Apparent intermediate K conductance channel hyposmotic activation in human lens epithelial cells

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Lauf PK, Misri S, Chimote AA, Adragna NC. Apparent intermediate K conductance (IK) channel hyposmotic activation in human lens epithelial cells. Am J Physiol Cell Physiol 294: C820–C832, 2008. First published January 9, 2008; doi:10.1152/ajpcell.00375.2007.—This study explores the nature of K fluxes in human lens epithelial cells (LECs) in hyposmotic solutions. Total ion fluxes, Na-K pump, Cl-dependent Na-K-2Cl (NKCC), K-Cl (KCC) cotransport, and K channels were determined by 82Rb uptake and cell K (Kc) by atomic absorption spectrophotometry, and cell water gravimetrically after exposure to ouabain ± bumetanide (Na-K pump and NKCC inhibitors), and ion channel inhibitors in varying osmolalities with Na, K, or methyl-th-gluconamide and Cl, sulfamate, or nitrate. Reverse transcriptase polymerase chain reaction (RT-PCR), Western blot analyses, and immunohistochemistry were also performed. In isosmotic (300 mosM) media ~90% of the total Rb influx occurred through the Na-K pump and NKCC and ~10% through KCC and a residual leak. Hyposmotic media (150 mosM) decreased Kc by a 16-fold higher K permeability and cell water, but failed to inactivate NKCC and activate KCC. Sucrose replacement or extracellular K to 57 mM, but not Rb or Cs, in hyposmotic media prevented Kc and water loss. Rb influx equaled Kc loss, both blocked by clotrimazole (IC50 ~25 μM) and partially by 1-(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34) inhibitors of the IK channel KCa3.1 but not by other K channel or connexin hemichannel blockers. Of several anion channel blockers (dihydroindenyl)oxalkanoic acid (DIOA), 4-2(butyl-6,7-dichloro-2-cyclopentylindan-1-on-5-yl)oxbutyric acid (DCPPIB), and phloretin totally or partially inhibited Kc loss and Rb influx, respectively. RT-PCR and immunohistochemistry confirmed the presence of KCC3.1 channels, aside of the KCC1, KCC2, KCC3 and KCC4 isoforms. Apparently, IK channels, possibly in parallel with volume-sensitive outwardly rectifying CI channels, effect regulatory volume decrease in LECs.

K-Rb fluxes; Kc loss, volume regulation; Na-K-2Cl and K-Cl cotransport isoforms; reverse transcriptase polymerase chain reaction; immunohistochemistry

VOLUME CONSTANCY (VC) is a fundamental property of all living cells, and its maintenance involves adjustments of transmembrane ion and obligatory water flow through channels and transporters, processes coined volume regulatory decrease (RVD) and increase (RVI) in response to physiological or biochemical stimuli causing cell swelling and shrinkage, respectively (16, 25, 31, 33). This paradigm is expected to apply to the human lens epithelial cell (LEC), which as part of a monolayer under the lens capsule covering the anterior lens portion, critically contributes to lens integrity and hence transparency through LEC to lens fiber cell (LFC) transdifferentiation. In general, ion transporters (electroneutral or electroneutral K conductance Ca-activated K channels (IK, KCa3.1), detected by reverse transcriptase polymerase chain reaction (RT-PCR), Western blot analyses, and immunohistochemistry. Accordingly, K loss was prevented by replacing external Na with K ions by clotrimazole (CTZ) and partially by 1-(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34). Blockage by

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(dihydro-indenyl)oxy]alkanoic acid (DIOA), and phloretin and partial inhibition by high concentrations of 4-(2-butyl-6,7-dichloro-2-cyclopentyl)indan-1-0-5-yl]oxy]butyric acid (DCPIB), niflumic acid (NA), and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) suggest involvement of commensurate anion fluxes, possibly via volume-sensitive outward rectifying (VSOR) Cl channels.

**MATERIALS AND METHODS**

**Reagents**

Chemicals. Analytic grade chemicals such as NaNO3, MgCl2, perchloric acid (PCA), DMSO, Tris, HEPES, KCl, NaOH, and fetal bovine serum (FBS) were procured from Fisher Scientific (Fair Lawn, NJ); ultrapure RBcI and RbNO3 were from Alfa (Danvers, MA); NaCl was from Calbiochem (La Jolla, CA); bovine serum albumin (BSA), CaCl2, NEM, sulfamic acid, N-methyl-d-glucamine (NMDG), and 3-[N-morpholino]propane sulfonic acid (MOPS) were from Sigma Chemical (St. Louis, MO); and CsCl, glucose, penicillin, streptomycin, and amphotericin were from Invitrogen Life Technologies (Carlsbad, CA).

Inhibitors. Ouabain, bumetanide, gadolinium-Cl (GdCl3), quinine, quinidine, DIDS, glibenclamide (Glib), 4-aminopyridine (4-AP), triethylammonium (TEA), apamin (AP), tamoxifen (TX), antarhene-9-carboxylate (9AC), 18β-glycerethinic acid (GA), flufenamic acid (FA), NPPB, mebendafir (MF), octanol, DIOA, phloretin, DCPIP, 1-(2-chlorophenyl) diphenylmethyl-Hyprazole (TRAM-34), and clotrimazole (CTZ) were from Sigma Chemicals, fursemide was from Hoechst Roussel Pharmaceuticals (Somerville, NJ), niflumic acid (NA) was from Calbiochem, and Ba was from J. T. Baker (Phillipsburg, NJ).

**Molecular and immunological tools.** RNAgent Total RNA Isolation System was purchased from Promega (Madison, WI), ThermoScript RT-PCR System plus Platinum Taq DNA polymerase was from Invitrogen (Carlsbad, CA), and human primers were from Integrated DNA Technologies (Corvaline, IA).

