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Functional interaction of the K-Cl cotransporter (KCC1) with the Na-K-Cl cotransporter in HEK-293 cells

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Gillen, Christopher M., and Bliss Forbush III. Functional interaction of the K-Cl cotransporter (KCC1) with the Na-K-Cl cotransporter in HEK-293 cells. *Am. J. Physiol. Cell Physiol.* 45: C328–C336, 1999.—We have studied the regulation of the K-Cl cotransporter KCC1 and its functional interaction with the Na-K-Cl cotransporter. K-Cl cotransporter activity was substantially activated in HEK-293 cells overexpressing KCC1 (KCC1-HEK) by hypotonic cell swelling, 50 mM external K, and pretreatment with *N*-ethylmaleimide (NEM). Bumetanide inhibited ^{86}Rb efflux in KCC1-HEK cells after cell swelling [inhibition constant (K_i) \sim 190 μM] and pretreatment with NEM ($K_i \sim$ 60 μM). Thus regulation of KCC1 is consistent with properties of the red cell K-Cl cotransporter. To investigate functional interactions between K-Cl and Na-K-Cl cotransporters, we studied the relationship between Na-K-Cl cotransporter activation and intracellular Cl concentration ($[\text{Cl}]_i$). Without stimulation, KCC1-HEK cells had greater Na-K-Cl cotransporter activity than controls. Endogenous Na-K-Cl cotransporter of KCC1-HEK cells was activated <2 -fold by low-Cl hypotonic prestimulation, compared with 10-fold activation in HEK-293 cells and >20 -fold activation in cells overexpressing the Na-K-Cl cotransporter (NKCC1-HEK). KCC1-HEK cells had lower resting $[\text{Cl}]_i$ than HEK-293 cells; cell volume was not different among cell lines. We found a steep relationship between $[\text{Cl}]_i$ and Na-K-Cl cotransport activity within the physiological range, supporting a primary role for $[\text{Cl}]_i$ in activation of Na-K-Cl cotransport and in apical-basolateral cross talk in ion-transporting epithelia.

intracellular chloride; epithelial transport; cell volume

THE SODIUM-POTASSIUM-CHLORIDE and potassium-chloride cotransporters are structurally related ion transport proteins that mediate electrically neutral transport of monovalent cations and Cl ions across plasma membranes (11, 20, 30). Two isoforms of each transporter have been cloned. The Na-K-Cl cotransporter NKCC1 is widely expressed in mammalian tissue (4, 32), whereas NKCC2 is kidney specific (7, 29). KCC1 is a widely expressed K-Cl cotransporter (8), whereas KCC2 is brain specific (28, 31). Na-K-Cl and K-Cl cotransporters are both inhibited by the sulfamoylbenzoic acid "loop" diuretics. However, bumetanide is a less effective inhibitor of the K-Cl cotransporter [inhibition constant (K_i) \sim 50–200 μM] than of the Na-K-Cl cotransporter ($K_i < 1 \mu\text{M}$) (11, 20). Bumetanide sensitivity can be used to differentiate between Na-K-Cl and K-Cl cotransporter activity in functional assays.

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In most cell types, Na-K-Cl and K-Cl cotransporters have opposing roles in cell volume regulation. The Na-K-Cl cotransporter mediates net influx of ions into cells under normal physiological conditions because of the inwardly directed chemical gradients for Na and Cl ions (11). In contrast, the well-studied red cell K-Cl cotransporter mediates net efflux of K and Cl from cells under normal conditions (20). Reciprocal regulation of the Na-K-Cl and K-Cl cotransporters might be expected based on their opposing physiological roles (14), and substantial evidence supports this hypothesis. For example, cell swelling is known to activate the K-Cl cotransporter in red blood cells and other tissues, whereas cell shrinkage activates the Na-K-Cl cotransporter in many cell types (11, 20).

The Na-K-Cl cotransporter is activated by agents that stimulate intracellular kinase activity or reduce phosphatase activity. An increase in the net phosphate content of the Na-K-Cl cotransporter accompanies its activation, demonstrating that phosphorylation of the cotransporter protein itself is an important event in its activation (13, 21, 23, 24). In contrast, the K-Cl cotransporter is inhibited by phosphatase inhibitors and activated by kinase inhibitors. Recent evidence suggests that activation of the K-Cl cotransporter by the sulfhydryl group reagent *N*-ethylmaleimide (NEM) occurs through the inactivation of a kinase (6, 18). Whether the K-Cl cotransporter itself is directly phosphorylated is not known.

Several models for transepithelial NaCl transport [e.g., that of Greger and Schlatter (9)] include Na-K-Cl and K-Cl cotransporters on opposing epithelial cell membranes. In these models, coordinated activation of the transporters is essential to achieving efficient transepithelial Cl movement. In many cell types, a reduction in intracellular Cl concentration ($[\text{Cl}]_i$) activates the Na-K-Cl cotransporter (3, 11, 22, 24), and activation of Na-K-Cl cotransport by reduced $[\text{Cl}]_i$ has been suggested as an important process in the coupling of apical and basolateral Cl fluxes in ion-transporting epithelia. Molecular cloning of the Na-K-Cl and K-Cl cotransporters has provided further opportunities to test this hypothesis.

The K-Cl cotransporter (KCC1) is widely expressed in mammalian tissues, including erythroid cells (33). This expression pattern, along with kinetic data (8), suggests that KCC1 may represent the widely studied red cell K-Cl cotransporter. In this paper, we report characterization of Na-K-Cl and K-Cl cotransporter activity in human embryonic kidney (HEK-293) cells overexpressing KCC1 or the human secretory Na-K-Cl cotransporter (NKCC1). We have examined regulation of KCC1, testing the hypothesis that regulation of

KCC1 is the same as regulation of the red cell K-Cl cotransporter. We have also studied the interaction between K-Cl and Na-K-Cl cotransporter activity, examining the possible role of $[Cl]_i$ in mediating a functional interaction between the K-Cl and Na-K-Cl cotransporters.

