Lithium Fluxes Indicate Presence of Na-Cl Cotransport (NCC) in Human Lens Epithelial Cells

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Key Words

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Abstract

During regulatory volume decrease (RVD) of human lens epithelial cells (hLECs) by clotrimazole (CTZ)-sensitive K fluxes, Na-K-2Cl cotransport (NKCC) remains active and K-Cl cotransport (KCC) inactive. To determine whether such an abnormal behavior was caused by RVD-induced cell shrinkage, NKCC was measured in the presence of either CTZ or in high K media to prevent RVD. NKCC transports RbCl + NaCl, and LiCl + KCl; thus ouabain-insensitive, bumetanide-sensitive (BS) or Cl-dependent (CID) Rb and Li fluxes were determined in hyposmotic high NaCl media with CTZ, or in high KCl media alone, or with sulfamate (Sf) or nitrate as Cl replacement at varying Rb, Li or Cl mol fractions (MF). Unexpectedly, NKCC was inhibited by 80% with CTZ ($IC_{50} = 31 \mu M$). In isosmotic (300 mOsm) K, Li influx was $\sim 1/3$ of Rb influx in Na, 50% lower in Sf, and bumetanide-insensitive (BI). In hypotonic (200 mOsm) K, only the CID but not BS Li fluxes were detected. At Li MFs from 0.1-1, Li fluxes fitted a bell-shaped curve maxing at ~ 0.6 Li MF, with

the BS fluxes equaling $\sim 1/4$ of the CID-Li influx. The difference, i.e. the BI/CID Li influx, saturated with increasing Li and Cl MFs, with K_{ms} for Li of 11 with, and 7 mM without K, and of ~ 46 mM for Cl. Inhibition of this K-independent Li influx by thiazides was weak whilst furosemide ($<100 \mu M$) was ineffective. Reverse transcription polymerase chain reaction and Western blots verified presence of both NKCC1 and Na-Cl cotransport (NCC). In conclusion, in hyposmotic high K media, which prevents CTZ-sensitive K flux-mediated RVD in hLECs, NKCC1, though molecularly expressed, was functionally silent. However, a K-independent and moderately thiazide-sensitive CID-Li flux, i.e. LiCC, likely occurring through NCC was detected operationally and molecularly.

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Introduction

During hyposmotic swelling, nucleated erythrocytes undergo a relatively slow regulatory volume decrease (RVD) whereby the Na-K-2Cl cotransporter (NKCC) becomes inactivated [1] and the K-Cl cotransporter (KCC) activated [1, 2]: the so called 'coordinate modulation of the two cotransporters' [1]. In contrast,

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hyposmotically swollen hLECs undergo a rapid RVD mediated by activation of clotrimazole (CTZ)-sensitive K-fluxes associated with obligatory anion and cell water loss leading to cell shrinkage, a process attenuated by increasing extracellular K. Upon completion of RVD, NKCC and KCC remain shrinkage-activated and -inactivated, respectively [3]. With the exception of work on KCC-transfected human epithelial kidney (HEK293) cells [4], little attention has been paid to the NKCC and KCC interaction during RVD in non-erythrocytic cells.

The initial purpose of the present work was to test in hLECs the behavior of NKCC by influx measurements of Rb (a K congener) when RVD is pharmacologically inhibited by CTZ. However, to our surprise, CTZ inhibited not only ouabain-plus bumetanide-insensitive K efflux and Rb influx but also 80% of the NKCC activity. To understand why exposure of hLECs to hypotonic media lead to activation instead of inactivation of NKCC as well as the mechanism of its CTZ inhibition, we used Li and Rb as Na and K congeners, respectively. Our results are the first report on the functional and molecular presence of Na-Cl cotransport (NCC) in hLECs. Earlier, NCC was found in flounder urinary bladder [5], mammalian renal distal convoluting tubules [6-8] and intestine [9], as NCC transcripts in the flounder eye [10] and various other tissues [11], and in human osteoblasts [12]. Part of this work was presented orally and as abstracts at the 2007 International Conference on Cell Volume and Signaling, Salzburg/Austria and the 2007 Cooperative Cataract Research Group, Kona, HI.

Materials and Methods

Reagents

A) Chemicals. Analytical grade chemicals such as NaNO₃, MgCl₂, perchloric acid (PCA), dimethyl sulfoxide (DMSO), trishydroxymethylaminomethane (TRIS), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), KCl, NaOH, and fetal bovine serum (FBS) were procured from Fisher Scientific (Fair Lawn, NJ); ultrapure RbCl and RbNO₃ from Alfa (Danvers, MA); LiCl, LiNO₃, and NaCl from Calbiochem (La Jolla, CA); bovine serum albumin (BSA), CaCl₂, N-methyl-D-glucamine (NMDG), sulfamic acid, and 3-[N-morpholino]propane sulfonic acid (MOPS) from SIGMA Chemical (St. Louis, MO); and CsCl, glucose, penicillin, streptomycin, and amphotericin from Invitrogen-Life Technologies, Inc. (Carlsbad, CA).

B) Inhibitors. Clotrimazole (CTZ), ouabain, bumetanide, hydrochlorothiazide (HCTZ) and metolazone (MTZ) were procured from Sigma Chemicals, St. Louis, MO, and furosemide from Hoechst Roussel Pharmaceuticals, Somerville NJ.

