Characterization of Glial Cell K-Cl Cotransport

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Key Words

K-Cl cotransport (KCC) • Glial cells • KCC isoforms (1, 3, and 4) • NKCC1 isoform • Ion flux studies • Hyposmotic shock • Hofmeister anion series • *N*-ethylmaleimide • Polymerase chain reaction

Abstract

Background: The molecular mechanism of K-Cl cotransport (KCC) consists of at least 4 isoforms, KCC 1, 2, 3, and 4 which, in multiple combinations, exist in most cells, including erythrocytes and neuronal cells. **Methods:** We utilized reverse-transcriptase-polymerase chain reaction (RT-PCR) and ion flux studies to characterize KCC activity in an immortalized *in vitro* cell model for fibrous astrocytes, the rat C6 glioblastoma cell. Isoform-specific sets of oligonucleotide primers were synthesized for NKCC1, KCC1, KCC2, KCC3, KCC4, and also for NKCC1 and actin. K-Cl cotransport activity was determined by measuring either the furosemide-sensitive, or the Cl⁻-dependent bumetanide-insensitive Rb⁺ (a K⁺ congener) influx in the presence of the Na/K pump

inhibitor ouabain. Rb⁺ influx was measured at a fixed external Cl⁻ concentrations, [Cl⁻]_e, as a function of varying external Rb concentrations, [Rb⁺]_e, and at a fixed [Rb⁺]_e as a function of varying [Cl⁻]_e, and with equimolar Cl replacement by anions of the chaotropic series. Results: RT-PCR of C6 glioblastoma (C6) cells identified mRNA for three KCC isoforms (1, 3, and 4). NKCC1 mRNA was also detected. The apparent K_m for KCC-mediated Rb⁺ influx was 15 mM [Rb⁺]_e, and V_{max} 12.5 nmol Rb⁺ * mg protein⁻¹ * minute⁻¹. The calculated apparent K_m for external Cl⁻ was 13 mM and V_{max} 14.4 nmol Rb⁺ * mg protein⁻¹ * minute⁻¹. The anion selectivity sequence of the furosemide-sensitive Rb⁺ influx was Cl⁻ >> Br⁻ = NO₃⁻ > I⁻ = SCN⁻ >> Sfm⁻ (sulfamate). Established activators of K-Cl cotransport, hyposmotic shock and *N*-ethylmaleimide (NEM) pretreatment, stimulated furosemide-sensitive Rb⁺ influx. A ~50% NEM-induced loss of intracellular K⁺ was prevented by furosemide. Conclusion: We have identified by RT-PCR the presence of three distinct KCC isoforms (1, 3, and 4) in rat C6 glioblastoma cells, and functionally characterized the anion selectivity and kinetics of their collective sodium-independent cation-chloride cotransport activity.

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Introduction

Potassium (K^+) transport across the plasma membrane of mammalian cells is mediated primarily by the Na/K pump, a multitude of dissipative K^+ ion channels, and secondary active electroneutral cation-anion coupled cotransporters. K^+ ions are involved in the generation of the neuronal resting membrane potential and the propagation of membrane electrical signals. Elevations of the extracellular K^+ concentration, $[K^+]_e$, in the central nervous system (CNS) arise from increases in the K^+ conductance of activated neurons and primary afferent fibers, and also from the K^+ release from injured neuronal and glial cells. Glial cells include astrocytes, oligodendrocytes, and microglia [1] and participate with neurons in maintaining cerebral volume homeostasis [2-4], neurotransmitter concentrations [5], synaptic plasticity [6], and repair [7]. Whereas, long-term regulation of extracellular K^+ depends upon the control of ion movement across the blood brain barrier, glial cells have been associated with short-term buffering of external K^+ from the extracellular space around neurons [8, 9]. Several studies have demonstrated a rapid and significant increase in K^+ and Cl^- , but not Na^+ , in astrocytes when the external K^+ concentration is raised. As this increase is sensitive to loop diuretics, the participation of potassium-chloride cotransport has been proposed [9].

K-Cl cotransport (K-Cl COT or KCC), initially identified in red blood cells as a Na-independent, Cl^- -dependent, ouabain-insensitive bi-directional K^+ transport, is now recognized as an intrinsic property of all cells [10]. The K-Cl COT utilizes the larger outwardly directed chemical driving force of K^+ to transfer Cl^- against its inwardly directed chemical gradient. Four distinct genes encoding KCC proteins have been identified: KCC1 (SLC12A4) on chromosome 16q22 [11-13]; KCC2 (SLC12A5) on chromosome 20q13 [14, 15]; KCC3 (SLC12A6) on chromosome 15q14 [16-18]; and KCC4 (SLC12A7) on chromosome 5p15 [16]. Each of the four KCC proteins have: 12 transmembrane domains (tmd), several N-glycosylation sites present within the large extracellular loop between tmd-5 and tmd-6, and cytoplasmic amino and carboxy terminal domains [11]. KCC1, KCC3, and KCC4 have been found expressed in several tissues including muscle, brain, heart, lung, and kidney, whereas, KCC2 is specifically localized in neurons [14-18]. The widespread expression of the different isoforms of the K-Cl COT in several different tissues correlates with their functional importance (for reviews, see [19, 20].