The Mem-Prep protein extraction kit, Halt protease inhibitors cocktail, and PAGElprep protein clean up kit were from Pierce Biotechnology (Rockford, IL). A horseradish peroxidase (HRP)-coupled donkey anti-rb IgG (H+L) for Western blot analysis and a cY3-labeled donkey anti-rb IgG for immunofluorescence were procured as secondary antibodies from Jackson Immunoresearch Laboratories (West Grove, PA), and fluorescein-labeled donkey anti-rb IgG secondary antibody was from Vector Laboratories (Burlingame, CA). Lumi-Light Western Blotting substrate was obtained from Roche Diagnostics (Indianapolis, IN), and Fujifilm Super RX autoradiography film was 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): 132 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, and 10 glucose. In BSS-NaNO3 and BSS-Na-Sf (sulfamate) media, NO3 or sulfamate-Mf, respectively, was substituted for Cl in K, Rb, and Na salts and gluconate in the Ca and Mg salts. BSS-NMDG-Cl or sulfamate-Containing NMDG-Cl or Sf substituting for Na on an equiosmolar basis. Stock solutions (in M) of 1 NEM, 2 × 10−10 ouabain, and 2 × 10−3 bumetanide were contained in DMSO or ethanol, and all other chemical reagents were dissolved in deionized water. The 300 mosM washing solution contained 112 mM MgCl2 and 10 mM Tris-MOPS (pH 7.4) at 4°C. In general, in the experiments with lower osmolalities, sucrose was used as filler solute while keeping the ionic strength constant. Osmolalities were determined with an Advanced Micro-Osmometer, model 330 (Advanced Instruments, Norwood, MA). For convenience, throughout the text, but not in the figures, osmolality (osmol/kg H2O) was abbreviated as mosM, the term for milliosmolarity (mosM/l H2O) as the density of water is close to one.

**Human Lens Epithelial Cell Cultures**

Primary human lens epithelial HFL124 cells were kindly donated by Professor John Reddan (Oakland University, MI). Their nature and relatedness to fresh human epithelial cells is emphasized in the first paragraph of RESULTS. Culture flasks were coated with liquefied 0.1–0.2 mg gelatin (G1393)cm2 and subsequently dried for at least 2 h. Cells from passages 16–25 were grown on gelatin in a humidified atmosphere with 5% CO2 at 37°C in a 1:1 mixture of KGM (CC300) from Clonetics-BioWhittaker and medium 199 (M199, M5017) from Sigma, in the presence of antibiotics (50 μg gentamicin/ml M199; GKM comes complete with antibiotics) and 10% of 1:1 mixture of heat-inactivated horse serum (Sigma H1138) and FBS (F4135), or 10% FBS. Cells were split after being rinsed with Ca-Mg-free phosphate-buffered saline (PBS, Sigma D8537). After being warmed to 37°C and addition of 1 ml trypsin-EDTA solution (T3924), cells were incubated at 37°C for 10 min, neutralized with 3–5 ml complete growth serum containing medium, and centrifuged for 3 min. The supernatants were discarded, and the cell pellets were resuspended to known volume for cell counting and seeded in gelatin-coated 12-well culture plates at required densities.

**Ion Fluxes**

The general strategy, adapted from previous publications (2, 34), was to remove the culture media from the 12-well plates, wash the confluent LC cultures with BSS-NaCl at 37°C, and equilibrate them with BSS-NaCl-BSA for 10 min at 37°C to permit manipulations needed to activate the transport rates such as replacing CI and NO3 with inhibitor or activators drugs like NEM, or preexposing the cells to BSS-NaCl-BSA with different osmolalities. Thereafter, cells were exposed to the actual flux media, usually BSS-Rb/NaCl-BSA, BSS-Rb/Na-Sf-BSA, or BSS-Rb/NaNO3-BSA before commencement of Rb uptake and K loss usually during a period of 5–15 min. BSA (0.1%) was included to stabilize cells during washings. The contaminating presence of 184 μM Na did not affect the outcome of the experiments. Details deviating from these procedures will be addressed in the description of the experiments and are also noted in the figures. The K congener 85Rb has been shown in many publications to accurately substitute for K in these uptake measurements. In the basal studies, 10 mM RB was used to maximize the signal and maintain a concentration close to the kM values for KCC as in previous studies (34), and RB uptake and K loss were stopped by washing the cells with the ice-cold washing solution (see Solutions and Media). Ions were extracted with 5% perchloric acid and protein determined after being solubilized in 1 N NaOH using the bicinchoninic acid (BCA) method. K was measured with a Na-K lamp and RB by flame emission using a Perkin Elmer 5000 atomic absorption spectrophotometer (Norwalk, CT).

Operationally, as shown in earlier kinetic studies, Kc loss was measured from the cis (inside) to the trans side (outside), whereas RB uptake was measured from the trans to the cis side in the absence of external K. RB uptake or Kc loss are determined as nanomoles of ions per milligram protein as function of time (flux). The flux components are defined as follows: Total flux (1) is without any inhibitor, Na/K pump flux (2) = (1) minus flux in presence of 0.1 mM ouabain (3), NKCC (4) = (3) minus flux in presence of 10 μM bumetanide (5), KCC (6) = (4) in Cl minus (4) in Sf or NO3 media. K channel-mediated fluxes were measured in CI or Sf/NO3 always in the presence of 0.1 mM ouabain and 10 μM bumetanide.