C) Molecular and Immunological Tools. A High Pure RNA Isolation Kit was purchased from Roche Diagnostics (Indianapolis IN), TaqMan® Reverse Transcription Reagents were obtained from Applied Biosystems (Branchburg, NJ). FastStart PCR Master Mix was purchased from Roche Diagnostics (Indianapolis IN). Human primers for NKCC1 and NCC were purchased from Integrated DNA Technologies (Coralville, IA). The sequences of the NKCC1 sense and antisense primers were TGGTTGTTTCGAGATGCCACTG and TGT AGC CAC GAA GAG GTT CA, respectively and the expected product size was 330 bp. The forward and reverse primers for NCC were TCC AGATGG CAC CTT CTT CGGAAT and TTG TTC TTG CCA TAG CCT TTG CCG, respectively, with a product size of 503 bp. Agarose for electrophoresis was obtained from Invitrogen (Carlsbad, CA). The Mem-PER protein extraction kit, Halt™ protease inhibitors cocktail and PAGEprep™ protein clean up kit were obtained from Pierce Biotechnology (Rockford, IL).

For Western blotting, a horseradish peroxidase (HRP)-coupled donkey anti-rb IgG (H+L) was procured as secondary antibody from Jackson Immunoresearch Laboratories (West Grove, PA). The primary rabbit antibodies (TF4 and anti-NTD) directed against the C- and N-termini of rat NKCC1 was a gift from Dr. Chris Lytle, Riverside, CA, whereas the commercial primary anti-NCC against the N-terminus of rtNCC was obtained from Chemicon International (Temecula, CA). Lumi-Light Western Blotting substrate was obtained from Roche Diagnostics (Indianapolis IN) and the Fujifilm Super RX autoradiography film from Fisher Scientific (Fair Lawn, NJ).

Solutions and Media

Balanced salt solution (BSS-NaCl) consisted of 20 mM HEPES/TRIS buffer pH 7.4 containing (in mM): NaCl (132), KCl (5), CaCl₂ (2), MgCl₂ (1), and glucose (10). In BSS-NaNO₃ and BSS-Na-Sf (sulfamate) media, NO₃ or methyl-Sf, respectively, replaced Cl in K, Rb, Li and Na salts, and gluconate in Ca and Mg salts. In BSS-NMDG-Cl or Sf NMDG substituted for Na on an equi-osmolar basis. Stock solutions (M) of ouabain (2x10⁻¹), bumetanide (2x10⁻³), and HCTZ and MTZ were made in DMSO or ethanol and all other chemical reagents dissolved in deionized water. The 300 mOsM washing solution contained 112 mM MgCl₂ and 10 mM TRIS/MOPS pH 7.4 at 4°C. Osmolalities were determined with an Advanced™ Micro-Osmometer, Model 330 (Advanced Instruments, Inc. Norwood, MA).

Human lens epithelial cells (hLECs)

Primary cultures of human lens epithelial cells (FHL124) were kindly donated by Professor John Reddan, Oakland University, MI. The characteristics and relatedness of these cells to fresh human epithelial cells and the exact procedure to culture them was recently reported [3]. Cells were used at passages between 7 and 22 and seeded on gelatin-coated 12-well culture plates at required densities.

Ion fluxes

It is well established that NKCC transports Rb for K as well as Li for Na ions [13-15]. Hence the economically and environmentally friendlier Li ion was chosen to monitor NKCC

activity in the presence of high extracellular K to reverse K efflux and RVD. Chloride and Cl-replacement by the non-transported anions Sf or NO₃ were used to either define the Cl-dependent Li flux, or, in addition to bumetanide, to calculate the BS Li flux through NKCC (CID-LiKCC and BS-LiKCC, respectively). The general strategy adapted from previous publications [16, 17] was to remove the culture media from the 12-well plates, wash the confluent FHL124 cell cultures with BSS-NaCl at 37°C and equilibrate them with BSS-NaCl/NaSf-BSA or BSS-NMDGCl/NMDGSf for 10 min at 37°C to permit manipulations needed to alter the transport rates such as replacing Cl with Sf or NO₃, adding inhibitors or pre-exposing the cells to BSS-NaCl-BSA with different osmolalities. Thereafter, cells were exposed, usually for a period of 5-15 min to the following flux media: For Rb influx, the K congener ⁸⁵Rb has been shown in many of our previous publications to accurately substitute for K in these uptake measurements, and the media were BSS-Rb/XCl-BSA or BSS-Rb/XSf-BSA where X stands for either Na or Li used at varying concentrations as described in Results. For Li influx, the non-isotopic ion was used as in previous studies in erythrocytes [14, 18, 19] and the flux solutions were BSS-LiYCl-BSA or BSS-LiYSf/NO₃-BSA where Y stands for K or NMDG used at concentrations mentioned in Results. Details deviating from these procedures are addressed in the description of the experiments and also noted in the Figure Legends. Rb and Li uptake were stopped by washing the cells with the ice-cold washing solution (see Media). Ions were extracted with 5% perchloric acid and protein was determined after solubilization in 1 N NaOH using the bicinchonic acid (BCA) method. Li was measured by atomic absorption at 670.8 nm and Rb by flame emission at 780 nm using a Perkin Elmer 5000 atomic absorption spectrophotometer [18]. Rb or Li uptake are expressed in nmoles/mg protein, and the fluxes in nmoles/mg protein per 5 and 15 min, respectively. To account for inter-experimental variability in primary cultures of lens epithelial cells, all experiments were carried out with the appropriate controls.

The Rb/Li flux components were determined as: (1) Total Rb/Li flux (Total), without inhibitors; (2) Ouabain-insensitive Rb/Li flux in presence of 0.1-1 mM ouabain (OI); (3) [Ouabain + bumetanide (10 μM)]-insensitive Rb/Li flux (O+B)I; (4) Na/K (Li/K) pump (OS) = (Total – OI); (5) Bumetanide-sensitive (BS) Na-K-2Cl cotransport (BS-NRbCC or BS-LiKCC) = (OI) – (O+B)I; (6) Chloride-dependent (CID) NRbCC or LiKCC = (OI in Cl) – (OI in Sf) without bumetanide; (7) K-Cl cotransport (RbCC) = (O+B)I in Cl – (O+B)I in Sf; (8) CIDLiCC = Li influx in MDGCl – Li influx in MDGNO₃ ± 10 μM bumetanide.