METHODS

Cell lines. Three cell lines were studied; control human embryonic kidney cells (HEK-293), HEK-293 cells transfected with the human secretory Na-K-Cl cotransporter (NKCC1-HEK), and HEK-293 cells transfected with the rabbit K-Cl cotransporter KCC1 (KCC1-HEK). KCC1-HEK cells express epitope-tagged (*c-myc*) KCC1; details of the production and initial characterization of this cell line have been described (8). Four separate clonal KCC1-HEK cell lines were studied in this paper; no qualitative differences were measured among these cell lines. We have found that KCC1 expression (measured by functional assays or by Western blotting) decreases in long-term cell culture; we have not observed this with NKCC1 expression (16). Therefore, we periodically assayed KCC1-HEK cells for KCC1 expression by Western blotting with a monoclonal antibody against the *c-myc* epitope (5), and we returned to original stocks when expression level decreased. NKCC1-HEK cells were generated as previously described (32), and expression of NKCC1 was verified by Western blotting with the T4 monoclonal antibody (27).

Cells were maintained under standard growth conditions in a humidified incubator in DMEM, 10% fetal bovine serum, 50 U/ml penicillin, 50 μ g/ml streptomycin, and 900 μ g/ml G418 (omitted in control HEK-293 cells) at 37°C and 5% CO₂. Cells were plated at a 1:5 or 1:10 dilution onto poly-D-lysine-coated 6-well or 96-well plates 3–5 days before an experiment. Experiments were performed with fully confluent cells.

⁸⁶Rb efflux assays. Efflux experiments were done as described in detail elsewhere (8). Cells were incubated for 60 min at ~22°C in tissue culture medium (DMEM, no additives) containing 2 μ Ci/ml ⁸⁶RbCl, cells were washed four times with PBS, and fresh efflux medium (in mM: 70 *N*-methyl-D-glucamine gluconate, 65 NaCl, 5 KCl, 1 CaCl₂, 1 Na₂HPO₄, 2 Na₂SO₄, 15 HEPES, pH 7.4, and 0.1 ouabain) was added to the cells. Hypotonic efflux medium was made by removing *N*-methyl-D-glucamine gluconate from the standard efflux medium, resulting in a solution with a calculated osmolality of ~50% of standard efflux medium. *N*-methyl-D-glucamine gluconate was replaced with KCl to achieve 50 mM K efflux medium. After a 10-min incubation, efflux medium was removed and assayed for ⁸⁶Rb content as Cerenkov radiation. Cells were lysed with 2% SDS and assayed for ⁸⁶Rb. Efflux was calculated as a percentage of total cellular ⁸⁶Rb (⁸⁶Rb content of medium + ⁸⁶Rb content of cells). For inhibition curves, bumetanide was added to the efflux medium; no preincubation with the inhibitor was used. When used, NEM was added to the loading medium 15 min before the end of the preincubation. Hypotonic and 50 mM K efflux media were added at the beginning of the flux period; no preincubation in these media was used. To determine whether any component of the loop-diuretic-sensitive loss was due to cell lysis rather than ⁸⁶Rb transport, we measured [¹⁴C]urea efflux. No furosemide-sensitive [¹⁴C]urea loss was observed from cells in regular efflux medium, hypotonic efflux medium, or 50 mM K efflux medium (data not shown).

⁸⁶Rb influx assays. Influx experiments to assay function of the Na-K-Cl cotransporter were done as described in detail elsewhere (32, 37), using cells plated onto 96-well dishes.

Experiments were done at ~22°C. Cells were preincubated for 1 h in standard preincubation medium (in mM: 135 NaCl, 5 KCl, 1 CaCl₂, 1 Na₂HPO₄, 2 Na₂SO₄, and 15 HEPES, pH 7.4). Extracellular Cl concentration ($[Cl]_o$) was varied by replacement with gluconate. To stimulate Na-K-Cl cotransport, cells were preincubated in hypotonic low-Cl preincubation medium (1:2 dilution of preincubation medium with water; Cl replaced with gluconate to achieve final Cl concentration of 3 mM). Influx was measured during a timed exposure of the cells to regular influx medium (in mM: 135 NaCl, 5 RbCl, 1 CaCl₂, 1 Na₂HPO₄, 2 Na₂SO₄, 15 HEPES, pH 7.4, and 0.1 ouabain) containing 1 μ Ci/ml ⁸⁶RbCl. Cells were washed five times with an ice-cold high-K solution containing 250 μ M bumetanide and lysed with 2% SDS. ⁸⁶Rb content was measured as Cerenkov radiation, and protein content was determined colorimetrically (Pierce, Microassay).

Cell volume, $[Cl]_i$, and K content. Cell volume, $[Cl]_i$, and K content were measured in cells plated onto six-well dishes. Cell volume was measured as [¹⁴C]urea space. Cells were incubated for 45 min in medium containing 1 μ Ci/ml [¹⁴C]urea (preliminary experiments showed equilibration of [¹⁴C]urea and ³⁶Cl to be complete after ~20 min), washed four times rapidly with ice-cold PBS, and lysed with 2% SDS or distilled water. [¹⁴C]urea content was determined by scintillation counting. Cell volumes were not corrected for trapped water. Thus reported values may be small overestimates of actual cell volumes. Cl content was measured in parallel to cell volume, using the same protocol. Incubation medium contained 1 μ Ci/ml ³⁶Cl. $[Cl]_i$ was calculated as Cl content per cell volume. To determine K content, cells were washed four times rapidly with K-free PBS and lysed overnight in distilled water. K concentration of the lysate was measured by flame photometry. Protein concentration was determined in each sample (Pierce Microassay), and reported cell volume measurements are corrected for protein content.

Statistics. All values are expressed as means \pm SE. Comparisons between cell lines were made using *t*-tests. We found considerable day-to-day variability in measurements of $[Cl]_i$ and cell volume. Thus we calculated differences between HEK-293 cells and transfected cells (KCC1-HEK and NKCC1-HEK) grown on the same 6-well plate and analyzed the statistical significance of these differences in an ANOVA.

RESULTS

Activation of KCC1. In a previous study (8), we showed KCC1-HEK cells have substantial K-Cl cotransporter activity after pretreatment with NEM or after hypotonic cell swelling. However, the magnitude of KCC1 activation in previous experiments was difficult to determine, largely because of substantial contribution of Na-K-Cl cotransport to resting ⁸⁶Rb fluxes. In this study, we accounted for a contribution of endogenous Na-K-Cl cotransporter to the observed ⁸⁶Rb fluxes, using 10 μ M bumetanide to abolish endogenous Na-K-Cl cotransport. This refinement is especially important because of the significantly elevated ⁸⁶Rb transport through the endogenous Na-K-Cl cotransporter in KCC1-HEK cells (see Fig. 4). In these studies, we used 2 mM furosemide as a general inhibitor of Na-K-Cl and K-Cl cotransporter; this concentration of furosemide completely abolishes K-Cl cotransport in influx (8) and efflux experiments (data not shown).

