was dramatically lowered by an increase in [K⁺]e. Almost no effect was observed at 2 mM [K⁺]e, while in 50 mM [K⁺]e, °86Rb⁺ influx was completely blocked by a 100 µM DIDS concentration. This relationship between [K⁺]e and the inhibition of the K⁺-Cl⁻ cotransporter by DIDS was previously observed in low potassium sheep red blood cells (34) and was explained by the existence of two sites for K⁺ in the cotransporter: a modifier site and a transport site. Of interest, DIDS can also inhibit the function of the thiazide-sensitive Na⁺-Cl⁻ cotransporter but has no effect on the bumetanide-sensitive Na⁺-K⁺-2Cl⁻ cotransporter (26). The acid alkaloid DIOA, considered a specific inhibitor of red cell K⁺-Cl⁻ cotransporter (27), also inhibited KCC4 and KCC1. However, in contrast to DIDS and loop diuretics, all of which primarily inhibit anion transporters and exchangers (35, 36), the higher the [K⁺]e, the lower the efficacy of DIOA. However, even in a very high [K⁺]e (50 mM), the inhibition of °86Rb⁺ influx by 100 µM DIOA was still greater than 50%.

The increased °86Rb⁺ uptake induced by KCC4 was also inhibited by about 20% in low and 40% in high [K⁺]e, by 2 mM concentration of the thiazide-diuretic trichlormethiazide. The members of the electroneutral cation chloride coupled cotransporters have been defined in part due to their sensitivity to diuretics. The Na⁺-K⁺-2Cl⁻ cotransporters are sensitive to loop diuretics, derivatives of sulfamoylbenzoic acid, and resistant to the benzothiadiazine derivatives, whereas the Na⁺-Cl⁻ cotransporter is inhibited by thiazides but not affected by loop diuretics (12). Our results suggest that KCC4, which exhibits a low degree of identity with the sodium-dependent cation-chloride cotransporters (~22%), can be inhibited not only by loop diuretics but also by thiazide-type diuretics. A similar observation has been reported by Harling et al. (37), who showed that the plant cation-chloride cotransporter AXI 4, which exhibits the highest sequence identity with the KCCs (36–38%), can also be inhibited by bumetanide, furosemide, and the thiazide-like diuretic metolazone.

We have found that KCC4 and KCC1 can be blocked by the addition of 10 mM BaCl₂ to the uptake medium, with a relative sensitivity of KCC4 > KCC1. Although red cell K⁺-Cl⁻ cotransport is at least partially sensitive to quinidine derivatives (38), this is the first indication that the cloned K⁺-Cl⁻ cotransporters are directly sensitive to BaCl₂. This observation is also consistent with the controversial proposal by Greger and Schlatter (7) that the basolateral membrane of renal thick ascending limb cells contains a barium-sensitive K⁺-Cl⁻ cotransporter. The observation by Amlal et al. (8) that a thick limb chloride-dependent, barium-sensitive NH₄⁺ transport mechanism is only modestly sensitive to furosemide suggests that KCC4 is the isoform present in the basolateral membrane of the mammalian thick ascending limb. In this regard, Liapis et al. (39) failed to detect KCC1 mRNA in human thick ascending limb.