Reverse Transcriptase Polymerase Chain Reaction

Total RNA was isolated from FHL124 cells using High Pure RNA Isolation Kit, as recommended by the manufacturer. 0.5 μg of DNase digested RNA was used to synthesize cDNA using TaqMan® Reverse Transcription Reagents as per manufacturer's instructions. PCR was performed using 1X FastStart PCR Master Mix, 100 ng cDNA and 300 nM each of forward and reverse primers. The mixture was transferred to an Applied Biosystems GeneAmp 2700 thermocycler and cycled as follows: denaturation by heating to 95°C for 4 min, followed

by 40 cycles each for denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and elongation at 72°C for 30 sec. A final extension was performed by heating to 72°C for 7 min. RNA and primers were denatured by incubating at 65°C for 5 min, and placed on ice. The PCR products were verified by electrophoresis in 1% agarose containing ethidium bromide.

Membrane protein extraction, SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting

Membrane proteins were extracted from cultured FHL-124 cells using Mem-PER® eukaryotic protein extraction reagent kit in the presence of the Halt™ protease inhibitors cocktail, and SDS PAGE and protein band luminescence development as described recently [3]. Anti-NCC and anti-NKCC antibodies were used at 1:2000 and 1:200 fold dilutions, respectively. HRP-coupled donkey anti-rabbit IgG (Jackson Laboratories) as secondary antibody was used in a 1:5000 dilution for 90 min.

Statistical Analysis. Unpaired or paired Student's t-tests and 1-way ANOVA tests for multiple intergroup differences were calculated with STATISTIX 7™ (Analytical Software, Tallahassee, FL) and Origin™ (Originlab, Northampton, MA). P-values are indicated in the figure legends with p<0.05 considered statistically significant. Error bars are in general SE values for a mean of 4 or greater as indicated in the legends.

Results

Human LECs undergo RVD in hyposmotic Na media, mediated apparently by intermediate conductance (I_K) K channels since CTZ inhibited ouabain- and bumetanide-insensitive K efflux and Rb influx [3]. Thus inhibition of I_K should have prevented K loss, cell shrinkage and, hence, would have been commensurate with the volume regulation paradigm [1], as outlined earlier [3], by causing NKCC inactivation (and KCC activation). However, as shown in Figure 1, the ouabain-insensitive BS-NKCC activity as measured by Rb influx was inhibited by CTZ by about 80% of the control in both 300 or 150 mOsM high Na flux media with an apparent K_i of 31 μM. This finding, though in itself interesting, made CTZ unsuitable to study the response of NKCC and KCC in hyposmotically-stressed hLECs.

As an alternate to CTZ, and since Li is accepted in place of Na by NKCC [13-15], we used this ion to study NKCC activity in low osmolality media with high external K concentrations, [K]_o, to counteract the I_K-mediated cell shrinkage [3]. Figure 2A shows the Li uptake time courses in hLECs exposed either to 300 or 200 mOsM high KCl or KSf media containing 10 mM Li, and 1 mM ouabain to inhibit the Na/K pump. Because Li uptake was near linear up to 15 min, Li fluxes were terminated at this time point

Fig. 1. Rb influx through bumetanide-sensitive NKCC as a function of clotrimazole (CTZ) concentration. LECs were incubated for 5 min in the presence of 0.1 mM ouabain, 10 μ M bumetanide with or without the indicated CTZ concentrations in 150 mOsM (closed squares) and 300 mOsM (open circles) Na flux media. Insert: Dixon plot to estimate the apparent K_i of 31 μ M. Mean $\pm$ standard error (SE), $n=4$. Non-linear and linear function plots by OriginTM. In some cases error bars were smaller than symbols.

as opposed to 5 min for the larger Rb influxes (see below). Results show a large Cl-dependent Li uptake in Cl and Sf in 300 but not in 200 mOsM media which appears to be based in part on an increased Li flux in 200 mOsM Sf media. Figure 2B shows more clearly these results. The hyperbolic Cl-dependent Li uptake presumably through NKCC in the form of LiKCC was abolished in 200 mOsM media suggesting that preventing I_{K^+} -channel-mediated RVD and associated shrinkage in high $[K]_o$, caused the expected inhibition of LiKCC/NKCC by hypotonic media.

Figure 3 compares the major components of both Rb and Li influxes at 5 min in 300 (open columns) and 200 (black columns) mOsM solutions in high Na (upper panel) and high K (lower panel) media. Total uninhibited Rb influx was ~ 3 -fold greater than Li influx, a difference in large part due to the expected absence of any sizable Li influx through the Na/K pump at high $[K]_o$ (lower panel), and consistent with a $\sim 50\%$ of the total Rb influx through the Na/K pump (upper panel), as shown earlier for B3 hLECs [17]. The BS-NRbCC in both 300 and 200 mOsM Na media matched the CID-NRbCC (upper panel), consistent with our hypothesis that, during RVD in Na media, NKCC remains active [3]. Rb flux through the calculated bumetanide-insensitive (BIS) KCC, i.e. BIS-RbCC, was larger in 300 than in 200 mOsM high Na media, which again supports the notion that, during RVD, KCC activity is reduced by shrinkage.