Activation of ⁸⁶Rb efflux by cell swelling. Bumetanide inhibited ⁸⁶Rb efflux from KCC1-HEK cells swollen in hypotonic medium with a K_i of $188 \pm 18 \mu$ M ($n = 5$;

Fig. 1). To assess the activation of KCC1 by cell swelling, we performed experiments in the presence of 10 μM bumetanide, which completely inhibits Na-K-Cl cotransport but does not affect K-Cl cotransport (based on preliminary experiments). Hypotonic swelling produced a large activation of furosemide-sensitive K-Cl cotransport in KCC1-HEK cells and no activation in control cells (Fig. 2). Furosemide-sensitive K-Cl cotransport in KCC1-HEK cells was also activated in medium containing 50 mM K. This effect may be due to cell swelling or to an increase in intracellular K concentration ($[\text{K}]_i$) and $[\text{Cl}]_i$. Furosemide-insensitive ^{86}Rb efflux was unchanged in hypotonic and reduced in 50 mM K medium (Fig. 2), indicating that these treatments produced specific activation of K-Cl cotransport, rather than nonspecific ^{86}Rb loss.

Activation of ^{86}Rb efflux by NEM. Bumetanide inhibited ^{86}Rb efflux in KCC1-HEK cells pretreated with 1 mM NEM with a K_i of $55 \pm 12 \mu\text{M}$ ($n = 6$; Fig. 3, top). The K_i for bumetanide inhibition of K efflux from KCC1-HEK cells was lower after NEM pretreatment ($\sim 60 \mu\text{M}$) compared with cell swelling in hypotonic medium ($\sim 190 \mu\text{M}$). Although the above data are from separate experimental series, the difference was also present in a side-by-side experiment in which external K concentration ($[\text{K}]_o$) and $[\text{Cl}]_o$ were identical between the two conditions (data not shown).

In control cells, no bumetanide-inhibitable ^{86}Rb efflux was observed after NEM pretreatment (Fig. 3, top). In ^{86}Rb efflux experiments performed in the presence of 10 μM bumetanide, a large activation of ^{86}Rb efflux is observed after NEM pretreatment in KCC1-HEK cells but not in controls (Fig. 3, bottom). Thus NEM activates the transfected cotransporter in KCC1-HEK cells.

Inhibitors of K-Cl cotransport. We tested the effect of several compounds reported to inhibit K-Cl cotransport on ^{86}Rb transport in KCC1-HEK cells. Previous work has shown that blockers of protein phosphatases inhibit K-Cl cotransporter activity in red blood cells.

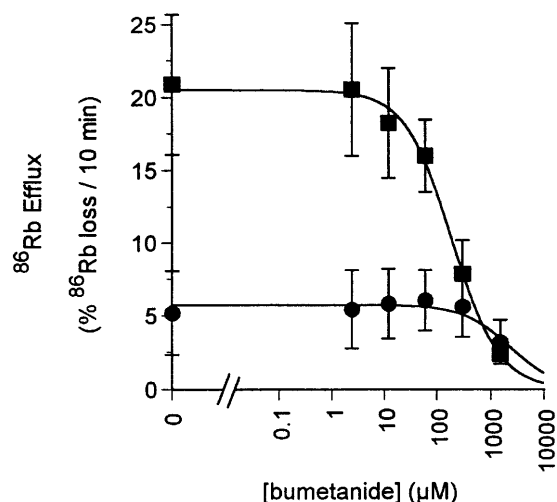


Fig. 1. Effect of cell swelling on ^{86}Rb efflux in KCC1-HEK cells. Inhibition by bumetanide of fractional ^{86}Rb loss (% of total cellular ^{86}Rb during a 10-min incubation) after hypotonic cell swelling is plotted. ■, KCC1-HEK cells; ●, HEK-293 cells. Values are means \pm SE ($n = 5$).

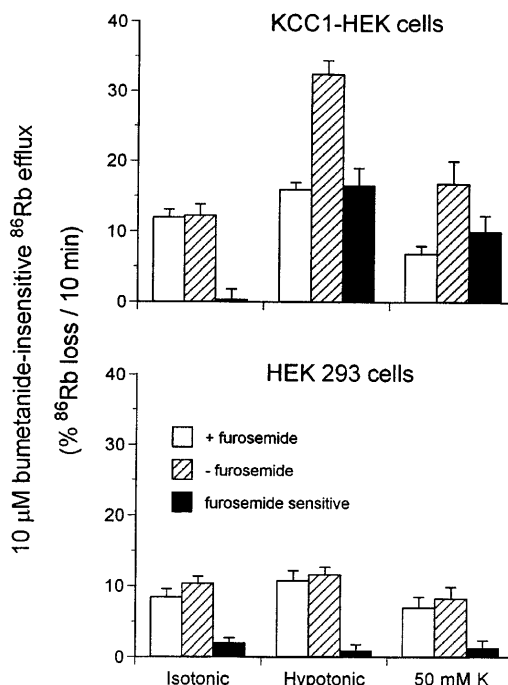


Fig. 2. Top: fractional ^{86}Rb loss from KCC1-HEK cells in presence of 10 μM bumetanide in 3 different conditions (isotonic, hypotonic, and 50 mM external K; see METHODS for composition of these media). Open bars, efflux with 2 mM furosemide present; hatched bars, efflux in absence of furosemide; solid bars, furosemide-sensitive efflux. Bottom: fractional ^{86}Rb loss from HEK-293 cells in presence of 10 μM bumetanide in same conditions as top. Values are means \pm SE ($n = 5$).

Calyculin A, an inhibitor of type 1 and type 2A protein phosphatases, inhibits activation of K-Cl cotransport by staurosporine and low Mg concentration in sheep red blood cells and by swelling in rabbit red blood cells (2, 35). Consistent with these studies, calyculin A (0.5 μM , 10-min preincubation) inhibited $52 \pm 13\%$ ($n = 3$) of the swelling-activated ^{86}Rb efflux in KCC1-HEK cells.