C6 cells exhibit several differentiated properties of *in vivo* fibrous astrocytes (GABA synthesis, expression of glial fibrillary acidic protein and glial calcium binding protein) [21, 22]. In terms of ion transport, previous studies have characterized the role of the Na-K-2Cl cotransporter in volume regulation and K^+ clearance in cultured glial cells [23-25]. However, there is no information regarding the identity or physiological role of KCC activity in glial cells. Thus the C6 cell could be useful model to study the role of multiple non-neuronal cells in the general support of neuronal function. The present study fills this knowledge gap by demonstrating molecular and functional evidence for the presence in C6 cells of three KCC isoforms (KCC1, KCC3, and KCC4), in addition to NKCC1. This work was presented in abstract form at the 2000 Society of General Physiology and the 2002 Experimental Biology Meetings.

Materials and Methods

Reagents

Analytic grade chemicals such as calcium gluconate and magnesium gluconate were obtained from Aldrich Chemicals (Milwaukee, WI); $RbCl_2$, $RbOH$, and $RbNO_3$ from Alfa (Danvers, MA); calyculin A from Calbiochem (La Jolla, CA); NaCl, NaBr, NaI, NaSCN, $NaNO_3$, KCl, KBr, KI, KSCN, KNO_3 , KOH, $MgCl_2$, glucose, perchloric acid (PCA), dimethyl sulfoxide (DMSO), Kaighn's modified F12K nutrient medium containing L-glutamine, fetal bovine serum (FBS), normal horse serum (NHS), and normal goat serum (NGS) from Fisher Scientific (Fair Lawn, NJ); furosemide from Hoechst-Roussel Pharmaceuticals (Somerville, NJ); TRIzol® Reagent from Invitrogen-Life Technologies, Inc. (Carlsbad, CA); Access RT-PCR kit, rat-specific actin primer set, and 100-bp DNA ladder from Promega Corp. (Madison, WI); NaOH, bovine serum albumin (BSA), bumetanide, $CaCl_2$, 4-(2-hydroxy-ethyl)piperazine-1-ethanesulfonic acid (HEPES), 3-(N-Morpholino)propanesulfonic acid (MOPS), N-ethylmaleimide (NEM), ouabain, poly-D-lysine, staurosporine, penicillin, streptomycin, and trimethylhydroxymethane (TRIS) from SIGMA Chemical (St. Louis, MO).

Solutions/stocks

The balanced salt solution (BSS) consisted of 20 mM HEPES/TRIS buffered to pH 7.4 containing (in mM): NaCl (130), KCl (5), $CaCl_2$ (2), $MgCl_2$ (1), and glucose (10). Balanced flux solution (BFS-0.01% BSA) consisted of 20 mM HEPES/TRIS buffered to pH 7.4 containing (in mM): NaCl (125), $RbCl$ (10), $CaCl_2$ (2), $MgCl_2$ (1), 0.01% BSA, and glucose (10). Final wash solution (FWS) consisted of 10mM MOPS buffered to pH 7.4 containing (in mM): $MgCl_2$ (114) and glucose (5). Stock solutions (in mM) of NEM (100), ouabain (200), bumetanide (2), and furosemide (20) were made in either DMSO or ethanol according to manufacturers instructions. All other chemical

reagents were dissolved in de-ionized water.

Culture of C6 glioblastoma cells

Rat C6 glioblastoma cells purchased from ATCC (American Type Culture Collection, Rockville, MD) were incubated in Kaighn's modified F12K nutrient medium (containing L-glutamine, 15% horse serum, 2.5% fetal bovine serum, penicillin and streptomycin) at 37°C, in a 95% O₂/5% CO₂ atmosphere. Monolayers of cells were grown to confluence in 6-well plates for RNA extraction and 12-well plates for Rb uptake experiments.

Total RNA extraction, RT-PCR, and KCC expression in C6 cells

Total RNA from confluent C6 cells was obtained by using TRIzol reagent following the instructions of the manufacturer. Specific sets of primers for all KCC mRNA isoforms were synthesized according to the following sequences: Actin sense, 5'-¹⁰³gtggccgcctagcaccag¹²³-3'; and antisense, 5'-⁸⁴³gat gtc aac gtc aca ctt cat gat g⁸⁶⁷-3'; NKCC1 sense, 5'-¹⁷⁶⁵tgg tgt cag gat ttg cac cc¹⁷⁸⁵-3'; and antisense, 5'-²¹⁶²gcc cac cag tta atg aca aac c²¹⁸³-3'; KCC1 sense, 5'-¹¹⁴cgg aca ggg taac cac aga g¹³⁴-3'; and antisense, 5'-³³⁹caa ggc tcc cag cat ggg cac a³⁶¹-3'; KCC2 sense, 5'-⁵⁰cgg tga tgg caa ccc caa ag⁷⁰-3'; and antisense, 5'-²⁷⁸ggc tcc tcg aat ggg cac ctt²⁹⁸-3'; KCC3 sense, 5'-²¹⁹²agg tca agg cag gaa agg gac²²¹³-3'; and antisense, 5'-²³⁸⁸cct cgg agg cat gaa aca caa²⁴⁰⁹-3'; KCC4 sense, 5'-¹⁵⁹gga gat gga aac ccc agg ga¹⁷⁹-3'; and antisense, 5'-³⁸⁵gca tgg gca cct tca tcg ga⁴⁰⁵-3' (a superscript indicates the base pairs upstream or downstream of the start codon, A¹TG). The first cDNA strand from brain and C6 cell mRNA was obtained using these primers and reverse transcriptase (RT), and polymerase chain reaction (PCR) was used for subsequent amplification of each KCC mouse cDNA isoform present in C6 cells. Briefly, RT: 45 min at 48°C; denaturation, 2 min at 94°C; and PCR: denaturation, 1 min at 94°C; annealing, 1 min at 55°C; and extension, 1 min at 68°C, during 40 cycles. After RT-PCR, the contents of each independent reaction tube were analyzed by 2% agarose gel electrophoresis. The bands (actin, 765 bp; NKCC1, 418 bp; KCC1, 247 bp; KCC2, 248 bp; KCC3, 217 bp; and KCC4, 246 bp) were visualized with ethidium bromide. Corroboration of the ability of these KCC-specific primers to amplify each isoform was demonstrated by RT-PCR using total RNA extracted from mouse brain.