The difference between the total Rb and Li influxes can be ascribed to the absence of both significant Li/K pump activity and a BS-LiKCC at both osmolalities (lower panel). However, about 50% of the total Li influx in 300 mOsM high $[K]_o$ was due to a CID-LiKCC, which, commensurate with the data of Figure 2, was abolished in 200 mOsM high K media ($[K]_o = 85$ mM) where cell shrinkage mediated by the I_{K^+} -channel was absent [3]. In contrast, the calculated BIS-LiKCC was not different from the CID-LiKCC in 300 mOsM high K media and was absent in hypotonic medium (200 mOsM). Whereas CID-LiKCC, as defined by relationship 6 in Material and Methods, behaves operationally as NKCC, the remaining

Fig. 2. Li uptake in FHL124 LECs versus time in 300 and 200 mOsM high KCl and K-Sf (Sulfamate) media. LECs were incubated for the indicated time periods at 37°C in iso-ionic high K flux solutions (see Material and Methods) of 300 mOsM (100 mOsM sucrose, squares) and 200 mOsM (sucrose-free, triangles) BSS-Li-KCl-BSA (closed symbols) or BSS-Li-KSf-BSA (open symbols) in the absence of inhibitors. A: Li uptake at 10 mM external Li, $[Li]_o$. B: Calculated CID-LiKCC at 300 mOsM (filled squares) and 200 mOsM (filled triangles). Mean $\pm$ SE, $n=4$. Non-linear function plots by OriginTM. See Methods for definition of the different flux components.

Fig. 3. Rb and Li influx in iso- and hyposmotic high Na or high K media. Rb or Li uptake (5 min at 37°C) in Cl or Sf flux media (see Material & Methods) with 10 mM Rb or 10 mM Li replacing Na or K, respectively, and 0.1 mM ouabain $\pm$ 5 μ M bumetanide for inhibition of the Na/K pump and NKCC. Upper and lower panels: Calculated Rb and Li influx components: Uninhibited (Total), Na/K pump (Pump), bumetanide-sensitive NKCC (BS-NRbCC or BS-LiKCC), chloride-dependent NKCC (CID-NRbCC or CID-LiKCC), bumetanide-insensitive and Cl-dependent KCC (BIS-RbCC) and LiCC (BIS-LiCC), and BIS- and Cl-insensitive Rb or Li leak (Leak) in 300 (white columns) and 200 mOsm (black columns) media. Mean $\pm$ SE, n=4. * for $p < 0.05$.

calculated BIS-LiKCC cannot be ascribed to the NKCC activity but must constitute a CID- and BIS Li flux in the presence of K through another coupled, K-independent transporter, perhaps the NCC. The “leak” is the unaccounted flux of both Rb and Li through a pathway not affected by the inhibitors used and by Cl replacement with Sf. The upper panel shows a minimal Rb leak influx, which was about two-fold higher in 200 than in 300 mOsm high Na media ($p < 0.05$), due to activation by hyposmotic swelling. In contrast, the Li leak flux rather decreased at lower osmolality ($p < 0.05$) but was still more than twofold larger than the Rb leak influx, a finding attributed to the presence of volume-independent Na channels and Na/Na (Li) exchange mechanisms [20].

The data in Figures 2 and 3 revealed that Li indeed can be effectively used to demonstrate that, once shrinkage induced by I_K channels in hyposmotic medium is prevented by high $[K]_o$, NKCC can be inactivated in hyposmotic high K media with $[K]_o$ values close to the reversal K concentration for these fluxes [3]. An unexplained finding was that only CID-LiKCC was present but not BS-LiKCC (Figure 3, lower panel), strongly contrasting with the presence of a BS-NKCC for Rb influx (Figure 3, upper panel). From work on erythrocytes [13, 21, 22], it is well established that BS-NKCC activity displays a bell-shaped function of the Na molar fraction (MF), with zero K fluxes in zero or all Na media, consistent with the mutual cotransport of Na and K through this system. If, indeed, BS- or CID-Li influx occurs only through NKCC, a similar dome-shaped dependence of LiKCC as a function of the Li MF ($[Li]_o/([Li]_o + [K]_o)$) should be found. Figure 4A shows that, as in the erythrocyte, indeed the BS-LiKCC, calculated as described in Methods from Li fluxes in 1 mM ouabain-containing media in the presence and absence of 10 μ M bumetanide, was zero at 0 and 1, with

Fig. 4. Bumetanide-sensitive and chloride-dependent Li influx as a function of Li MF in isosmotic media. LECs, pre-equilibrated at 37°C for 10 min in 300 mOsm BSS-KCl or -KNO₃ solutions, were fluxed at 37°C in either Cl or NO₃, containing 0–1 Li MF in exchange for K, and 1 mM ouabain $\pm$ 10 μ M bumetanide for inhibition of the Na/K pump and NKCC, respectively. A. Bumetanide-sensitive Li influx through NKCC (BS-LiKCC, squares) is the calculated difference between Li influx in the presence of ouabain and [ouabain+bumetanide]. Chloride-dependent Li influx (CID-LiKCC, triangles and circles from 2 independent experiments) was calculated from the difference between ouabain-insensitive Li influx in Cl and NO₃. Results are expressed as mean $\pm$ SE (n=4) for each independent experiment. B. Calculated bumetanide-insensitive Cl-dependent Li influx (BIS-CID-LiKCC) as a function of Li MF with K replacement. Insert: Lineweaver-Burke plot of 1/LiKCC versus 1/[Li]_o MF: $V_m = 26.6$ nmol Li/[mg protein $\times$ 15 min], $K_m = 10.9$ mM [Li]_o, $r = 0.979$. Mean $\pm$ SE, n=8.