**Cell Water**

Four 35-mm diameter gelatin-coated culture plates per condition were dried at 80°C until tare weight (TAW) constancy. FHL124 cells

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were then seeded onto these plates and grown to confluence, the growth medium was removed, the cells were washed with isosmotic BSS-NaCl-BSA and exposed for 15 min to BSS-Rb/NaCl-BSA or BSS-Rb/KCl-BSA flux media of different osmolalities (150 to 300 mosM, with sucrose filling the difference between the two values). Supernatants were quantitatively removed with a micropipette. Plates were immediately weighed for total weight (TOW) and then dried at 80°C for 48 h until weight constancy to obtain the wet weight.

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Molecular Biology (RT-PCR)

cDNA synthesis was performed with the Thermoscript RT-PCR system. Total RNA was isolated from FHL124 cells using RNAgent Total RNA extraction kit, as recommended by the manufacturer. After 5 μg of DNase digested RNA to random hexamer primers was annealed, cDNA was prepared using ThermoScript reverse transcriptase enzyme following the manufacturer’s instructions. Briefly, RNA and primers were denatured by incubating at 65°C for 5 min and placed on ice. To the sample tube containing denatured RNA and primers, the following were added: 4 μl of 5× cDNA synthesis buffer, 1 μl 0.1 M dithiothreitol (DTT), 1 μl RNAseOUT (40 U/μl), 1 μl DEPC-treated water, and 1 μl ThermoScript RT (15 μl/μl). The mixture was transferred to a thermal cycler preheated to the appropriate cDNA synthesis temperatures and conditions. To verify the presence of calcium-activated K channels at the mRNA level, oligonucleotide primers were chosen against the human sequences of Kc,3,1 (IK) channels: sense and anti-sense primers were TCTCAATACGTTCCGGTCTCC and AGCATGACTCTCCTTCCTGC, respectively, predicting a product of 457 bp. Human β-actin served as control. Two sets of primers were used to identify the presence of KCC isoforms as listed in Table 1. Products were then verified with ethidium bromide after 2% agarose gel electrophoresis.

SDS-PAGE and Western Blotting

Membrane proteins were extracted with the Mem-PER eukaryotic protein extraction reagent kit in the presence of the Halt protease inhibitors cocktail, following manufacturer’s instructions. Primary antibodies against Kc,3,1 (IK) channels were obtained from Alomone Laboratories (Jerusalem, Israel) and used in a 1:200 dilution as per manufacturer’s protocol together with HRP-coupled donkey anti-rabbit IgG in a 1:5000 dilution. The blot was exposed 5 min to Lumi-Light Western Blotting substrate and subsequently to X-ray film (Fisher Scientific, Fair Lawn, NJ).

Immunofluorescence Staining and Microscopy

FHL-124 cells were plated on a Lab-Tek Chamber-Slide Culture Chambers (NUNC) at a density of 6 × 10⁴ cells/well as previously described (40), simultaneously permeabilized and fixed in a freshly prepared 4% paraformaldehyde and 0.01% saponin solution for 30 min at 4°C, washed three times with PBS (0.5 ml/well) for 5 min each, incubated at 4°C for 1 h with a nonspecific blocking agent (3% normal goat serum in PBS), and then incubated overnight at 4°C with a 1:100-fold diluted primary antibody followed by the secondary antibody, a Cy3-conjugated donkey anti-rb IgG (1:250). Images were obtained with a Nikon Labophot epifluorescence microscope (Nikon) under a ×10 objective using SPOT digital color camera (Diagnostic Instruments, Sterling Heights, MI) and analyzed using SPOT Advanced image analysis software (Diagnostic Instruments).

Statistical Analysis

Unpaired or paired Student’s t-tests and one-way ANOVA tests for multiple intergroup differences were calculated with STATISTIX 7 (Analytical Software, Talahassee, FL) and Origin (Originlab, Northampton, MA). P values are indicated in the figure legends, and P < 0.05 was considered statistically significant.

RESULTS

Choice of Primary Human Lens Epithelial Cells (FHL124 Cell Line)

Based on several publications (13, 37, 61, 64, 65) and an unpublished gene chip analysis given to us for comparison by the late Professor George Duncan, School of Biological Sciences, University of East Anglia, Great Britain, the properties of the FHL124 cells are 99.5% close to freshly obtained human LECs [presence of crystallins, PAX6, FOXE3, TGFβ receptors, phospholipids synthesis, other factors and growth response to thrombin (26)].

Basic K Influx Components

The K uptake kinetics in FHL124 cells are unknown and were first established using Rb as K congener (32). Figure 1A shows the time course of Rb uptake in uninhibited (total), and in 0.1 mM ouabain, and ±5 μM bumetanide-treated LECs. Because Rb uptake was nonlinear, with a 5-min point closest to initial rates without compromising the Rb signal, Rb uptake was stopped at 5 min in most experiments, and Rb influx data were calculated in terms of nanomoles Rb per [milligram protein × 5 min].

Figure 1B defines the major K influx components and their response to NEM, a well-characterized stimulator of KCC (1). Shown are total (white columns), Na-K pump (vertically striped column), NKCC- (diagonally striped column) and KCC- (black columns) mediated Rb influxes as well as [ouabain + bumetanide]-resistant and Cl-independent Rb inward leak in Sm media (horizontally striped columns) before (None) and after NEM treatment. Consistent with an earlier report on K (Rb) fluxes in immortalized human B3 LECs (34),
the Na-K pump, NKCC, and KCC constituted about 49, 40, and 6% of the total Rb influx. Hence, >95% of K influx was carrier-mediated K transport. The presence of KCC was further secured by applying the pharmacological modifier NEM. As shown recently in B3 LECs (34), 0.05 mM NEM inactivated NKCC and activated KCC ($P < 0.05$) also in FHL124 cells, presumably by the inverse regulation proposed for both transporters by protein kinases and phosphatases (27, 33, 34).