Kinetic analyses reveal that KCC4 and KCC1 exhibit very similar affinities for extracellular Cl⁻ (Kₑ values in 16.1 ± 4.2 and 17.2 ± 8.3 mM, respectively), whereas the differences in the affinity for extracellular K⁺ approached statistical significance (Kᵢ values 17.5 ± 2.7 and 25.5 ± 3.2 mM, respectively; p = 0.08). Our results for KCC1 agree with those reported by Gillen et al. (13). It is known that the central core of 12 transmembrane (TM) segments determines the kinetic properties of the cation-chloride cotransporters (40). In this regard, the otherwise identical TM segments of the four KCC proteins differ primarily at amino acid residues within TM2, TM4, and TM7. An elegant series of studies have implicated TM2 in the determination of cation (Na⁺ and K⁺) affinity in the Na⁺-K⁺-2Cl⁻ cotransporters, whereas residues within TM4 and TM7 appear to affect anion affinity (23, 24, 40, 41). However, sequence comparisons of the entire gene family indicate that only TM7 is particularly conserved between the Na⁺-dependent and Na⁺-independent cation-chloride cotransporters; thus, these observations may not translate to the K⁺-Cl⁻ cotransporters. Of particular significance, however, the reported affinity of KCC2 for K⁺ (~5 mM) is closer to that of KCC4 than KCC1, and KCC2 shares significant identity with KCC4 within TM2. Therefore, as in the Na⁺-K⁺-2Cl⁻ cotransporters, TM2 may play a major role in the determination of cation affinity. The K⁺ affinity of individual K⁺-Cl⁻ cotransporters may be of major physiological significance, since under conditions wherein extracellular K⁺ increases, such as cardiac ischemia (9) and neuronal activity (4), the higher affinity KCCs may function as K⁺-Cl⁻ influx pathways.

KCC4 and KCC1 exhibit surprisingly similar affinities for chloride. However, they do differ in another parameter of anion transport, the anion selectivity or “anion-series” of K⁺-Cl⁻ cotransport. KCC4 and KCC1 thus differ in the profile of °86Rb⁺ transport that can be sustained by different anions. In KCC4, about 50% of the function can be observed in the presence of Br⁻, and some transport is still present with PO₄³⁻ whereas in KCC1 70% of °86Rb⁺ uptake can be obtained in the absence of Cl⁻, while when either Br⁻ or SCN⁻ are present in the extracellular medium. Whether these differences are encoded by subtle variation in TM4 and TM7 will require further study; however, differences in anion selectivity were crucial for the identification of the anion channel pore in the CLC chloride channels (42, 43).

The functional properties of KCC4 and KCC1 observed in the present study suggest that it is unlikely that either of these isoforms is the predominant K⁺-Cl⁻ cotransporter expressed in red blood cells. On the one hand, Delpire and Lauf (44) observed that the K⁺-Cl⁻ cotransporter in hyposmotically swollen low K sheep erythrocytes exhibited a Kᵢ for extracellular K⁺ of ~55 mM, which is very different from the Kᵢ obtained in the present study for KCC4 and KCC1. It has also been shown in sheep red blood cells (45) that K⁺ influx is higher in the presence of Br⁻ than in the presence of Cl⁻; neither KCC4 nor KCC1 exhibited this behavior. Moreover, although it has been shown that KCC1 mRNA is expressed in mouse erythrocytes, it is not present in circulating reticulocytes (46), and the KCC2 isoform is expressed exclusively in the central nervous system (14). Thus, taking all the information together, our kinetic and anion substitution experiments suggest that the major K⁺-Cl⁻ cotransporter in erythrocytes is either KCC3 or...
an as-yet-unidentified isoform.