Fig. 5. Lack of significant inhibition of ouabain-insensitive and Cl-dependent Li influx (LiCC) by loop diuretics. Human LECs, pre-equilibrated at 37°C for 10 min in 300 mOsM BSS-NaCl solutions, were washed in isosmotic N-methyl-D-glucamine (NMDG) prior to 15 min flux in solutions with 0.6 Li/0.4 NMDG MF ratios with either Cl (squares) or NO₃ (circles) containing bumetanide (A) and furosemide (B). LiCC is the calculated Li flux difference between Cl and NO₃ (triangles). Mean $\pm$ SE, n=4.

a maximum around 0.5 Li MF, and thus behaved as predicted for NKCC cotransporting Na+K in stoichiometric unity with 2 Cl [13, 23]. The Cl-dependent Li influx (Cl-LiKCC) also was low near zero Li MF, but then increased to ~ 3 times larger values than the BS-LiKCC at 0.5-0.6 Li MF (~ 40 versus 12 nmol/mg protein $\times$ 15 min) without ever declining to zero at 1 Li MF, i.e. exhibiting an apparent bumetanide-insensitive Cl-dependent Li influx (BIS-Cl-LiKCC). This apparent BIS-Cl-LiKCC, plotted in Figure 4B, clearly saturated with increasing Li MF, and from the Lineweaver-Burke plot in the insert, a V_m of 26.6 nmol/mg protein $\times$ 15 min and a K_m value of 10.9 mM Li were calculated. Since the apparent BIS-Cl-LiKCC saturated with Li and decreasing K MF, the K_m must represent mainly a value for Li alone, with small, if any, interference by K. Since BS-LiKCC = 1/3 Cl-LiKCC, i.e. the majority of NKCC-mediated Li influx was bumetanide-insensitive, and apparently K-independent. Hence Li must have been cotransported with Cl through a Na-Cl cotransport (NCC) mechanism, i.e. as LiCC, instead as LiKCC.

In the absence of K, Li influx in either Cl or NO₃ was insensitive to both loop diuretics, bumetanide (Figure 5A) and furosemide (Figure 5B). The resulting calculated Cl-dependent Li fluxes (LiCC) were not affected by bumetanide or furosemide at concentrations well known to inhibit NKCC as well as KCC.

Because hLECs appeared to possess a Cl-dependent, K independent and loop diuretic-insensitive Li influx that may involve NCC, it was decided to measure the affinity for Li and Cl ions supporting the Li influx through this putative NCC/LiCC system. Figure 6A shows the [Na+K]-independent and 1 mM ouabain-insensitive Li influx saturating as a function of the Li MF in Cl with (filled triangles) and without 10 μ M bumetanide (filled squares), and also in NO₃ (filled circles). The difference between Li influx in Cl and in NO₃ is the Cl-Li influx or LiCC which, shown in Figure 6B, saturated with the Li MF yielding a V_m of 22 nmol/mg protein $\times$ 15 min and a K_m of 7.7 mM Li (see insert in B). The fact that saturation kinetics occurred even in NO₃ indicates that the so-called Li leak influx mentioned earlier indeed may involve other

Fig. 6. (Na + K)-independent Li Influx in N-MDG-Cl or -NO₃ with 0-1 Li MFs and effect of 10 μ M bumetanide. HLECs were exposed to 300 mOsM NMDG-Cl or -NO₃ with 0-100% Li MFs $\pm$ 10 μ M bumetanide, and 1 mM ouabain, for 15 min at 37°C. A. Ouabain-insensitive Li influx in Cl (squares), and with bumetanide (triangles), and in NO₃ (circles). B. Cl-dependent Li influx, LiCC is the calculated difference between Cl (minus bumetanide) and NO₃. Insert: Lineweaver-Burke plot of 1/Li influx versus 1/[Li]_o mol fraction. V_m = 22.6 nmol Li/[mg protein $\times$ 15 min] and K_m = 7.7 mM [Li]_o, r = 0.992. Mean $\pm$ SE, n=8.

Fig. 7. Cl-dependence of Li influx in the absence of K and inhibitors. A: Total Li influx in Cl as function of Cl MF. B: Calculated Cl-dependent Li influx, LiCC, and C: 1/LiCC versus $1/[Cl]_o$ (in mmol/L). $V_m = 30 \text{ nmol Li/mg protein} \times 15 \text{ min}$, and $K_m = 37 \text{ mM}$ external Cl, $[Cl]_o$. Mean $\pm$ SE, $n=4$.

carrier-mediated mechanisms, such as Na/Na(Li) exchange [20]. Figure 7A shows that the total Li influx in Cl and in the absence of K can be separated into a larger Cl-independent and a smaller Cl-dependent fraction, the former an unknown Cl-independent Li transporter not further pursued in this study (Li/Na exchange), whereas the latter appears to be LiCC. Figure 7B shows a hyperbolic saturating behavior of the calculated LiCC versus the Cl MF, resulting in a V_m of near $30 \text{ nmol/mg protein} \times 15 \text{ min}$ and a K_m of 37 mM for the activation of Li influx by Cl (Figure 7C). Under slightly different experimental conditions, K_m values for Cl activation of LiCC were $46 \pm 12 \text{ mM}$ (mean $\pm$ SE, $N=3$) suggesting that the apparent affinity for Cl may be at least 5-6 times lower than for Li.