Mercury chloride inhibits the shark rectal gland cotransporter, sNKCC1, with a K_i of $\sim 25 \mu\text{M}$ and inhibits mammalian NKCC isoforms with lower affinities (50–200 μM) (17). In this study, mercury chloride inhibited swelling-activated ^{86}Rb efflux in KCC1-HEK cells with a K_i of 12–15 μM ($n = 2$). The relatively strong inhibition of mammalian KCC1 compared with mammalian NKCC1 by inorganic mercury presumably reflects the distribution of cysteine residues in the transporter proteins. It is interesting that there is a series of four consecutive cysteine residues in the predicted second transmembrane domain of KCC1; these cysteines are conserved among mammalian KCC1 isoforms but are not present in NKCC proteins.

The alkanolic acid [(dihydroindenyl)oxy]alkanoic acid (DIOA) has been proposed as a specific inhibitor of K-Cl cotransport (36). DIOA inhibited NEM-stimulated ^{86}Rb influx in KCC1-HEK cells with a K_i of $57 \pm 21 \mu\text{M}$ ($n = 5$). However, DIOA also inhibited Na-K-Cl cotransport in control cell lines with a K_i of $23 \pm 12 \mu\text{M}$ ($n = 3$), indicating that DIOA is not a specific inhibitor of K-Cl cotransport in HEK-293 cells.

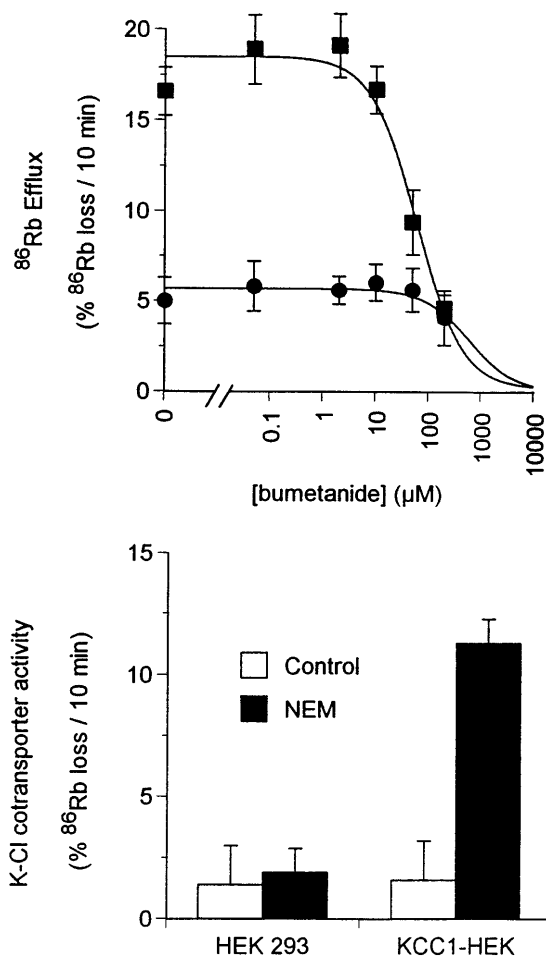


Fig. 3. Effect of *N*-ethylmaleimide (NEM) on ⁸⁶Rb efflux in KCC1-HEK cells. *Top*: inhibition by bumetanide of fractional ⁸⁶Rb loss (% of total cellular ⁸⁶Rb during a 10-min incubation) after a 15-min pretreatment with 1 mM NEM. ■, KCC1-HEK cells; ●, HEK-293 cells. *Bottom*: furosemide-sensitive (2 mM) fractional ⁸⁶Rb loss from KCC1-HEK and HEK-293 cells in presence of 10 μM bumetanide. Control, efflux in absence of pretreatment; NEM, efflux after a 15-min pretreatment with 1 mM NEM. Values are means ± SE (*n* = 6).

Activation of Na-K-Cl cotransporter by low-Cl hypotonic preincubation. Because Na-K-Cl cotransport and K-Cl cotransport have opposing roles in cell volume and intracellular Cl regulation, we investigated whether overexpression of KCC1 in HEK-293 cells influences activity of endogenous and expressed Na-K-Cl cotransporters. Na-K-Cl cotransporter activity (functionally defined as Na-dependent and 10 μM bumetanide-sensitive ⁸⁶Rb influx) was studied in KCC1-HEK, NKCC1-HEK, and control HEK-293 cells. Reduction of [Cl]_i by preincubation in hypotonic low-Cl medium (see ⁸⁶Rb influx assays) produced activation of Na-K-Cl cotransporter by ~10-fold above control in untransfected HEK-293 cells and >20-fold above control in NKCC1-HEK (*n* = 3; Fig. 4). This result confirms previous findings (32, 37) and supports the importance of [Cl]_i in activation of Na-K-Cl cotransport. Compared with control cells, KCC1-HEK cells showed a significantly increased level of baseline Na-K-Cl cotransporter activity (unpaired *t*-test, *P* < 0.05) and much smaller activation of endogenous Na-K-Cl cotrans-

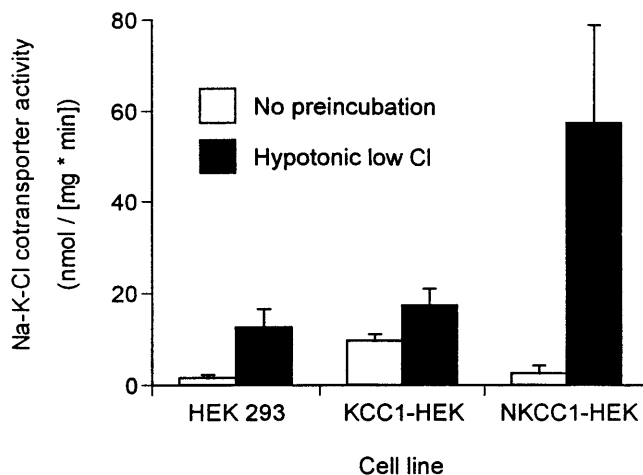


Fig. 4. Na-dependent ⁸⁶Rb uptake (nmol · mg⁻¹ · min⁻¹) into HEK-293 (*n* = 3), KCC1-HEK (*n* = 5), and NKCC1-HEK (*n* = 3) cells is plotted. Bars represent bumetanide-sensitive (200 μM) component of Na-dependent ⁸⁶Rb uptake in absence of pretreatment or after a 1-h preincubation in low-Cl, hypotonic medium (see METHODS). Values are means ± SE.

porter activity after hypotonic low-Cl preincubation. The average activation of Na-K-Cl cotransport by hypotonic low-Cl preincubation in KCC1-HEK cells was less than twofold (*n* = 5). Thus the majority of endogenous Na-K-Cl cotransporter in KCC1-HEK cells is activated in the absence of exogenous stimulation.