Ion Flux Experiments

The Rb⁺ (as K⁺ congener) uptake procedure previously used for measurement of KCC activity in vascular smooth muscle and human embryonic kidney cells [26, 27] was modified for experiments with C6 cells. Briefly, confluent cell cultures were washed with a warm (37°C) balanced salt solution (BSS) and then equilibrated in the same BSS with the addition of 0.01% bovine serum albumin (BSS-0.01%BSA) for 10 min. C6 cells were pretreated an additional 10 min with warm (37°C) BSS-0.01%BSA containing different pharmacological agents (ouabain, bumetanide, furosemide, and NEM). The pretreatment solutions were then aspirated and replaced with a

balanced flux solution (BFS-0.1%BSA) containing 125 mM NaCl + 10 mM ⁸⁵Rb in place of 130 mM NaCl + 5 mM ⁴¹K. Ion uptake (or loss) was stopped after 6 min by adding an ice-cold isotonic final wash solution (FWS). Four subsequent washes removed any residual ⁸⁵Rb prior to ion extraction with 5% perchloric acid (PCA). Aliquots of the PCA supernatants were quenched with 10 µl of 400 mM CsCl and measured with a Perkin Elmer 5000 Atomic Absorption Spectrophotometer.

Cellular protein was determined with a bicinchonic acid (BCA) protein assay kit. Rb⁺ uptake and cellular K⁺ content were calculated in nmol Rb⁺ (K⁺) x mg protein⁻¹ for any time point. The difference between the total and ouabain-insensitive Rb⁺ uptake represents the Na/K pump flux. The difference between the ouabain-insensitive and (ouabain + bumetanide)-insensitive Rb⁺ uptake (ie. bumetanide-sensitive) represents the Na-K-2Cl cotransporter flux. KCC activity (expressed as nmol Rb⁺ x mg protein⁻¹ x minute⁻¹) was calculated in two separate ways: the difference between the (ouabain + bumetanide)-insensitive Rb⁺ uptake in Cl⁻ and Sfm⁻-based solutions (ie. Cl⁻-dependent bumetanide-insensitive); and the difference between the (ouabain + bumetanide)-insensitive and (ouabain + bumetanide + furosemide)-insensitive Rb⁺ uptake (ie. furosemide-sensitive) Rb⁺ influx.

Results and Discussion

KCC mRNA isoform expression in rat C6 cells

Previous *in situ* hybridization studies have shown the presence of KCC1, KCC2, and KCC3 mRNA in neurons within the mammalian central nervous system [28-30]. However, little is known about the glial expression of the different cation-chloride cotransporter isoforms. Several properties of *in vivo* fibrous astrocytes have been demonstrated in the immortalized rat C6 glioblastoma cell line [21]. Thus, we performed RT-PCR reactions with specific sets of primers for NKCC1, KCC1, KCC2, KCC3, and KCC4 to determine which cotransporter isoforms C6 cells express. We also used a specific set of actin primers as a positive control of RNA extraction from mouse brain and rat C6 glioma cells. We confirmed the capability of each sense/anti-sense primer set to amplify a product of the correct size by using mRNA extracted from whole mouse brain (Figure 1a, upper panel). We demonstrate the RT-PCR amplification of actin, NKCC1, KCC1, KCC3, and KCC4 in C6 cell mRNA (Figure 1a, lower panel). Although, the expression level of KCC4 is much less than that of KCC1 and KCC3, the identification of three K-Cl cotransporter isoforms (at the RNA level) agrees with similar findings in other non-neuronal cell types, such as the sheep [31], and human erythrocytes [32].

Fig. 1. Molecular identification and functional activity of KCC isoforms in C6 cells. A) Upper panel: RT-PCR of mouse brain demonstrating amplification of cDNA for Actin, NKCC1, KCC1, KCC2, KCC3, and KCC4; Lower panel: RT-PCR of rat C6 cells demonstrating bands of the expected size for Actin, NKCC1, KCC1, KCC3, and KCC4. These agarose gels are representative of four experiments with identical results. B) Rb uptake in the presence of 1 mM ouabain + 5 μ M bumetanide as a function of time in chloride (filled circles) and sulfamate (open circles). Data represent means $\pm$ standard error of two separate experiments (n=6).

Fig. 2. Dose-dependence of typical inhibitors for NKCC and KCC. A) Rb influx in the presence of 1 mM ouabain and bumetanide concentrations from 0-50 μ M. B) Rb influx in the presence of 1 mM ouabain, 5 μ M bumetanide, and furosemide concentrations ranging from 0-5 mM. Data represent means $\pm$ standard error of three separate experiments (n=9).