Pharmacological proof for Li flux through NCC is shown in Figures 8 and 9. In Figure 8A, the thiazide derivative metolazone (MTZ) inhibited in a dose dependent manner LiCC calculated as the difference of the fluxes in Cl and NO₃ (closed triangles) at concentrations $< 1 \mu\text{M}$, but was less effective at higher concentrations. Similar, but somewhat weaker inhibition was obtained with hydrochloride thiazide (HCTZ) as shown in Figure 8B. Both drugs have been shown to inhibit in nano- and micro-molar concentrations ratNCC and fNCC in transfected *Xenopus* oocytes [6]. Hypothesizing that the biphasic dose response curve of LiCC to MTZ could be due to modulation by Li, the effect of varied external Li concentrations was tested at constant MTZ

Fig. 8. Thiazide inhibition of K-independent Li influx. Li influx in 300 mOsm Cl (squares) and NO₃ (circles) with 20 mM Li versus $0.1\text{--}50 \mu\text{M}$ metolazone (MTZ) (A) and $0\text{--}100 \mu\text{M}$ hydrochlorothiazide (HCTZ) (B) concentrations. LiCC (triangles) is the calculated Li flux difference in Cl and NO₃. Mean $\pm$ SE, $n=4$. * $p<0.05$.

Fig. 9. Metolazone (MTZ) ($1 \mu\text{M}$) inhibition of K-independent Li influx in isosmotic Cl and NO₃ with increasing Li concentrations. Li fluxes in Cl (squares) and NO₃ (circles) and calculated LiCC (triangles) in controls (A, open symbols) and $1 \mu\text{M}$ MTZ-treated (B, closed symbols) hLECs. C: Comparison of LiCC before (open triangles) and after $1 \mu\text{M}$ MTZ (closed triangles). Non-linear plots by Origin™. Mean $\pm$ SE, $n=4$.

Fig. 10. K-independent Li influx in Cl (closed squares) and NO₃ (open squares) media of different osmolalities. LiCC = calculated difference of Li influx in Cl and NO₃ with 0.1 mM ouabain and 10 μ M bumetanide (filled circles). Mean $\pm$ SE, n=4.

(1 μ M). Figure 9A shows Li influx in Cl and NO₃ and LiCC, the calculated difference between the two, and Figure 9B the same fluxes in the presence of 1 μ M MTZ. Note that the expected hyperbolic saturation of LiCC as a function of the external Li concentration (panel A) changed to an inverse hyperbolic function in the presence of the drug. Figure 9C compares the calculated LiCC in the absence (open triangles, Figure 9A) and presence of MTZ (closed triangles, Figure 9B): the greatest difference between these two components, i.e. the MTZ-sensitive LiCC was between 10 and 30 mM external Li, and higher Li concentrations reduced the inhibition by MTZ.

Human LECs on gelatin support base were resilient to hyposmotic but not hyperosmotic solutions which tended to displace these cells from their gelatin base. Thus only studies in hyposmotic solutions were performed. Figure 10 shows ouabain- and bumetanide- insensitive Li influx in Cl and NO₃ media at different osmolalities and at equal ionic strength with sucrose compensating for the osmolality gaps. Whereas there was little osmolality dependence of Li influx in Cl, a sharp rise of Li influx in NO₃ at 150 mOsM was responsible for the negative LiCC values calculated from the differences in the fluxes between Cl and NO₃. In this experiment, LiCC was of smaller magnitude than shown in previous ones addressing different modalities of the transporter. This variability may be related to the late passage number (usually >20) used in this case.

To further document that hLECs possess, in addition to NKCC, a K-independent, Cl-dependent and loop diuretic-insensitive Li influx, possibly mediated by NCC, i.e. a LiCC transport mechanism, molecular studies were

Fig. 11. Molecular evidence for simultaneous presence of NKCC1 and NCC in FHL124 LEC membrane extracts. A: RT-PCR of hNKCC1. Lane (bp): 1, ladder (100); 2&3, NKCC1 (331); 4, β -actin (297); 5, -RT (negative control); and 6, ladder (1 kb). B: Western blot with rabbit anti-rtNKCC1 (TEFS-1, 1:250, NT (N-terminus) 1:250) + HRP-coupled donkey anti-rb IgG (1:4000). C: RT-PCR of hNCC. Lane (bp): 1, ladder (100); 2, β -actin (297); 3, -RT (negative control); 4, hNCC (504); 5, ladder (1 kb). D: Western blot with commercial rabbit anti-serum (1:2000) against the N-terminus of rtNCC + HRP-coupled donkey anti-rb IgG (1:5000). A rat kidney extract (left lane) served as positive control for the FHL cells (right two lanes). Numbers in B and D: Molecular weight standards in kDa.

performed. Figure 11A shows the 331 bp RT-PCR product expected for the hNKCC1 using sense and antisense primers listed in Material and Methods, and for β -actin as internal control (297bp). Figure 11B shows the corresponding Western blot on a hLEC membrane fraction with primary rb anti-rabbit anti-rtNKCC antibodies (TEFS-1/2 and NT, kindly provided by Dr. Lytle, UC Riverside, CA) and secondary HRP-coupled donkey anti-rb IgG. Both antibodies revealed a major protein band of >150 kDa which is consistent with the molecular weight of NKCC1 reported earlier [24], and a smaller, about 130 kDa protein breakdown product, which either is the deglycosylated form or some degradation product of the largest molecular weight peptide. Figure 11C shows the

results for the NCC RT-PCR product (504bp, lane 4) using sense and anti-sense primers listed in Materials and Methods and β -actin (lane 2) as positive internal and – RT (lane 3) as negative controls. The Western blot in Figure 11D demonstrates in two lanes the presence of anti-NCC positive protein bands, of which the large ca 170 kDa band was commensurate with the estimated molecular weight of a well glycosylated protein (see Discussion) based on the reported primary sequence (ca 1021 amino acids) whereas the smaller ca 100 kDa bands were likely proteolysis-derived peptides that were also present in the rat kidney sample (lane 1).