Na-K-Cl cotransport in efflux experiments. Without pretreatment, bumetanide-sensitive ⁸⁶Rb efflux in HEK-293 and KCC1-HEK cells was predominantly through the Na-K-Cl cotransporter; bumetanide inhibited efflux with a *K*_i of <1 μM (Fig. 5). Consistent with our measurements of Na-K-Cl cotransporter-mediated ⁸⁶Rb influx (Fig. 4), we observed larger Na-K-Cl-mediated

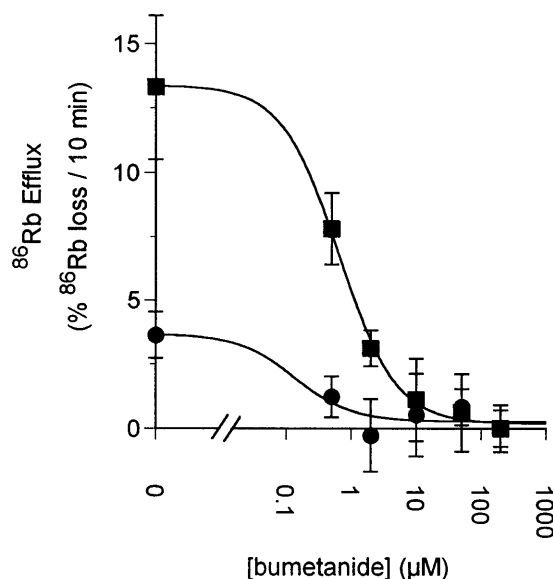


Fig. 5. Inhibition by bumetanide of fractional ⁸⁶Rb loss (% of total cellular ⁸⁶Rb during a 10-min incubation) in absence of prestimulation is plotted. ■, KCC1-HEK cells; ●, HEK-293 cells. Values are means ± SE (*n* = 6).

^{86}Rb efflux in the absence of pretreatment in KCC1-HEK cells than in HEK-293 cells.

[Cl]_i of transfected HEK-293 cells. Because a reduction in $[\text{Cl}]_i$ is known to activate Na-K-Cl cotransporter activity in HEK-293 cells, we investigated the possibility that elevated Na-K-Cl cotransporter activity in unstimulated KCC1-HEK cells is a response to reduced $[\text{Cl}]_i$ caused by KCC1 activity. Resting $[\text{Cl}]_i$ was 42 ± 3 mM in KCC1-HEK cells, 48 ± 4 mM in NKCC1-HEK cells, and 45 ± 4 mM in control HEK-293 cells. In 12 experiments, $[\text{Cl}]_i$ of KCC1-HEK cells was 3.1 ± 1.2 mM lower than control cells ($P < 0.05$), and $[\text{Cl}]_i$ of NKCC1-HEK cells was 3.3 ± 1.6 mM higher than control cells ($P < 0.05$).

Cell volume of transfected HEK-293 cells. Cell volume was 6.5 ± 0.3 $\mu\text{l}/\text{mg}$ protein in control HEK-293 cells, 6.1 ± 0.3 $\mu\text{l}/\text{mg}$ protein in NKCC1-HEK cells, and 7.2 ± 0.6 $\mu\text{l}/\text{mg}$ protein in KCC1-HEK ($n = 8$). These differences were not significant when analyzed in the same manner as for the $[\text{Cl}]_i$ measurements (the sample size for this measurement is smaller than for our $[\text{Cl}]_i$ measurements because protein content was not determined for 4 trials). It is worth noting that large changes in cell volume produce only small changes in activation of the endogenous Na-K-Cl cotransporter in HEK-293 cells, even when $[\text{Cl}]_o$ is reduced (17). These measurements of $[\text{Cl}]_i$ and cell volume are consistent with the hypothesis that reduced $[\text{Cl}]_i$ is responsible for the observed activation in Na-K-Cl cotransporter activity of KCC1-HEK cells.

Relationship between $[\text{Cl}]_o$ and Na-K-Cl cotransporter activity. We further evaluated the differences in Na-K-Cl cotransporter activation among these cell lines by measuring Na-K-Cl activity in cells preincubated for 60 min in isotonic media with a range of Cl concentrations (Fig. 6). Consistent with the results in Fig. 4, fractional activation of the endogenous Na-K-Cl cotransporter without prestimulation was greater in KCC1-HEK cells than in HEK-293 cells. Additionally, in the absence of stimulation, the fractional activation of Na-K-Cl cotransporter in NKCC1 cells was less than that of HEK-293 cells. This result is consistent with Fig. 4 and with previous studies (17). Significant stimulation of Na-K-Cl cotransport in all three cell lines required preincubation with < 50 mM $[\text{Cl}]_o$.

Relationship between $[\text{Cl}]_i$ and Na-K-Cl cotransporter activity. To determine the quantitative relationship between $[\text{Cl}]_i$ and Na-K-Cl cotransporter activity, we measured $[\text{Cl}]_i$ in the control condition (140 mM Cl), in the maximally stimulated condition (3 mM Cl), and at the inflection point in the activation curves (31 mM Cl) (Figs. 6 and 7). Incubation in isotonic medium with 31 mM Cl produced small reductions in $[\text{Cl}]_i$, whereas incubation in 3 mM Cl produced large reductions in $[\text{Cl}]_i$. The relationship between $[\text{Cl}]_i$ and activation of Na-K-Cl cotransport was similar for KCC1-HEK cells and HEK-293 cells (Fig. 7). Importantly, small reductions in $[\text{Cl}]_i$ resulted in substantial increases in Na-K-Cl cotransporter activation.