Response of Major K Influx Pathways to Hyposmotic Stress

After equilibration of LECs for a total of 10 min in BSS-NaCl-BSA with osmolalities ranging from 300 to 150 mosM, total Rb influx, measured during subsequent 5 min in BSS-RbCl-NaCl-BSA media, increased significantly at 200 and 150 mosM ($P < 0.05$) as shown in Fig. 2A. This increase appeared to be due to a small but significant increase ($P < 0.05$) of the Na-K pump but even more to a doubling ($P < 0.05$) of the “leak” Rb influx in Cl media probably mediated by ion channels since the low activity of KCC was practically unaltered. Whereas KCC was not stimulated by hyposmotic swelling, NKCC activity, rather than being silenced as expected, increased significantly at 250 and 200 mosM, whereas at 150 mosM it fell to near the starting values at 300 mosM. To gain further insight into the time dependence of the response of NKCC to hyposmotic stress, Fig. 2B shows the bumetanide-sensitive (filled circles and squares) and Cl-dependent (open symbols) Rb influx through NKCC as well as KCC after 10- versus 30-min preincubation at decreasing extracellular osmolalities. The Cl-dependent NKCC activity (open symbols) was significantly higher than the bumetanide-sensitive NKCC (closed symbols) due to inclusion of the osmolarity-independent Cl-dependent KCC seen at the bottom of Fig. 2B. It is readily apparent that significantly higher NKCC activities were obtained at 150 mosM after 30 min rather than after 10 min incubation. Since cell swelling in hyposmotic media should have inactivated NKCC and activated KCC, the data suggest these LECs already had completed RVD with ion loss and cell shrinkage greater after 30 min than after 10 min preequilibration, the apparent cause of the seemingly paradoxical activation of NKCC and lack of KCC response.

To secure that FHL124 cells indeed possess the mRNA for the KCC1, three and four isoforms previously shown in B3 cells, RT-PCR was performed using two different sets of human primers. Figure 3 shows that FHL124 cells indeed possess these isoforms but in addition, and unexpectedly, strong evidence for KCC2, the isoform thus far restricted only to neurons (45). Preliminary experiments with the respective antibodies confirmed the presence of isoform-specific protein bands (not shown). Interestingly, a second KCC2 spliced variant, KCC2a, has been recently reported (58) in rat testis, which is different from the previously reported KCC2(b) isoform by 40 NH2-terminal amino acid residues. The primers used in our studies are downstream from exon 1 encoding for this isoform variance, and future studies are planned to verify this isoform in FHL124 cells.

Response of Cell K and Water to Hyposmotic Stress

In Na media, loss of cellular KCl and water should have occurred during RVD. Figure 4 shows an experiment in which

Fig. 1. Rb uptake (A) and major Rb influx components (B) in FHL124 cells. A: Rb uptake and influx were determined as described in MATERIALS AND METHODS. Nonlinear function plots by Origin. B: Rb influx (5 min) without (None) and with 50 $\mu$M N-ethylmaleimide (NEM). Flux components as defined in MATERIALS AND METHODS. NKCC, Na/K pump Cl-dependent Na KCl; KCC, K-Cl cotransport. Error bars for $n = 4$; values are means $\pm$ SE. *$P < 0.05$.

Fig. 2. Response of Rb influx components in lens epithelial cells (LECs) to lower medium osmolalities. A: total, Na/K pump, NKCC, leak, and KCC. B: bumetanide-sensitive (closed symbols) and [bumetanide + chloride]-sensitive (open symbols) NKCC and KCC after 10 and 30 min preequilibration in 300, 250, 200, and 150 mosM BSS-NaCl-BSA before 5 min exposure to the 37°C BSS-RbCl-NaCl-BSA or BSS-Rb-NaCl-BSA influx solutions of identical osmolalities. Leak is Rb influx in BSS-Rb-NaCl-BSA with all inhibitors, and KCC is the calculated chloride-dependent and [ouabain + bumetanide]-insensitive Rb influx. $n = 4$; values are means $\pm$ SE. *$P < 0.05$. 

Fig. 3. RT-PCR showing expression of KCC1, KCC2, and KCC2a in FHL124 cells. Analysis by agarose gel electrophoresis. A: expression of KCC1 mRNA in FHL124 cells B: expression of KCC2 mRNA in FHL124 cells. C: expression of KCC2a mRNA in FHL124 cells. Western blot analysis of KCC2 protein in FHL124 cells.
Kc was determined 15 min after exposure to Cl (Fig. 4A) or Sf (Fig. 4B) media with osmolalities from 300 to 150 mosM, in the absence and presence of ouabain and of [ouabain]/H11001 bumetanide]. Independently of the anion, at lower than 250 mosM, Kc fell significantly by/45%, and, as expected, was independent of Na-K pump and NKCC inhibitors present. By definition, the Kc loss in Sf minus that in Cl constitutes KCC activity (Fig. 4B, inset C), which was insignificantly small and osmolality independent. To decide whether osmolality or ionic strength or both were responsible for the K loss, Kc was measured after 15 min equilibration in 300 mosM full ionic strength and half ionic strength (sucrose replacement). Figure 5 shows that Kc was identical in isosmotic sucrose Cl flux media and was lowered by ~43% only in 150 mosM hypoosmotic media without sucrose, indicating that indeed, the Kc loss was due only to osmotic and not to low ionic stress. Results were similar in Sf, although Kc was somewhat, but not statistically significant, lower than in Cl.