These molecular data establish for the first time that, in addition to NKCC1 predicted from a report in rabbit LECs [25], NCC is present in hLECs at both the mRNA and protein level matching the functional data of the existence of a K-independent and Cl-dependent Li flux through the NCC mechanism.

Discussion

The present study extends previous work on volume regulation in human LECs [3]. Our first attempt to measure NKCC activity in hyposmotic Na media in the presence of CTZ to block the rapid RVD mediated by K fluxes, apparently carried by I_K channels [3], could not be achieved due to the unexpected collateral inhibition of this transporter by the drug (Figure 1). To the best of our knowledge this is the first report that CTZ inhibits NKCC in a mammalian cell line. When CTZ was tested in NKCC1-expressing *Xenopus* oocytes (courtesy of Eric Delpire, Vanderbilt University), no such inhibition was seen (data not shown), a finding indicating that the action of CTZ depends on the cell model used. The effect of CTZ on NKCC (and possibly on the I_K channel) could be of indirect biochemical nature, for example through a transduction mechanism involving P450 cytochromes [26] modulating Ca channels during contraction in rat singular ventriculocytes [27], that are perhaps absent or less functional in *Xenopus* oocytes. This finding then raises the question whether cytochromes P450 controls in a yet to be defined manner the activity of NKCC1.

Lithium has been employed long time ago to assess NKCC in human enucleate and nucleated duck erythrocytes [14, 21, 28] and more recently in quail erythrocytes [29], and at least two Li transport routes (a Na/Li exchanger and a conductive Li pathway) have been found in the turtle colon [20]. Although Li reabsorption through Na/Li exchange and ENaC in the distal tubule has

been studied extensively [30], no reports exist, to our knowledge, on Li-cotransport in non-erythrocytic, cultured mammalian nucleated cells. From the uptake kinetics of Figure 2 and the comparison of Li influx with Rb influx in Figure 3, it may be concluded that Li served as a useful tool to study NKCC in hLECs and eventually supports the paradigm established in erythrocytes [1, 28], that NKCC is inactivated (and KCC activated) in swollen cells. However, at identical cation concentrations, the signal of Li was only about 1/3 of that of Rb which can be explained, in part, by the virtual absence of Na/K pump mediated Li influx and, in part, by a lower binding and transport rate than for Na as shown for NKCC [15]. Even though the affinity of NKCC in human erythrocytes for Li is greater than that for Na [13], we had to compensate for the lower Li fluxes in hLECs by extending the flux times from 5 to 15 min in most of the experiments shown.

It is commonly accepted that measurements of CID-NKCC and BS-NKCC yield similar values, the former usually somewhat larger due to the additional presence of KCC. However, in the experiments of Figures 2 and 3B, CID-LiKCC and, by definition, BIS-LiKCC were inactivated in hyposmotic high K media and thus contrasted clearly with the CID-NRbCC remaining active in high Na media (Figure 3A). This means that the high external K concentration (>57 mM), effectively reversing the outward flux of K through I_K channels, obligatory water loss and hence RVD [3] also prevented the activation of NKCC as measured by CID-Li influx. Thus the absence of a BS-LiKCC was surprising and contrary to the accepted paradigm stated above. This puzzle was resolved by the results of Figure 4A where minimal BS-LiKCC activity was found at the low Li MF used in Figure 3. As the Li MF was raised, BS-LiKCC behaved in a classic manner, as described for Na in human red blood cells [3, 13, 21, 22], in that its activity was maximal at 50 Li MF and fell to near zero in only Li media consistent with the established concept of this cotransporter that, in the absence of Na, cotransports both Li and K in a stoichiometric fashion [13, 21]. The new feature in Figure 4A is the emergence of a two-fold greater bumetanide-insensitive CID-LiKCC (BIS-CID-LiKCC) that was saturable. The saturation with increasing Li and decreasing K MF suggests the presence of a K-independent Cl-dependent mechanism, most likely through a constitutive Na-Cl cotransporter, NCC, i.e. via a BIS-LiCC.

Additional experiments in Figures 5-9 strengthen our proposal for the presence of NCC in hLECs. In the absence of external K, neither bumetanide nor

furosemide, at concentration sufficient to block NKCC as well as KCC, inhibited Li influx in Cl or NO₃ and hence had no effect on the calculated Li influx through the presumed NCC, i.e. LiCC (Figure 5A and B). Bumetanide also did not lower the Na-K-independent Li Influx in Cl to that observed in NO₃ as the Li MF was raised from 0 to 1 (Figure 6A) yielding a saturable CID-Li influx (CID-LiCC) (Figure 6B) with a V_m of 22 nmol/mg protein x 15 min, similar to that of Figure 3B in the presence of external K, and a K_m of 7.7 mM [Li]_o, a value lower than that of Figure 4B and closer to the K_m values reported for Na of NCC-cRNA transfected *Xenopus* oocytes [7]. In another experiment (not shown), a K_m of 8 mM [Li]_o was determined. These apparent Li affinities are close to the Na affinities reported in mammalian (rat) NCC but higher than in teleost urinary bladder NCC expressed in oocytes [5-7, 31], and distinguish the Li transporter described here from the transfected endogenous NKCC of HEK293 cells with K_m values for Na at least twice higher [32], and the Li/Na exchanger with almost one order of magnitude higher both, external and internal, Li affinities [33]. Finally, at similar V_m, the aggregate K_m value for external Cl required for activation of Li influx through the LiCC was 46 ± 12 mM (see Figure 7B and C) which is higher than the K_m values reported for Cl for the teleost urinary NCC in the *Xenopus* model [7, 10], however, within the range reported for endogenous NKCC1 in HEK293 cells [32]. Thus, the bulk of the flux data in Figures 1-8 support the notion of the presence of Li influx through a K-independent and Cl-dependent mode, i.e. through NCC rather than NKCC.