K⁺-Cl⁻ cotransport was initially defined as a red cell transport pathway that is activated by the alkylation agent NEM. Multiple laboratories have since found that pretreatment of erythrocytes from several species with 1 mM NEM results in significant activation of this Cl⁻-dependent K⁺ transport pathway (2). It is still unclear if the activating effect of NEM is related to NEM-induced dephosphorylation, via activation of an upstream kinase, or to direct modification of the thiol groups on the cotransporter. There are reports supporting both possibilities (47) (for reviews, see Refs. 2 and 3). However, like the activation by cell swelling, NEM-activated transport is prevented by phosphatase inhibitors, suggesting a positive effect of NEM on upstream signaling pathways (48–52). In this regard, our data show a very interesting behavior of the KCCs when exposed to NEM. Under isotonic conditions, NEM stimulated the function of KCC4 and KCC1, as well as the endogenous K⁺-Cl⁻ cotransporter of the oocytes. Su et al. (18) also observed KCC1 activation by NEM in oocytes. In contrast, in hypotonic conditions, when we exposed oocytes to NEM, both KCC4 and KCC1 were inhibited. The fact that KCC4 and KCC1 were activated by NEM in isotonic conditions suggests that oocytes possess the intracellular pathways that NEM requires for activation of the cotransporters. The mechanisms by which swelling-activated KCC4 and KCC1 are inhibited by NEM are still unclear. It has been shown in sheep red cells that K⁺-Cl⁻ cotransporters can be activated or inhibited by NEM through high and low affinity stimulatory thiols, respectively (47, 53, 54). Further experiments will be required to clarify this issue; however, a reconciliation of these and previous observations is that there are direct inhibitory sites on the transporter proteins themselves and stimulatory sites on upstream kinases. In this regard, there are several transmembrane or juxtamembrane cysteines in the predicted KCC proteins, and transmembrane cysteines were recently implicated in the differential sensitivity of Na⁺-K⁺-2Cl⁻ cotransporters to cysteine-reactive compounds (55).

Over the last decade or so, several laboratories have suggested that dephosphorylation of the K⁺-Cl⁻ cotransporter is required for its activation, since inhibition of protein phosphatases prevents the swelling- and NEM-induced activation (48–50, 56, 57). Our data support this hypothesis, since the protein phosphatase inhibitor calyculin A completely abrogates hypotonic activation of KCC4 and KCC1. Calyculin A is known to inhibit both protein phosphatase 1 and 2A (58). To discriminate between phosphatases, we also tested the effect of okadaic acid in a concentration of 1 nM, which inhibits only protein phosphatase 2B, and cypermethrin, which inhibits only protein phosphatase 2A, and protein phosphatase 1 also is responsible for activating the basolateral Na⁺-K⁺-ATPase (63); protein phosphatase 1 may thus function to couple the pump to basolateral Cl⁻ and K⁺ exit through the KCCs. Of more specific relevance to K⁺-Cl⁻ cotransport, the combined data for NEM, phosphatase inhibition, and cell swelling dissociates for the first time these various control points for this transport pathway.

In conclusion, we have found significant regulatory, kinetic, and pharmacological differences between KCC4 and KCC1.

Despite differences in their relative activation by cell swelling, KCC4 and KCC1 share a requirement for dephosphorylation by a protein phosphatase for swelling-induced activity. As previously shown for K⁺-Cl⁻ cotransport in red cells (48, 50, 56, 64), the relevant protein phosphatase is probably protein phosphatase 1. The two KCC isoforms in this study differ slightly in affinity for K⁺, presumably due to variation within transmembrane 2, a region of the cation-chloride cotransporter proteins previously implicated in cation affinity (22–24). Ion affinity may also be of physiological relevance, in that lower affinity K⁺-Cl⁻ cotransporters, such as KCC1 and the red cell K⁺-Cl⁻ cotransporter (potentially KCC3), may function exclusively as efflux mechanisms. As proposed initially by Payne (19), the higher affinity isoforms (KCC2 and now KCC4) may function as both efflux and influx pathways. Such a duality has been verified experimentally in neurons (65), where synaptic activity may increase extracellular K⁺ to the point that KCC2 mediates K⁺-Cl⁻ influx. KCC4 transcript is in turn particularly abundant in heart, where K⁺ efflux during ischemia appears to involve K⁺-Cl⁻ cotransport (9); again, a higher affinity isoform may play a role in reclaiming this intracellular K⁺ in the postischemic myocardium. Finally, the pharmacological characterization of KCC4 fulfills the prediction, based on observations of the physiology of renal thick ascending limb cells (7, 66), that K⁺-Cl⁻ cotransporters may be sensitive to barium, widely considered a specific inhibitor of K⁺ channels.

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