C6 cell KCCs accounts for 13% of total K⁺ influx

In order to establish an endpoint for Rb⁺ uptake in C6 cells which was still linear, we measured the initial velocity of Rb⁺ uptake from 2 to 10 min in chloride- and sulfamate-based solutions in the presence of 1 mM ouabain and 10 μ M bumetanide (Figure 1b). Based on these preliminary results, we chose 6 min as a reasonable endpoint for all our subsequent uptake experiments. The role of Na-K-2Cl cotransport (NKCC) in C6 cells was initially investigated with respect to volume regulation by exposing C6 cells to iso-, hypo-, and hyper-osmotic

conditions in the presence of 1 mM ouabain and 100 μ M bumetanide [23]. Previous studies have found low concentrations of bumetanide (2-10 μ M) inhibit NKCC activity without significantly affecting KCC activity [11, 12]. In Figure 2a, an initial inhibition of ouabain-sensitive Rb⁺ uptake occurred between 5 μ M to 10 μ M (i.e. NKCC activity), and another reduction between 10 and 30 μ M bumetanide (i.e. KCC activity). The 2 nmol * mg protein⁻¹ * minute⁻¹ decrease in Rb⁺ uptake shown in Figure 2b as a result of increasing concentrations (0-2 mM) of furosemide, another 'loop diuretic,' indicates that each of

Fig. 3. Fractionation of C6 cell Rb⁺ influx and anion selectivity. A) The total Rb⁺ influx activity is broken up into Na⁺/K⁺ pump, NKCC, KCC, and ground permeability. Data represent means $\pm$ standard error of twelve separate experiments done in triplicate (n=36). B) 5 μ M Bumetanide-sensitive anion selectivity sequence: Cl⁻>Br⁻>SCN⁻=I⁻>NO₃⁻>> Sfm⁻. C) 2 mM Furosemide-sensitive anion selectivity sequence: Cl⁻>Br⁻=NO₃⁻>I⁻=SCN⁻>>Sfm⁻. D) 2 mM Furosemide-insensitive (background) anion selectivity sequence: Cl⁻= Sfm⁻>SCN⁻=Br⁻=NO₃⁻=I⁻. Data represent means $\pm$ standard error of three separate experiments (n=9).

the CCC components of chloride-dependent Rb⁺ uptake can be functionally isolated. Figure 3a represents the breakdown of the total Rb⁺ influx into the Na/K pump (20%), NKCC (37%), KCC (13%), and residual fluxes in the presence of all inhibitors, here called background permeability (30%). The net Cl⁻-dependent furosemide (2 mM)-sensitive Rb⁺ influx (basal activity) in C6 cells measured in the presence of 1 mM ouabain and 5 μ M bumetanide, was 2.4 nmol $\times$ mg protein⁻¹ $\times$ minute⁻¹, consistent with previous KCC studies in other mammalian cell systems [33, 34].

Glial NKCC1 and KCCs exhibit different anion selectivity

KCC activity requires both K⁺ and Cl⁻ to bind on the protein prior to commencement of electroneutral cotransport across the cell membrane [35]. We replaced Cl⁻ with bromide (Br⁻), iodide (I⁻), thiocyanate (SCN⁻), nitrate (NO₃⁻), and sulfamate (Sfm⁻) in the wash, pre-treatment, and flux solutions to determine the anion selectivity of glial KCC function. The differential bumetanide-sensitive Rb⁺ influx (nmol $\times$ mg protein⁻¹ $\times$ minute⁻¹) calculated from the fluxes measured in the

presence of '1 mM ouabain' and of '1 mM ouabain + 5 μ M bumetanide' revealed an anion selectivity sequence ($\text{Cl}^- > \text{Br}^- > \text{SCN}^- = \text{I}^- > \text{NO}_3^- \gg \text{Sfm}^-$) characteristic for the Na-K-2Cl cotransporter (figure 3b) [36]. The differential furosemide-sensitive Rb^+ influx calculated from the fluxes measured in the presence of '1 mM ouabain + 5 μ M bumetanide' and of '1 mM ouabain + 5 μ M bumetanide + 2 mM furosemide' (figure 3c) revealed an anion selectivity sequence ($\text{Cl}^- \gg \text{Br}^- = \text{NO}_3^- > \text{I}^- = \text{SCN}^- \gg \text{Sfm}^-$) characteristic of the anion dependence of KCC1 in *Xenopus laevis* oocytes [37]. Earlier studies in sheep erythrocytes found Br^- promoting 'furosemide-sensitive' K^+ flux greater than Cl^- [38, 39]. This differential anion sensitivity of 'furosemide-sensitive' K^+ transport might be due to functional dominance of one of the three KCC isoforms present in C6 cells. In contrast to the anion selectivity of the cation-chloride cotransporters, the furosemide-insensitive background permeability (figure 3d) did not discriminate between Cl^- , Sfm^- and SCN^- suggesting that this Rb^+ flux component is not Cl^- -dependent (i.e. channel-mediated).

NEM stimulates C6 cell KCC

For over 25 years, NEM pre-treatment has been known to stimulate KCC activity [40]. This 'NEM effect' on K-Cl cotransport is time, temperature and concentration dependent [19]. Interestingly, at 0°C, NEM stimulates red blood cell cotransport activity, whereas, at physiological temperatures (ie. 37°C), different concentrations of NEM have been shown to both stimulate and inhibit KCC activity in cultured HEK293 cells over expressing rabbit KCC1 cDNA [19, 27]. In figure 4a, exposure of C6 cells to 1 mM NEM for 10 min stimulated the Na/K pump (2-fold), K-Cl cotransporter (5-fold), and background permeability (6-fold). The 2-fold stimulation of the Na/K pump following NEM treatment, although not statistically significant, is likely a result of a higher Na^+ load elicited by an increased Na^- inward leak or some other unknown factor, which also has recently been demonstrated in human lens epithelial cells [41]. The 'transitory effect' of NEM on KCC activity is apparent when the length of NEM incubation prior to measurement of Rb^+ uptake is increased from 10 to 20 min. Both the 5- and 6-fold increases in KCC and background 'channel' activity, respectively, are abrogated by the longer NEM pretreatment (Figure 4a). These results are consistent with previous findings in trout hepatocytes, HEK293 cells, and rat aortic smooth muscle cells [19, 42]. Interestingly, no significant difference in NKCC activity was observed between control and 10