Water follows K and Cl efflux during RVD. Figure 6A shows net determination of cell water in LECs exposed for 15 min to hypotonic Na media with the Na concentrations indicated in parentheses. Consistent with the data in Fig. 5, at osmolalities lower than 250 mosM, cell water decreased by about 40% in zero-trans-K Na media, a result that did not change significantly at 30 min of incubation (Fig. 6B, open triangles vs. open circles). However, in the presence of only external K (zero trans-Na), cell water fell insignificantly by/10% as the osmolality and external K concentrations (numbers in parentheses are in mM) decreased (Fig. 6B, closed squares). Hence, the RVD was significantly attenuated (P < 0.005) in high K media, commensurate with involvement of a K channel.

The kinetics of osmotically induced Kc loss through the putative K channel are shown in Fig. 7, which compares Kc loss within 15 min after change to either 300 (Fig. 7A) or 150 (Fig. 7B) mosM BSS-NaCl-BSA or BSS-NaSf-BSA with and without 10 mM external Rb ([Rb]o). Whereas replacement with the same osmolality exhibited little change in Kc over 15 min,
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A

B

Fig. 6. Cell water in LECs challenged with hyposmotic high Na (A) or high K (B) media. B contains in addition two repeat data for cell water in 150 mosM media sampled at 15 (open circle) and 30 (open triangle) min. The numbers in parentheses in both panels refer to the actual external Na or K concentrations (in mM). See MATERIALS AND METHODS for details. n = 4; values are means ± SE with * and ** indicating significant differences (P < 0.05 and <0.005, respectively) at 150 and 200 mosM Na vs. 300 mosM Na in A and at 150 mosM K vs. 150 mosM Na after 15 and 30 min in B.

switching to 150 mosM caused an exponential fall of $K_c$ that reached 50% of the initial value at the end of the incubation and apparently was independent of the anions and the trans $[\text{Rb}]_o$ present. From a plot of ln $K_c$ versus time (see insets in Fig. 7, A and B), the relative individual $K_c$ efflux rate coefficients ($c_{kK}$) in 300 mosM Cl/$\text{H}^+$ media were 0.0035/min ($n = 8$) and 0.0015/min ($n = 4$), respectively, and 0.0046/min in 300 mosM Sf. In contrast, the corresponding $c_{kK}$ values in 150 mosM Cl, Cl-Rb, and Sf media were 0.047/min, 0.057/min, and 0.055/min, indicating a >10-fold increase in the $K$ permeation rate upon hyposmotic stress that was apparently independent on the anion present, suggesting the absence of a Cl-dependent mechanism in this process. The $c_{kK}$ values 0.0031 and 0.051 given in the insets of Fig. 7 are means of pooled experiments ($n = 12$) either in 300 or 150 mosM solutions, respectively. Since the time constant for $K$ equilibration ($\tau$) equals $1/c_{kK}$, the calculated $\tau$ was 323 min, i.e., >5 h, for cells in isosmotic and only ~20 min in hyposmotic solutions. Hence, swollen cells exchanged $K$ at least 16 times faster than isosmotically suspended cells.

Independent of its transport of $K$, the putatively RVD-mediating $K$ channel also recognized $\text{Rb}$ as shown in Fig. 8A, where the $\text{Rb}$ uptake over 10 min in 200 mosM media with increasing $[\text{Rb}]_o$ displayed hyperbolic behavior; i.e., saturated with time. Recalculation of the data in terms of $\text{Rb}$ influx at the three time points tested versus the applied $[\text{Rb}]_o$ (Fig. 8B) yielded perfectly linear relationships with slopes declining with time suggesting that, like $K$, $\text{Rb}$ traversed a $K$ channel and not a carrier-mediated system. The inset in Fig. 8B shows that the ln $\text{Rb}$ influx calculated from the slopes declined with time, yielding an apparent or relative inward rate coefficient ($k_i$) of ~0.059/min; i.e., a time constant $\tau = 1/k_i$ equal to 17 min, which is similar to that measured for $K_c$ loss.

Functional and Pharmacological Nature of the Hyposmotically Stimulated $K$-Selective Channel

The evidence thus far suggests that $K$ efflux or $\text{Rb}$ influx is elevated by at least an order of magnitude in response to hyposmotic swelling. According to Ussing’s flux ratio equation, $K$ influx/$K$ efflux = $[K]_o/[K]_c \times e^{-zFE/RT}$, where $[K]_o$ and $[K]_c$ are the respective extra- and intracellular $K$ concentrations and $e^{-zFE/RT}$ is the membrane potential term with $z$, $F$, $E$, $R$ and $T$ with their usual meanings. This relationship only holds for one particular ion and not for any related ion (57). To test the “Ussing” behavior of the hyposmotically activated $K$ channels, $[K]_o$ was raised in exchange for Na from 0 to 56 mM main-
taining the total osmolality at 200 mosM during 10 min hyposmotic challenge, and $K_c$ was determined after all external $K( K_o)$ was removed by washing with a buffered Mg solution as described in MATERIALS AND METHODS. Figure 9A shows that $K_c$ loss in media of increasing [K]o was sharply reduced. The rate of $K_c$ loss was calculated from the $\ln K_c$ as described in Fig. 7 and plotted as a function of [K]o (mM external cations) as shown in Fig. 9B. The $c_k$ fell from just under 0.10 to 0.02/min over the range of [K]o applied. Linear transformation of the single exponential fall of $c_k$ yielded an intercept of 87 mM [K]o interpreted as the reversal [K]o. Consistent with the Ussing independence principle in the equation above, neither Rb, which is transported independently as shown above, nor Cs reversed the $K_c$ loss, establishing unequivocally the hyposmotically activated cation channel as K selective.