The relationship between $[\text{Cl}]_i$ and Na-K-Cl cotransporter activation was significantly different in NKCC1-

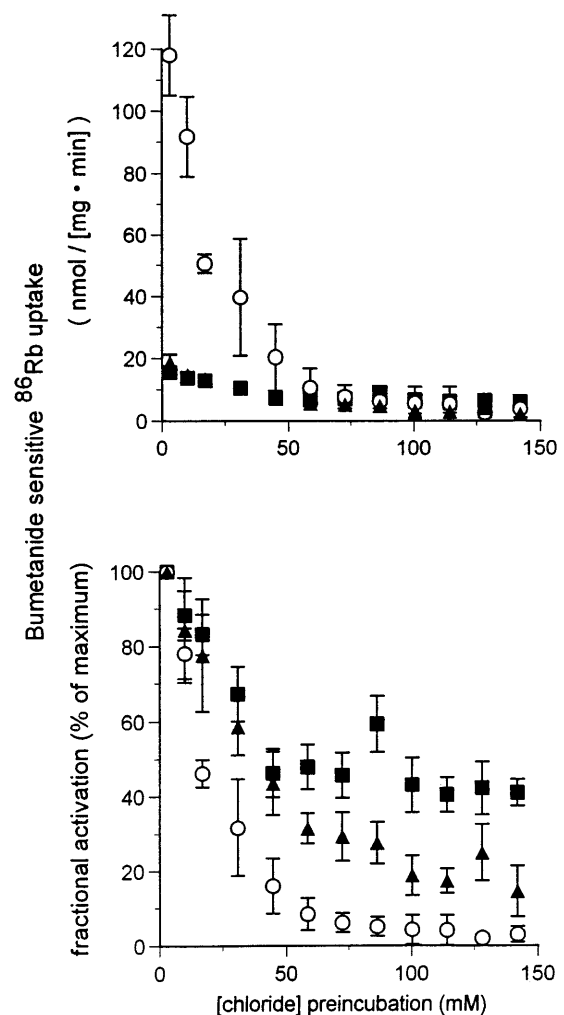


Fig. 6. Bumetanide-sensitive (200 μM) ^{86}Rb uptake (% of maximum) after a 1-h preincubation with a range of external Cl concentrations is plotted. ■, KCC1-HEK cells; ▲, control HEK-293 cells; ○, NKCC1-HEK cells. *Top*: absolute values. *Bottom*: fractional activation. Values are means \pm SE ($n = 4$).

HEK cells compared with control HEK-293 and KCC1-HEK cells. Incubation in 31 mM $[\text{Cl}]_o$ produced similar reductions in $[\text{Cl}]_i$ among all three cell lines. After incubation in 31 mM $[\text{Cl}]_o$, the absolute magnitude of Na-K-Cl cotransporter activity was greater in NKCC1-HEK cells than in KCC1-HEK or HEK-293 cells (Fig. 6, *top*). However, fractional activation of Na-K-Cl cotransporter was lower in NKCC1-HEK cells than in KCC1-HEK or HEK-293 cells (Figs. 6, *bottom*, and 7).

Regulation of cell volume in NKCC1-HEK and KCC1-HEK cells. To determine whether overexpression of KCC1 and NKCC1 influences the ability of cells to regulate cell volume, we measured cell volume after preincubation in various media (Table 1). These measurements of cell volume were made 45 min after cells were placed in various media. Transient changes in cell volume such as swelling followed by regulatory volume decrease would not be detected. Control HEK and NKCC1-HEK cells remained swollen by $\sim 20\%$ above their original volumes 45 min after exposure to hypotonic medium; KCC1-HEK cells were swollen $< 10\%$

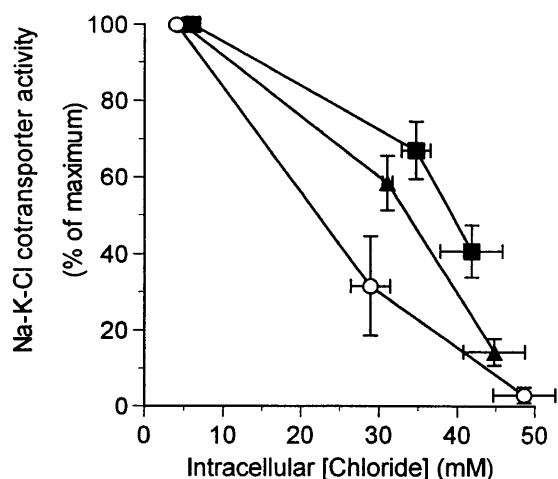


Fig. 7. Bumetanide-sensitive (200 μ M) 86 Rb uptake (% of maximum) is plotted as a function of intracellular Cl concentration. Each cell line was preincubated for 1 h in 140, 31, or 3 mM external Cl. ■, KCC1-HEK cells; ▲, control HEK-293 cells; ○, NKCC1-HEK cells. Values are means \pm SE ($n = 4$).

after the same treatment. Similar responses were observed after low-Cl, hypotonic preincubation. KCC1-HEK cells demonstrated a small reduction in cell volume, compared with \sim 15% increases in the cell volume of control HEK-293 and NKCC1-HEK cells. Thus overexpression of KCC1 allows cells to regulate volume more efficiently during cell swelling.

No increases in cell volume were observed after 45 min in high-K medium (Table 1), and moderate increases (Table 2) in cellular K content were observed after this treatment. All cell lines lost large amounts of K during hypotonic swelling (Table 2), consistent with the role of K efflux in the volume regulatory process. K losses were greatest from KCC1-HEK cells, consistent with a role of KCC1 in the improved volume regulation in these cells.

All cell lines showed decreases in cell volume after incubation in $[Cl]_o$ of 3 and 31 mM (Table 1). However, at 31 mM $[Cl]_o$, NKCC1-HEK cells demonstrated smaller (not statistically significant) decreases in volume than HEK control and KCC1-HEK cells. Incubation with 250 μ M bumetanide (in normal $[Cl]_o$) produced shrinkage in all three cell lines. These results confirm the importance of Na-K-Cl cotransport in normal cell volume maintenance and in volume regulation in response to cell shrinkage.

Table 1. Changes in cell volume after experimental treatments

Cell Line	Cell Volume, % of control					
	Hypotonic	50 mM K	3 mM Cl	31 mM Cl	3 mM Cl hypotonic	Bumetanide
HEK-293	123 \pm 5	105 \pm 6	65 \pm 3	70 \pm 4	114 \pm 4	91 \pm 7
NKCC1-HEK	121 \pm 4	99 \pm 6	72 \pm 3	86 \pm 7	117 \pm 9	83 \pm 6
KCC1-HEK	108 \pm 7	105 \pm 6	70 \pm 3	78 \pm 5	91 \pm 6*	83 \pm 8
<i>n</i>	10	6	10	6	4	4

Values are means \pm SE; *n*, no. of trials. * $P < 0.05$ compared with HEK-293 (control).