Fig. 4. C6 cell activation by NEM and hypotonicity. A) The calculated Na^+/K^+ pump, NKCC, KCC, and background Rb^+ influx components in control (black bars), after 10 min pre-treatment with 1 mM NEM (light grey bars), and after 20 min pre-treatment with 1 mM NEM (dark grey bars). B) Measurement of intracellular K^+ content following NEM pretreatment with 1 mM ouabain, 5 μ M bumetanide, and 2 mM furosemide. C) The Na^+/K^+ pump, NKCC, KCC, and background Rb^+ influx components in isosmotic (black bars) and hyposmotic (grey bars) balanced salt solution. Data represent means $\pm$ standard error of four separate experiments done in triplicate (n=12).

min NEM exposure. However, pre-treatment with NEM for 20 min completely inhibited NKCC activity. Although the molecular mechanism of the stimulatory 'NEM effect' has been ascribed to inhibition of a protein kinase reciprocally controlling the activities of both NKCC and KCC, the mechanism of complete inhibition of both transporters is still unknown. One possibility is that NEM reacts with a variety of nucleophilic groups such as thiols and histidines, and irreversibly modifies the underlying protein structures of both transporters [19, 42] rather than their common regulatory pathways. Recent work from the Delpire Laboratory (Vanderbilt University, Nashville) suggests that NEM interferes with the autophosphorylation of SPAK (Ste20p-like proline-alanine rich kinase) a key enzyme controlling the phosphorylation/regulation of both transporters [43].

NEM exposure causes 30% decrease in intracellular K^+

Net inward KCC activity in C6 cells was determined by measuring Rb^+ uptake, however, the cotransporter has also been shown in erythrocytes to simultaneously extrude intracellular K^+ [35, 44]. We therefore measured the intracellular K^+ content at the end of the uptake period to estimate the net changes of cellular K^+ . Intracellular K^+ content at 6 min was consistently about $\sim 850 \text{ nmol} \times \text{mg protein}^{-1}$ (see figure 4b, black bars). We have recalculated this value in terms of intracellular K^+ concentration, i.e. in terms of mequivalents/L cell water and found values close to 100 mM, the expected range for high K^+ glial cells. However, 10 min pre-treatment with 1 mM NEM caused intracellular K^+ content to decrease by 30-50% below control levels. Ouabain and bumetanide did not prevent the loss of intracellular K^+ , suggesting neither Na/K pump, nor the Na-K-2Cl cotransporter, participated in the K^+ loss (see figure 4b, grey bars). The addition of furosemide, however, completely prevented the loss of intracellular K^+ , suggesting the involvement of the K-Cl cotransporter. The calculated 'furosemide-sensitive' K^+ loss induced by NEM exposure ($\sim 300 \text{ nmol} \times \text{mg protein}^{-1}$) is three-fold greater than the measured Rb^+ influx over the same period ($\sim 90 \text{ nmol} \times \text{mg protein}^{-1}$) at an approximate K^+_i/K^+_o (Rb^+ replacement) ratio >10 . Although the intracellular Cl^- concentration is unknown, if we assume it to be $\sim 40 \text{ mM}$, the Cl^-_o/Cl^-_i ratio would be about 3-4 fold, still substantially smaller than the K^+ ratio. Based on thermodynamic considerations we developed for KCC activity in erythrocytes [44], we therefore assume that the approximated three-fold K^+ -efflux/ Rb^+ influx ratio is mainly due to the difference in the product

Fig. 5. Rb^+ influx as a function of external Rb^+ and Cl^- concentrations. A) 'Ouabain + bumetanide'-insensitive Cl^- -dependent Rb^+ influx as a function of external Rb^+ (0-50 mM). Inset) Hanes-Woolf plot with the $V_{max} = 12.5 \text{ nmol} \times \text{mg protein}^{-1} \times \text{minute}^{-1}$ and $K_m(Rb^+) = 15 \text{ mM}$. B) 'Ouabain + bumetanide'-insensitive Rb^+ influx as a function of external Cl^- concentration from 0-150 mM. Inset) Hanes-Woolf plot with $V_{max} = 14.4 \text{ nmol} \times \text{mg protein}^{-1} \times \text{minute}^{-1}$ and $K_m(Cl^-) = 13 \text{ mM}$. Data represent means $\pm$ standard error of four separate experiments done in triplicate ($n=12$).

of K^+ and Cl^- driving forces, commensurate with an outward-directed K-Cl cotransporter and less with a furosemide-sensitive K^+ channel [45].