Table 2 shows that neither inhibitors of the Na/K pump (ouabain) nor of NKCC and KCC (furosemide, bumetanide) reduced hyposmotically induced $K_c$ loss, and various inhibitors of Ca-activated K channels listed here in accordance with the nomenclature recently published by the International Union of Pharmacology (63), such as high conductance (KCa1.1, BK) K channels (TEA, Ba, Gd, Quinine), small conductance (KCa2.1, KCa2.2, KCa2.3; i.e., SK1, SK2, SK3) K channels (apamin), and stretch- and voltage-activated K channels (glibenclamide, 4-AP) were without effect, as were four inhibitors reported for connexin hemichannels (18βGA, FA, NPPB, 2-octanol) (15).

Clotrimazole (CTZ), which like charybdotoxin also inhibits KCa1.1 channels (63), has been documented as a potent inhibitor (Kd in the nanomolar range) of the Gardos K channel in human red blood cells (7, 36) that, based on work in mouse erythroleukemia cells (59), has been classified as an intermediate conductance (KCa3.1 or IK) channel. In the experiment in Fig. 10A, $K_c$ was determined at 2.5, 5, and 10 min after exposure to 150 and 300 mosM zero-trans K with 10 mM trans-Rb media with increasing CTZ concentrations. Results show that CTZ progressively inhibited and completely prevented K loss at 50 μM with an IC50 of about ~25 μM, in the presence of 0.1 mM ouabain ± 5 μM bumetanide in 150 mosM media but had no effect on the K loss in 300 mosM media (open symbols). Figure 10B shows that CTZ had no effect in 300 mosM media (open symbols), whereas it inhibited with high significance the swelling-induced Rb influx as a function of concentration, almost completely at 50 μM CTZ. Figure 11 shows that TRAM-34, a more recently recognized selective inhibitor of IK channels (63), reduced the loss of cell K in 150 mosM Cl or NO3 media ($P < 0.005$) at concentrations of 50 μM and higher while exerting weak, but significant, effects on Rb influx above 30 μM consistent with a partial and perhaps asymmetrical inhibition of the hyposmotically induced K/Rb flux.

That K loss and Rb influx occur through both a CTZ-sensitive and partially TRAM-34-sensitive mechanism suggests KCa3.1 or IK channels are the primary cation transport systems during RVD of hyposmotically challenged FHL124.
cells, since the BK channel inhibitors Ba and TEA failed to affect the Kc loss or Rb entry (not shown).

Role of Anion Channels in Hyposmotically Induced K Loss by Putative IK Channels

Several anion channel inhibitors such as DIDS, furosemide, TX, 9AC, and MF failed to block Kc loss (Table 2, experiments 5–10), although DIDS inhibited Rb influx in 150 mosM media by 32% (*P < 0.005), an effect most likely due to inhibition of KCC-mediated influx (data not shown). However, DIOA abolished Kc loss (Fig. 12A) better in NO3 than in Cl media and

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Kc, cell K; Ouab, ouabain; Bum, bumetanide; 9AC, anthracene-9-carboxylate; NA, niflumic acid; 4-AP, 4-aminopyridine; TEA, tetraethylammonium; TX, tamoxifen; NPPB, 5-nitro-2-(3-phenyl-propylamino) benzoic acid; MF, mibefradil; DCPiB, 4-2(butyl-6,7-dichloro-2-cyclopentylindan-1-on-S-yl) oxybutyric acid; GA, 18β-glycerethinic acid; FA, flurenamic acid.

Role of Anion Channels in Hyposmotically Induced K Loss by Putative IK Channels

Several anion channel inhibitors such as DIDS, furosemide, TX, 9AC, and MF failed to block Kc loss (Table 2, experiments 5–10), although DIDS inhibited Rb influx in 150 mosM media by 32% (*P < 0.005), an effect most likely due to inhibition of KCC-mediated influx (data not shown). However, DIOA abolished Kc loss (Fig. 12A) better in NO3 than in Cl media and

Fig. 10. Kc (A) and Rb influx (B) as function of clotrimazole concentrations present during 2.5, 5, and 10 min flux incubation in 300 and 150 mosM media. Open rhomboids for 10 min in 300 mosM and filled squares, circles, and triangles for 2.5, 5, and 10 min in 150 mosM solutions. Lines in A are Lorentzian and in B linear fits generated by the Origin Software. n = 4; values are means ± SE. *P < 0.05 and **P < 0.005 vs. zero drug concentration.
highly significant ($P < 0.005$) at concentrations higher than 100 μM. This clearly means that DIOA did not inhibit a Cl-dependent K loss through KCC as recently shown in corneal epithelial cells (10). DIOA reduced Rb influx in hyposmotic Cl but appeared to stimulate in NO₃, as shown in Fig. 12B, producing negative values as indicated by dCl (filled circles), again consistent with absence of an effect on the KCC mechanism.

A widely used, yet highly nonselective, anion channel blocker is phloretin. Figure 13A reveals that this drug, at 0.25 mM and higher, significantly inhibited hyposmotically induced K loss in NO₃ but failed to do so in Cl media. In addition, it apparently accelerated K loss even in 300 mosM isotonic media, suggesting unspecific effects on the membrane permeability. Nevertheless, a significant inhibition (up to >50%) of hyposmotically stimulated Rb influx occurred in both Cl and NO₃ between 0.25 and 0.75 mM, as shown in Fig. 13B. Table 2, experiment 79, shows data from a dose-response study in which only the highest concentrations (100 μM) of two anion channel blockers NA and NPPB inhibited hyposmotic Kᵢ loss by 61% at 50 μM (experiment 70).

Molecular and Immunochemical Evidence for the Presence of Ca-Activated IK Channels in FHL124 Cells

Figure 14 shows the RT-PCR product of 457 bp for the primers listed in MATERIALS AND METHODS to detect KCa3.1 channels in these FHL124 cells. As a positive internal control, β-actin was used and −RT served to eliminate the presence of genomic DNA. Thus the mRNA for IK was confirmed in FHL124 cells.