Table 2. Change in intracellular K content during hypotonic swelling and 50 mM external K

Cell Line	Intracellular K, % of control	
	Hypotonic	50 mM K
HEK-293	59 \pm 5	111 \pm 4
NKCC1-HEK	43 \pm 3	127 \pm 8
KCC1-HEK	34 \pm 7	125 \pm 2

Values are means \pm SE; $n = 3$ trials.

DISCUSSION

Overview. In the studies reported here, we have used cell lines that overexpress KCC1 to investigate the regulation of both the Na-K-Cl cotransporter and KCC1. Two general questions have been asked: 1) Does KCC1 demonstrate the same pattern of regulation as the well-studied red cell K-Cl cotransporter? and 2) Does overexpression of the K-Cl cotransporter influence the activity of endogenous Na-K-Cl cotransporter by altering $[Cl]_i$ and/or cell volume?

Regulation of KCC1. Many studies in red blood cells have shown that K-Cl cotransport is activated by cell swelling and by NEM [reviewed by Lauf et al. (20)]. The apparent ion and inhibitor affinities of KCC1 are the same as the red cell K-Cl cotransporter (8), and recent molecular evidence (33) indicates that red blood cells express KCC1. In this study, we have characterized the activation of K-Cl cotransport in KCC1-HEK cells, testing the hypothesis that regulation of KCC1 is the same as regulation of the red cell K-Cl cotransporter.

Our results clearly show that both NEM and cell swelling produce large activations of K-Cl cotransport in KCC1-HEK cells (Figs. 2 and 3). Importantly, cell swelling specifically activates K-Cl cotransport in KCC1-HEK cells. Furosemide-insensitive transport is not significantly increased by hypotonic swelling and is somewhat decreased in 50 mM $[K]_o$, whereas furosemide-sensitive transport is substantially increased in both conditions. Interestingly, the brain-specific K-Cl cotransporter, KCC2, is activated by NEM but is not activated by cell swelling (28). We have not investigated the mechanism of reduced furosemide-insensitive 86 Rb loss in 50 mM K medium; one possibility is that an endogenous HEK-293 cell K efflux pathway is inhibited by high extracellular K.

Differential bumetanide sensitivity. KCC1 activity in cells activated by cell swelling was inhibited by bumetanide with a lower affinity than inhibition in cells activated by NEM. This differential bumetanide sensitivity might be a result of altered $[K]_i$ or $[Cl]_i$ during swelling. Changes in $[K]_i$ or $[Cl]_i$ after hypotonic swelling may affect the membrane orientation of transporter ion and inhibitor binding sites, potentially altering accessibility of bumetanide to its binding site. Previous work supports this explanation. Lauf (19) found that furosemide inhibited K-Cl cotransport in sheep red blood cells with much higher affinity in the presence of Rb (23 mM) than in the absence of Rb. Similarly, Lytle and McManus (25) found that increased $[K]_o$ enhances inhibition of the red cell K-Cl cotransporter by bu-

metanide. Alternatively, NEM may influence the conformation of the transporter and thereby influence bumetanide affinity.

Functional interaction of KCC1 and Na-K-Cl cotransporter. Transepithelial movement of Cl ions is an important component of both secretory and absorptive processes. Efficient transepithelial Cl movement requires coordinated regulation of apical and basolateral Cl transport pathways. Two signals have been proposed to mediate apical-basolateral cross talk in secretory cells: cell volume and $[Cl]_i$. Recent evidence supports an important role of $[Cl]_i$ in this cross talk process. An increase in $[Cl]_i$ has been shown to inhibit Na-K-Cl cotransport in squid giant axon (3). Robertson and Foskett (34) found that a fall in $[Cl]_i$ is required for activation of Na transport pathways in secretory acinar cells. Haas and McBrayer (12) demonstrated activation of Na-K-Cl cotransport in nystatin-treated tracheal cells by a reduction in apical Cl concentration (and therefore $[Cl]_i$). Haas et al. (13) demonstrated a direct relationship between $[Cl]_i$ and Na-K-Cl cotransporter phosphorylation in dog tracheal epithelial cells. Finally, Lytle and Forbush (23, 24) showed a clear relationship between $[Cl]_i$, sNKCC1 phosphorylation, and activation of Na-K-Cl cotransport in secretory tubules isolated from dogfish shark rectal gland.

Cell lines overexpressing NKCC1 or KCC1 are a simple model to examine the relationship between transporter activity and $[Cl]_i$. The results presented here show that overexpression of KCC1 increases baseline endogenous Na-K-Cl cotransporter activity. KCC1-HEK cells have increased activity of the endogenous Na-K-Cl cotransporter in the absence of stimulation (Fig. 4). The increased ^{86}Rb influx in KCC1-HEK cells is not due to activity of the transfected K-Cl cotransporter because it is dependent on external Na (Fig. 4) and inhibited by bumetanide with a K_i of $<1 \mu M$ (Fig. 5). The measured increase is not due to increased capacity of the cells for Na-K-Cl cotransport. Low-Cl hypotonic stimulation of KCC1-HEK cells produced little further activation of endogenous Na-K-Cl cotransport, and Na-K-Cl cotransporter activity was the same in KCC1-HEK and control HEK-293 cells after maximal stimulation by low-Cl hypotonic preincubation (Fig. 4). Measurements of Na-K-Cl cotransporter activity by ^{86}Rb efflux (Fig. 5) are consistent with influx measurements (Fig. 4), supporting an increased Na-K-Cl cotransporter activity in KCC1-HEK cells. These results clearly show that expression of KCC1 in HEK-293 cells causes activation of the endogenous Na-K-Cl cotransporter. Similar findings have been noted by others (15).