The Na-Cl cotransporter (NCC) belongs to the SLC12A family of solute carriers (SLC12A3) with its gene located on human chromosome 16q13 [34]. Its putative molecular structure of 1021 amino acids constitutes a > 150 kDa protein with 12 transmembrane domains (TMDs) containing a large double-glycosylated loop between TMDs 7 and 8, and cytoplasmic amino-terminal and carboxy-terminal domains (NTD and CTD, respectively) [7, 8, 10, 34]. Loss of NCC function in the renal distal convoluted tubule leads to Gitelman's syndrome with hypocalciuria, hypomagnesemia and hypokalemic alkalosis causing hypotension [8], and higher bone mean density that also appears in NCC-null mice [35]. A characteristic property of NCC is its inhibition by thiazide derivatives such as HCTZ and MTZ which exert their effect by involving in a concerted manner the TMDs 8-12 [7]. Figure 8A shows that MTZ, the most potent of these thiazide analogues (and less HCTZ (Figure 8B)) inhibited Li influx in Cl in a biphasic manner. The MTZ

action was attenuated by higher Li concentrations as shown in Figure 9.

There are at least *five* possible explanations for the paradoxical biphasic behavior of LiCC inhibition by MTZ. *First*, Li, a Na ion replacement (with a generally lower affinity for the Na binding site of the NKCC [15]), in combination with Cl, alters sterically the postulated shared binding sites for Cl as well as for thiazides: Lowering extracellular Na decreased the Cl affinity and lowering Cl decreases the Na affinity in rat NCC expressed in oocytes in support of a random binding order [7, 36]. *Second*, like NKCC1, regulation of NCC may involve unknown Cl/volume sensitive kinases (WNK protein kinases) [37, 38] where Li may affect signaling cascade components like in the K-Cl cotransport mechanism of red blood cells [39]. *Third*, the low Cl and biphasic MTZ affinity could herald a change in glycosylation known to affect thiazide binding: double-deglycosylated NCC mutants have a greater affinity for MTZ than single or double-glycosylated forms [6, 40]. The strong high molecular weight signal (>150 kDa) obtained (Figure 11) supports the conclusion that also in hLECs the glycosylation level of NCC may be a determinant of both thiazide and Cl affinities. *Fourth*, the presence of a NCC isoform different from that reported thus far for flounder and mammalian kidneys cannot be entirely excluded and molecular studies need to verify this possibility. Interestingly, several experimental mutations have shown to affect affinity as well as turnover of the NCC in the oocyte expression model [7]. The primers used to verify the presence of NCC by RT-PCR (Figure 11C, 504 bp product) encompass bp 1039 of the 5' sense primer and bp 1542 of the 3' antisense primer. Hence, no differences from the renal NCC isoform should be expected. However, the thiazide binding site encompasses molecular regions in the NCC that are down stream from the above regions [6, 7] and thus it is not possible to correlate at this point the low Cl affinity and the biphasic response of MTZ to inhibit LiCC, i.e. NCC, with a molecular aberration. However, if this is the case, LECs would be endowed with a relatively thiazide-insensitive NCC which is of interest in regard to the reported hypocalciuria combined with Ca retention in thiazide-treated patients [41, 42]. Teleologically, this would protect the lens from Ca overload, activation of its RVD-inducing I_K channels, and possibly apoptotic volume decrease, potentially contributing to the development of cataract. However, data on the incidence of cataract in thiazide-treated patients are controversial [43, 44]. *Lastly*, it is possible that the thiazide action involves regulation of NCC at the metabolic level since it

has been long known that thiazides inhibit mitochondrial carbonic anhydrase in human liver tissue [45].

There is little information available on the response of NCC to anisotonic challenge. In Figure 10, LiCC activity fell to negative values below 200 mOsm, a result entirely due to a further increase of a putatively unrelated Li influx in NO₃ by a mechanism still to be explored. This result would mean that, unlike the NKCC, the NCC system is resilient to cell swelling and exhibits more of a tonic activity, a conclusion at variance with rat NCC that seems to be inactivated by hypotonicity but activated by cell shrinkage [7]. Since at higher osmolalities these FHL124 cells tend to detach easily from their supporting gelatin, no further studies were carried out.

The molecular and immunochemical data of Figure 11 on the presence of the NKCC1 isoform in hLECs is novel in regard to hLECs in culture and consistent with the flux data, confirming the independent demonstration of NKCC1 in animal lens tissues [25].

In conclusion, in our search to understand the behavior and contribution of electroneutral cotransporters NKCC (and KCC) to volume control of hLECs, especially during inhibition of an I_K-channel-mediated RVD by high K media, we succeeded to inactivate Cl-dependent LiKCC1 activity as a measure of NKCC. In addition, we encountered an unexpected bumetanide-insensitivity of the majority of the Cl⁻ Li influx in K media (LiKCC). This flux persisted in the absence of K and exhibited K_m values for Li in the range of those reported

by others for Na in the NCC expressed in NCC-cRNA-transfected *Xenopus* oocytes [7], suggesting that LiCC is mediated by NCC. It remains to be seen whether the newly discovered NCC resides in the basolateral or luminal membrane of LECs *in vivo*. Conceivably within the lens, NCC inhabits the luminal membrane and thus by transporting inwardly NaCl would aid the Na/K pump in the movement of this solute from the lens fiber cell compartment toward the LEC layer at the anterior face of the lens, in accordance with general transport models provided for Na, Cl and water flow through the lens [46-48].

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