Figure 15A displays the Western blot analyses obtained with FHL124 membrane protein extracts and commercially available IK-antibodies. The presence of IK channels is demonstrated by ~75- and 50- and 37-kDa protein bands in the FHL124 cells (lanes 1 and 2) compared with the positive control of rat brain extract (lane 3). The sizes of the protein bands suggest that the larger molecular weight protein is commensurate with the approximate molecular weight of the IK channel based on the number of amino acids present in the native or cloned proteins (63), whereas the smaller peptides most likely constitute breakdown products.

Figure 15B shows the immunohistochemical evidence IK channels in FHL124 cells with the same antibodies used in the Western blot analyses. There was cytoplasmic as well as membrane staining, with special intensity of channel proteins
present in mitotic cells (as indicated by arrows). An increased staining of ion channels and transporters during mitosis signifies volume changes probably requiring upregulation of these transporters.

DISCUSSION

This study originated in the attempt to determine swelling-activated K channels and inactivated NKCC under hyposmotic conditions, constituting a logical followup of our earlier work on chemical modification of these transporters under isosmotic conditions (34). Like B3 cells, FHL124 cells possess both a robust Na/K pump, the NKCC and a small, but NEM-augmented, KCC activity, together accounting for >80% of Rb influx activity (Fig. 1). One explanation for the persistency of NKCC activity and lack of hyposmotic response of KCC (Fig. 2) was to assume these LECs possess powerful RVD mechanisms eliciting NKCC activation and KCC inactivation through its putative signaling cascades. An alternate interpretation of the unexpected inverse behavior of NKCC and KCC (Fig. 2), to be detailed in future studies, is the possible contribution of KCC2 (Fig. 3), presumably KCC2a, to the overall K-Cl co-transport activity perhaps through hetero- or homo-oligomerization with NKCC1 or its own isoform partners KCC1, KCC3 and KCC4. This type of protein-protein interaction, involving COOH-terminal protein segments of these transport isoforms, has been recently reported in the oocyte model injected with cRNA of KCC1-4 and NKCC1 isoforms (52).

Our study presents novel evidence for the presence of swelling-activated K channels mediating cell water loss and hence RVD (Figs. 4 – 8) not yet reported for human LECs. This increase in K membrane permeability was solely due to osmotic differences (Fig. 5) and was associated with commensurate cellular water loss (Fig. 6A), which at 150 mosM was about 30%, and hence somewhat smaller than the Kc loss seen in Fig. 4, probably due to the robust NKCC inward flux activity. The aggregate mean rates of Kc loss (\( \frac{dKc}{dt} \)) (Fig. 7, A and B), indicated a >16-fold stimulation in hyposmotically challenged FHL124 cells compared with isosmotic controls, translating into apparent K permeability coefficients of about \( 4 \times 10^{-5} \) and \( 5 \times 10^{-4} \) cm/s, for 300 and 150 mosM, respectively. The estimated K permeability in isosmotic media is about one order of magnitude higher than that reported for bovine lenses (\( 3 \times 10^{-6} \) cm/s) using tracer K efflux measurements (11), which may be explained by the uncertainty in cell volume and surface area estimates as well as by species differences. The hyposmotically activated Rb influx and K loss as a function of time were quite similar (Fig. 8), which can be interpreted as due to the same process, namely K channel-mediated RVD. Whereas these flux studies do not assess opening and closing kinetics of individual channels, they constitute a macroscopic measure of various K channels, most likely IK channels, as well as of the process of RVD inactivation due to gradient dissipation and activation of NKCC inward flux by a variety of shrinkage-induced biochemical processes.

Voltage-gated (Kv) as well as Ca-activated (\( \text{K}_{\text{Ca}} \)) K channels have been studied by electrophysiological methods in human and animal LECs, especially BK channels (49). Outward and

![Fig. 13. Effect of increasing phloretin concentrations on cell K (A) and Rb influx (B) in isosmotic (300 mosM) and hyposmotic (150 mosM) Cl and NO3 media. Phloretin was present throughout the flux period and exposure to the two osmolalities. Squares: 300 mosM Cl; circles: 300 mosM NO3; triangles: 150 mosM Cl; inverted triangles: 150 mosM NO3. For each condition, n = 4; values are means ± SE with * indicating significance levels at P < 0.05.]

![Fig. 14. Reverse transcriptase products (bp) of calcium-activated KCa3.1, IK channel, and \( \beta \)-actin in total RNA isolated from FHL124 cells. Lane 1, 100 bp marker; lanes 2 and 3, \( \beta \)-actin (297, control); lanes 4 and 5, KCa3.1, IK (457); lane 6, –RT (negative control).]
inward rectifying K channels (46, 47), and electrically silent K_v channels (48, 51). To the best of our knowledge, there is no report yet available on the presence of RVD-mediating K_Ca,3.1 (IK) channels in human LECs. However, there are several recent electrophysiological studies on the role of K_Ca,3.1 channels in RVD of other mammalian cells such as in human T lymphocytes (29), human parotid gland cells (5, 55), human intestinal epithelial cells (60), human embryonic kidney (HEK293) cells (28), and mouse erythroleukemic cells (59).

Optical or Coulter counter-type volume measurements were used to study RVD, for example, in Ehrlich ascites cells (24), or in human corneal epithelial cells (10) and K fluxes to show NEM- and swelling-activated KCC in SV40-transformed mouse LECs (12). Our results suggest the quick onset of the K-channel-mediated RVD overrides the KCC activity in FHL124 cells. Only external K, but not Na, Rb nor Cs ions, reduced the *k* _k_ values in hyposmotic media (Fig. 9), which means that the flux of K through the RVD-mediating K channels obeyed the Ussing flux ratio equation (see RESULTS). Thus swelling of human lens epithelial cells activated K-selective channels but not nonselective cation channels as reported for other model systems (31, 62).