Hypotonicity inhibits NKCC and stimulates KCC

The opposing activities of NKCC and KCC to maintain and regulate cell volume have been well characterized in many other systems [46, 47]. C6 cells exposed to 300 mOsm and 100 mOsm balanced salt solutions revealed a complete loss of bumetanide-sensitive Rb^+ influx (Na-K-2Cl cotransport), and a nearly 2-fold increase in the furosemide-sensitive Rb^+ influx (K-Cl cotransport), whereas there was no significant effect on

the Na/K pump flux (figure 4c). Cells exposed to hyposmotic conditions increase in size due to the osmotically driven high water entry. The physiological response to cell swelling consists, amongst others, of inactivation of ion influx (i.e. NKCC) and stimulation of ion efflux (i.e. KCC). KCC activation results in K⁺ and Cl⁻ ion movement across the plasma membrane accompanied by water loss to balance osmotic forces and a return to normal cell size [10, 48]. The 2-fold increase in KCC activity and complete inhibition of NKCC activity observed in C6 cells exposed to 100 mOsM (see figure 4c) was consistent with previous studies of hypotonic KCC activation in a variety of animal and human erythrocytes [10, 48].

Kinetics of Rb⁺ and Cl⁻ dependence indicate KCC activity

The driving forces for KCC are determined by the product of the chemical gradients of K⁺ and Cl⁻ [44], with Cl⁻ binding to the carrier prior to K⁺, both with relatively low apparent affinities [49]. To ascertain the apparent K_m and V_{max} values of glia C6 cell KCC, we measured the 'ouabain + bumetanide'-insensitive Cl⁻-dependent Rb⁺ influx and the 'ouabain + bumetanide'-insensitive Rb⁺ influx as a function of extracellular Rb⁺ and Cl⁻, respectively. In figure 5a, 'ouabain + bumetanide'-insensitive Cl⁻-dependent Rb⁺ influx saturated at ~30 mM extracellular Rb⁺ and ~9.5 nmol x mg protein⁻¹ x minute⁻¹. The apparent K_m for Rb⁺ was 15 mM, and the V_{max} 12.5 nmol Rb⁺ x mg protein⁻¹ x minute⁻¹ (insert figure 5a). We previously reported K_m values for K⁺ of 41.8 ± 16.2 mM for sheep and pig erythrocytes, and 36.2 ± 24 mM for thiol-activated K-Cl cotransport [34]. Gillen and coworkers measured a K_m for K⁺ of > 50 mM for rabbit KCC1 when over-expressed in transiently transfected HEK293 cells [11]. An apparent K_m for K⁺ of 17.5 mM for KCC activity in *Xenopus laevis* oocytes was reported [20]. The variability of these K_m values suggests not only multiple isoforms contributing to the measured KCC activity, but also depending on cell type, one isoform exhibiting functional dominance over the others.

Measuring Rb⁺ influx as a function of extracellular Cl⁻ concentration provides another means to assess its carrier-mediated nature. In Figure 5b, we demonstrate that the bumetanide-insensitive Rb influx saturated at ~ 60 mM extracellular Cl⁻ and ~10 nmol x mg protein⁻¹ x minute⁻¹. The apparent K_m for the activation of Rb⁺ influx by Cl⁻ was 13 mM, and the V_{max} 14.4 nmol x

mg protein⁻¹ x minute⁻¹ (inset figure 5b). It needs to be understood that this K_m value is by no means an indicator of the Cl⁻ affinity per se, but rather documents that the K⁺ transport mediated presumably by C6 cell KCCs' shows saturation as function of external Cl⁻. This finding is consistent with our model of K-Cl cotransport in sheep erythrocytes where Cl⁻ binds prior to K⁺ and translocation of the loaded KCC carrier [35]. Although a rigorous kinetic analysis of the binding order of KCC activity in glia cells would require setting cellular zero-trans conditions, the data obtained suggest a hyperbolic (i.e. saturating) dependence of glia cell KCC activity on extracellular Cl⁻ which might well be the rate limiting step for K⁺ (Rb⁺) binding prior to translocation. In addition, the similarity of the K_m and V_{max} values for K⁺ and Cl⁻ maybe merely coincidental and not indicative of the true K⁺ and Cl⁻ affinities of the system. Given the fact that the presumable KCC influx exhibited K_m values for extracellular K⁺ and Cl⁻ of 15 mM and 13 mM, respectively, C6 cell KCCs' likely function as a K-Cl efflux pathway under physiological conditions. However, when external K⁺ rises under pathophysiological conditions, C6 cells possess the capacity to switch to an influx pathway for K-Cl.

Summary

In this study evidence for the molecular and functional existence of three KCC isoforms in an immortalized cell line (rat C6 glioblastoma) is presented. Ouabain- and bumetanide-resistant K⁺ influx with Rb as congener in rat C6 cells possesses several of the characteristic attributes found in other cells, such as Cl⁻-dependence, furosemide sensitivity, moderately low affinities for external K⁺ and Cl⁻ ions, stimulation by NEM and cell swelling, and inhibition by prolonged NEM incubation. Whereas over the past, the erythrocyte has been extensively used as a convenient cell model for physiological study of the K-Cl cotransporter [10, 34, 50-52], the immortalized rat C6 glioblastoma, may now serve as another mammalian cell model for this purpose.