Transfection of the K-Cl cotransporter KCC1 into HEK-293 cells could result in reduction of cell volume and/or a reduction in $[Cl]_i$; either of these effects could potentially activate Na-K-Cl cotransport. Previous data argue against changes in cell volume as the primary stimulus for activation of Na-K-Cl cotransport. In the shark rectal gland, large changes in cell volume are required to activate the Na-K-Cl cotransporter fully (22). Furthermore, the endogenous Na-K-Cl cotransporter of HEK-293 cells is relatively insensitive to cell

shrinkage, even when shrinkage is coupled with lower $[Cl]_i$ (17). The results presented here are consistent with these previous studies. No significant difference in resting cell volume among cell lines was measured, indicating that changes in cell volume are not the cause of Na-K-Cl cotransport activation in KCC1-HEK cells.

Previous studies show that reduction in $[Cl]_i$ activates the Na-K-Cl cotransporter in HEK-293 cells (17, 37). We found that $[Cl]_i$ was 3.1 ± 1.2 mM lower than control in KCC1-HEK cells and 3.3 ± 1.6 mM greater than control in NKCC1-HEK cells. These results are consistent with the direction of net Cl ion transport through the K-Cl and Na-K-Cl cotransporters. Our data support the hypothesis that a reduction in $[Cl]_i$ is responsible for the measured differences in Na-K-Cl cotransporter activation between KCC1-HEK cell and control cell lines (Figs. 4 and 5).

We measured $[Cl]_i$ and Na-K-Cl cotransport activation after incubation in isotonic media containing a range of Cl concentrations (Fig. 6). The relationship between $[Cl]_i$ and Na-K-Cl cotransporter activity was similar in KCC1-HEK cells and HEK-293 control cells (Fig. 7) and was very steep in the physiological range. Relatively small reductions in $[Cl]_i$ were measured when $[Cl]_o$ was reduced from 140 to 31 mM; these reductions were accompanied by activation of Na-K-Cl cotransport to 60–70% of its maximal activity in both KCC1-HEK and HEK-293 control cells (Figs. 6 and 7). Importantly, the data point for resting KCC1-HEK cells falls within the steep portion of this relationship, providing strong support for the role of $[Cl]_i$ in the activation of Na-K-Cl cotransport in KCC1-HEK cells. The functional consequence of the steep relationship between $[Cl]_i$ and Na-K-Cl cotransporter activity is that $[Cl]_i$ is tightly regulated by changes in Na-K-Cl cotransporter activity. This tight regulation of $[Cl]_i$ is consistent with studies performed in shark rectal glands that have shown that $[Cl]_i$ changes very little during secretion (10).

Although the mechanistic basis for the steep relationship between $[Cl]_i$ and Na-K-Cl cotransporter activity is not known, there are two Cl binding sites on the Na-K-Cl cotransporter, and a Cl-sensitive kinase has been suggested as a possible regulatory molecule. Thus it is possible that the steep relationship is created by Cl acting at several separate sites.

Our data support a role of $[Cl]_i$ in the functional interaction between KCC1 and the endogenous Na-K-Cl cotransporter of HEK-293 cells, but it is possible that other factors are also involved. Based on Fig. 7, the measured decrease in $[Cl]_i$ in KCC1-HEK cells cannot account for the entire activation of Na-K-Cl cotransporter in these cells. Furthermore, a question remains as to the mechanism of $[Cl]_i$ reduction in KCC1-HEK cells. Although the most straightforward explanation is increased Cl efflux through KCC1, we did not measure significantly increased ^{86}Rb efflux in nonstimulated KCC1-HEK cells. One explanation for this finding is that we have measured ^{86}Rb efflux under conditions different from cell growth conditions (importantly, efflux studies were performed at $\sim 22^\circ C$, whereas growth

was at 37°C) and that KCC1 is in fact activated under our growth conditions. Given the above discussion, it is appropriate to consider alternate explanations for the observed activation of Na-K-Cl cotransporter in KCC1-HEK cells. It does not appear that there are differences in Na-K-Cl cotransporter expression between KCC1-HEK and HEK-293 cells, because the maximal level of Na-K-Cl cotransporter-mediated ^{86}Rb flux is the same in KCC1-HEK and HEK-293 cells (Fig. 4) and because we did not observe differences in Na-K-Cl cotransporter expression level in Western blot comparisons of these lines using the T4 monoclonal antibody (27) (data not shown). Other possibilities include 1) the possibility that $[\text{K}]_i$ or intracellular Na concentration is altered in KCC1-HEK cells, leading to kinetic effects on Na-K-Cl cotransport (26), 2) possible direct or indirect physical interactions between KCC1 and Na-K-Cl cotransporters (1), and 3) competition of overexpressed KCC1 and the Na-K-Cl cotransporter for regulatory molecules (e.g., kinases and phosphatases).

NKCC1-HEK cells displayed a different relationship between $[\text{Cl}]_i$ and Na-K-Cl cotransporter activity than did KCC1-HEK and control cells. NKCC1-HEK cells showed smaller fractional activation of Na-K-Cl cotransporter activity at 31 mM $[\text{Cl}]_o$, although the reduction in $[\text{Cl}]_i$ was similar to that of the other cell lines. One explanation for this reduced sensitivity to reductions in $[\text{Cl}]_i$ is that the HEK-293 cell regulatory mechanism is not able to efficiently activate the exogenously expressed Na-K-Cl cotransporter. This inefficiency might be due to insufficient quantity of regulatory proteins or incompatibility of the native HEK-293 regulatory mechanism with the transfected NKCC1 protein. A second explanation for the reduced sensitivity is that overexpression of NKCC1 affects another variable that influences the regulatory process. For example, the reduction in cell volume is smaller in NKCC1-HEK cells than in control cells at 31 mM $[\text{Cl}]_o$; this may contribute to the observed differences between cell lines.

In conclusion, we used stable HEK-293 cell lines overexpressing the Na-K-Cl cotransporter, NKCC1, and the K-Cl cotransporter, KCC1, to investigate regulation of these transporters and their interaction. Our results show conclusively that KCC1 is activated by cell swelling and by NEM and is similar in this regard to the well-studied red cell K-Cl cotransporter. We have found that overexpression of KCC1 activates the endogenous Na-K-Cl cotransporter of HEK-293 cells and that a reduction in $[\text{Cl}]_i$ is at least partly responsible for this activation. This finding strengthens the hypothesis that changes in $[\text{Cl}]_i$ mediate apical-basolateral cross talk in ion-transporting epithelia by demonstrating that overexpression of K-Cl cotransporter can activate Na-K-Cl cotransport by lowering $[\text{Cl}]_i$.

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