Neither typical inhibitors of Big conductance (BK), other voltage-gated K channels, Small conductance (SK) channels, nor known blockers of the connexin family were effective (Table 2) (15). Only CTZ with an approximate IC_50_ of 25 µM and completely at 50 µM prevented K_c loss (Fig. 10). This concentration range is comparable to published CTZ doses in other epithelial cell systems, with intermediate conductance K_Ca,3.1 (IK) channels involved in RVD (29, 60). TRAM-34, an inhibitor of IK channels not involving the P450 protein like CTZ (56, 66), also reduced K_c loss by ~50% and less Rb influx suggesting that the two inhibitors act through different mechanisms either at the channel or regulatory level.

Like SK channels, IK channels are regulated by intracellular Ca ions (63). However, our preliminary studies addressing the external Ca dependence using media chelators such as EDTA were inconclusive suggesting intracellular Ca ions may play during RVD, a commonly held notion. However, preliminary experiments with high (mM) concentrations of external Ni, an inhibitor of Ca-dependent RVD in renal epithelial cells (54), significantly reduced K loss (data not shown). It should also be noted that K_Ca channels may be stretch activated and thus swelling of human lens epithelial cells activated K channels. Other Cl channels cannot be excluded since several blockers of VSOR Cl channels (41). Involvement of these VSOR CI channels cannot be excluded since several blockers of VSOR either completely (DIOA), partially (phloretin, DCIPB), or barely (NA and NPPB) inhibited. The complexity of the use of DIOA can be appreciated in the fact that it inhibited hyposmotically activated K efflux or Rb influx apparently was not rate limiting, as Cl replacement with SF was without major macroscopic effect, in contrast to findings in other cell types (31). This finding may be attributed to the fact that, to maintain electroneutrality, CI channels are also activated by cell swelling, in particular volume-sensitive outwardly rectifying (VSOR) CI channels (41). Involvement of these VSOR CI channels cannot be excluded since several blockers of VSOR either completely (DIOA), partially (phloretin, DCIPB), or barely (NA and NPPB) inhibited. The complexity of the use of DIOA can be appreciated in the fact that it inhibited hyposmotically activated K_c loss in LECs at similar concentrations blocking KCC in primary cultures of rat vascular smooth muscle cells (3), yet at some 10-fold greater concentrations than used for inhibition of human erythrocyte K-Cl cotransport (19). Phloretin, known to inhibit volume-sensitive and cAMP-activated but not Ca-activated CI channels (17) on the other hand, inhibited >50% of Rb influx, reduced K loss by 30% in isosmotic media (Fig. 13). Phloretin is known to inhibit other cation and anion exchangers (14) as well as K channels (53) and hence cannot be considered a diagnostically specific drug. Thus, in comparison, the overall asymmetric inhibition by DIOA and phloretin and partial inhibition by DCIPB, NA, and NPPB suggest indirectly electroneutrality is maintained by swelling activation of presumably VSOR anion channels accompanying K fluxes through IK channels. Other CI channels...
inhibited by DIDS, furosemide, TX, 9AC, and MF (see Table 2) are excluded since none of these drugs retarded Kc. loss. Clearly, a more detailed study of the biophysical, pharmacological, and molecular nature of the anion channel accompanying the apparent IK-channel-mediated RVD is warranted in future studies on LECs.

The RT-PCR, Western blot, and immunochemistry data in Figs. 14 and 15 show unequivocally that FHL124 cells possess Kcnn.3.1 (IK) channels, accompanied by Cl fluxes perhaps through VSOR Cl channels (47, 49). Clearly identified by molecularly and immunological studies (data not shown), a functional role during RVD of other Ca-activated K channels, such as BK and SK1.2, and 3 channels, previously reported in human LECs, was excluded pharmacologically (Table 2).

In summary, the studies reported here shed light on basic mechanisms involved in the maintenance of human LEC VC. Understandably, these mechanisms may play crucial roles also in the LEC to LFC trans-differentiation, involving obliteration of intracellular organelles, reduction of cytoplasm, and maintenance of the plasma membrane with apparently altered passive K permeabilities not yet fully understood in terms of VC (43). In FHL124 cells, hyposmotically induced RVD was mediated primarily by IK channels putatively coupled to VSOR channels, a powerful mechanism to respond to osmotic challenge. RVD-mediated activation of NKCC1, the predominant isoform in LECs (4), most likely will offset swelling-triggered K loss through IK channels, and thus preserve VC by RVI if these cells would be returned to isosmotic media, a mechanism at play in volume recovery of hyperosmotically stressed corneal epithelial cells (10). The clinical significance of VC is underscored by an increased apamin-sensitive KCa2.3 (SK3) channel expression in human LECs from patients with myotonic dystrophy type 1 where osmotic perturbation could influence the apparent occurrence of cataract in these patients (50). Another example of unexplored mechanisms is the observation that dehydration due to intestinal illnesses also correlates with a greater incidence of cataract (39).

ACKNOWLEDGMENTS

The superb execution of the ion flux experiments by our research assistant Kathy Leonard and the arduous help of Amber McCurdy to reference-edit this work is gratefully acknowledged. We appreciate the interest of and discussions with 3 second- and third-year medical students Shaminde Bhullar, Pooja Fattahi, and Sameer Ali of Boonshoft School of Medicine.

GRANTS

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REFERENCES

29. Jorgensen NK, Pedersen SF, Rasmussen HB, Grunnet M, Klaeke DA, Olesen SP. Cell swelling activates cloned Ca2+-activated K+ chan-
IK CHANNEL HYPOSMOTIC ACTIVATION IN HUMAN LENS EPITHELIA