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References

- 1 Aschner M, Allen JW, Kimelberg HK, LoPachin RM, Streit WJ: Glial cells in neurotoxicity development. *Annu Rev Pharmacol Toxicol* 1999;39:151-173.
- 2 Bourke RS, Kimelberg HK, Nelson LR, Barron KD, Auen EL, Popp AJ, Waldman JB: Biology of glial swelling in experimental brain edema. *Adv Neurol* 1980;28:99-109.
- 3 Kempinski O, Volk C: Neuron-glial interaction during injury and edema of the CNS. *Acta Neurochir Suppl (Wien)* 1994;60:7-11.
- 4 Kimelberg HK, Rutledge E, Goderie S, Charniga C: Astrocytic swelling due to hypotonic or high K⁺ medium causes inhibition of glutamate and aspartate uptake and increases their release. *J Cereb Blood Flow Metab* 1995;15:409-416.
- 5 Ye ZC, Sontheimer H: Glioma cells release excitotoxic concentrations of glutamate. *Cancer Res* 1999;59:4383-4391.
- 6 Newman EA: Regional specialization of the membrane of retinal glial cells and its importance to K⁺ spatial buffering. *Ann N Y Acad Sci* 1986;481:273-286.
- 7 Martin DL: Synthesis and release of neuroactive substances by glial cells. *Glia* 1992;5:81-94.
- 8 Hertz L: An intense potassium uptake into astrocytes, its further enhancement by high concentrations of potassium, and its possible involvement in potassium homeostasis at the cellular level. *Brain Res* 1978;145:202-208.
- 9 Walz W, Hinks E: Carrier-mediated KCl accumulation accompanied by water movements is involved in the control of physiological K⁺ levels by astrocytes. *Brain Res* 1985;343:44-51.
- 10 Lauf P, Adragna N: K-Cl cotransport: Properties and molecular mechanism. *Cell Physiol Biochem* 2000;10:341-354.
- 11 Gillen CM, Brill S, Payne J, Forbush B, III: Molecular cloning and functional expression of the K-Cl cotransporter from rabbit, rat, and human. A new member of the cation-chloride cotransporter family. *J Biol Chem* 1996;271:16237-16244.
- 12 Holtzman EJ, Kumar S, Faaland CA, Warner F, Logue PJ, Erickson SJ, Ricken G, Waldman J, Dunham PB: Cloning, characterization, and gene organization of K-Cl cotransporter from pig and human kidney and *C. elegans*. *Am J Physiol* 1998;275:F550-564.
- 13 Su W, Shmukler BE, Chernova MN, Stuart-Tilley AK, de Franceschi L, Brugnara C, Alper SL: Mouse K-Cl cotransporter KCC1: cloning, mapping, pathological expression, and functional regulation. *Am J Physiol* 1999;277:C899-912.
- 14 Payne J, Stevenson T, Donaldson L: Molecular characterization of a putative K-Cl cotransporter in rat brain. A neuronal-specific isoform. *J Biol Chem* 1996;271:16245-16252.
- 15 Song L, Mercado A, Vazquez N, Xie Q, Desai R, George AL, Jr., Gamba G, Mount DB: Molecular, functional, and genomic characterization of human KCC2, the neuronal K-Cl cotransporter. *Mol Brain Res* 2002;103:91-105.
- 16 Mount DB, Mercado A, Song L, Xu J, George AL, Jr., Delpire E, Gamba G: Cloning and characterization of KCC3 and KCC4, new members of the cation-chloride cotransporter gene family. *J Biol Chem* 1999;274:16355-16362.
- 17 Race JE, Makhlof FN, Logue PJ, Wilson FH, Dunham PB, Holtzman EJ: Molecular cloning and functional characterization of KCC3, a new K-Cl cotransporter. *Am J Physiol (Cell Physiol)* 1999;277:C1210-1209.
- 18 Hiki K, D'Andrea RJ, Furze J, Crawford J, Woollatt E, Sutherland GR, Vadas MA, Gamble JR: Cloning, characterization, and chromosomal location of a novel human K⁺-Cl⁻ cotransporter. *J Biol Chem* 1999;274:10661-10667.
- 19 Lauf PK, Adragna NC: Twenty-five years of K-Cl cotransport: From stimulation by a thiol reaction to cloning of the full-length KCCs. In: *Cell Volume and Signaling*, edited by Lauf PK and Adragna NC. Dayton: Springer Verlag, 2004, pp 11-28.
- 20 Gamba G: Molecular Physiology and Pathophysiology of Electroneutral Cation-Chloride Cotransporters. *Physiol Rev* 2005;85:423-493.
- 21 Benda P, Lightbody J, Sato G, Levine L, Sweet W: Differentiated rat glial cell strain in tissue culture. *Science* 1968;161:370-371.
- 22 Kukes G, De Vellis J, Elul R: A linked active transport system for Na⁺ and K⁺ in a glial cell line. *Brain Res* 1976;104:93-105.
- 23 Chassande O, Frelin C, Farahifar D, Jean T, Lazdunski M: The Na⁺/K⁺/Cl⁻ cotransport in C6 glioma cells. Properties and role in volume regulation. *Eur J Biochem* 1988;171:425-433.
- 24 Walz W: Role of Na/K/Cl cotransport in astrocytes. *Can J Physiol Pharmacol* 1992;70:S260-262.
- 25 Mongin AA, Akseptsev SL, Orlov SN, Kvacheva ZB, Mezen NI, Fedulov AS, Konev SV: Swelling-induced activation of Na⁺,K⁺,2Cl⁻ cotransport in C6 glioma cells: kinetic properties and intracellular signalling mechanisms. *Biochim Biophys Acta* 1996;1285:229-236.
- 26 Adragna NC, White RE, Orlov SN, Lauf PK: K-Cl cotransport in vascular smooth muscle and erythrocytes: possible implication in vasodilation. *Am J Physiol Cell Physiol* 200;278:C381-390.
- 27 Lauf P, Gagnon K, Delpire E, Fyffe R, Adragna N: K-Cl cotransport: Immunohistochemical and ion flux studies in human embryonic kidney (HEK293) cells transfected with full-length and C-terminal-domain-truncated KCC1 cDNAs. *Cell Physiol Biochem* 2001;11:143-160.
- 28 Clayton GH, Owens GC, Wolff JS, Smith RL: Ontogeny of cation-Cl⁻ cotransporter expression in rat neocortex. *Brain Res Dev Brain Res* 1998;109:281-292.
- 29 Kanaka C, Ohno K, Okabe A, Kuriyama K, Itoh T, Fukuda A, Sato K: The differential expression patterns of messenger RNAs encoding K-Cl cotransporters (KCC1,2) and Na-K-2Cl cotransporter (NKCC1) in the rat nervous system. *Neuroscience* 2001;104:933-946.
- 30 Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH: Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* 2001;22:153-183.
- 31 Lauf P, Zhang J, Delpire E, Fyffe R, Mount D, Adragna N: Erythrocyte K-Cl cotransport: Immunocytochemical and functional evidence for more than one KCC isoform in HK and LK sheep red blood cells. *Comp Biochem Physiol* 2001;130 Pt A:499-509, 2001.
- 32 Crable SC, Hammond SM, Papes R, Rettig RK, Zhou GP, Gallagher PG, Joiner CH, Anderson KP: Multiple isoforms of the KCl cotransporter are expressed in sickle and normal erythroid cells. *Exp Hematol* 2005;33:624-631.
- 33 Lauf P, Valet G: Cation transport in different volume populations of genetically low K⁺ lamb red cells. *J Cell Physiol* 1980;104:283-293.
- 34 Lauf P, Bauer J, Adragna N, Fujise H, Zade-Oppen A, Ryu K, Delpire E: Erythrocyte K-Cl cotransport: Properties and regulation. *Am J Physiol* 1992;263:C917-C932.
- 35 Delpire E, Lauf P: Kinetics of Cl⁻-dependent K fluxes in hyposmotically swollen low K sheep erythrocytes. *J Gen Physiol* 1991;97:173-193.
- 36 Owen NE, Prastein ML: Na/K/Cl cotransport in cultured human fibroblasts. *J Biol Chem* 1985;260:1445-1451.

- 37 Mercado A, de los Heros P, Vazquez N, Meade P, Mount DB, Gamba G: Functional and molecular characterization of the K-Cl cotransporter of *Xenopus laevis* oocytes. *Am J Physiol Cell Physiol* 2001;281:C670-680.
- 38 Lauf PK: Volume and anion dependency of ouabain-resistant K-Rb fluxes in sheep red blood cells. *Am J Physiol* 1988;255:C331-339.
- 39 Lauf PK: Foreign anions modulate volume set point of sheep erythrocyte K-Cl cotransport. *Am J Physiol* 1991;260:C503-512.
- 40 Lauf P, Theg B: A chloride dependent K⁺ flux induced by N-ethylmaleimide in genetically low K⁺ sheep and goat erythrocytes. *Biochem Biophys Res Comm* 1980;92:1422-1428.
- 41 Lauf PK, Warwar R, Brown TL, Adragna NC: Regulation of Potassium Transport in human lens epithelial cells. *Exp Eye Res* 2006;82:55-64.
- 42 Bianchini L, Fossat B, Porthe-Nibelle J, Lahlou B: Activation by N-ethylmaleimide of a Cl-dependent K⁺ flux in isolated trout hepatocytes. *J Exp Biol* 1991;157:335-348.
- 43 Gagnon KB, England R, Delpire E: Characterization of SPAK and OSR1, regulatory kinases of the Na-K-2Cl cotransporter. *Mol Cell Biol* 2006;26:689-698.
- 44 Lauf PK, Adragna N: A thermodynamic study of electroneutral K-Cl cotransport in pH- and volume-clamped low K sheep erythrocytes with normal and low internal magnesium. *J Gen Physiol* 1996;108:341-350.
- 45 Johnson JH, Dunn DP, Rosenberg RN: Furosemide-sensitive K⁺ channel in glioma cells but not neuroblastoma cells in culture. *Biochem Biophys Res Commun* 1981;109:100-105.
- 46 Gillen C, B IF: Functional interaction of the K-Cl cotransporter (KCCl) with the Na-K-2Cl cotransporter in HEK-293 cells. *Am J Physiol* 1999;276:C-328-C336.
- 47 Lytle C, McManus T: Coordinate modulation of Na-K-2Cl cotransport and K-Cl cotransport by cell volume and chloride. *Am J Physiol Cell Physiol* 2002;283:C1422-1431.
- 48 Lauf PK: Evidence for chloride-dependent potassium and water transport induced by hyposmotic stress in erythrocytes of the marine teleost *Opsanus Tau*. *J Comp Physiol* 1982;143:9-16.
- 49 Delpire E, Lauf P: Magnesium and ATP dependence of K:Cl cotransport in low K sheep red cells. *J Physiol (Lond)* 1991;441:219-231.
- 50 Lauf PK: K⁺:Cl⁻ cotransport: sulfhydryls, divalent cations, and the mechanism of volume activation in a red cell. *J Membr Biol* 1985;88:1-13.
- 51 Kaji D, Kahn T: Kinetics of Cl-dependent K influx in human erythrocytes with and without external Na: effect of NEM. *Am J Physiol* 1985;249:C490-496.
- 52 Garay RP, Nazaret C, Hannaert PA, Cragoe EJ Jr: Demonstration of a [K⁺,Cl⁻]-cotransport system in human red cells by its sensitivity to [(dihydroindenyl)oxy]alkanoic acids: regulation of cell swelling and distinction from the bumetanide-sensitive [Na⁺,K⁺,Cl⁻]-cotransport system. *Mol Pharmacol* 1988;33:696-701.