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Functional and molecular characterization of multiple K-Cl cotransporter isoforms in corneal epithelial cells

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Abstract

The dependence of regulatory volume decrease (RVD) activity on potassium–chloride cotransporter (KCC) isoform expression was characterized in corneal epithelial cells (CEC). During exposure to a 50% hypotonic challenge, the RVD response was larger in SV40-immortalized human CEC (HCEC) than in SV40-immortalized rabbit CEC (RCEC). A KCC inhibitor—[(dihydroindenyl)oxy] alkanoic acid (DIOA)—blocked RVD more in HCEC than RCEC. Under isotonic conditions, *N*-ethylmaleimide (*NEM*) produced KCC activation and transient cell shrinkage. Both of these changes were greater in HCEC than in RCEC. Immunoblot analysis of HCEC, RCEC, primary human CEC (pHCEC), and primary bovine CEC (BCEC) plasma membrane enriched fractions revealed KCC1, KCC3, and KCC4 isoform expression, whereas KCC2 was undetectable. During a hypotonic challenge, KCC1 membrane content increased more rapidly in HCEC than in RCEC. Such a challenge induced a larger increase and more transient p44/42MAPK activation in HCEC than RCEC. On the other hand, HCEC and RCEC p38MAPK phosphorylation reached peak activations at 2.5 and 15 min, respectively. Only in HCEC, pharmacological manipulation of KCC activity modified the hypotonicity-induced activation of p44/42MAPK, whereas p38MAPK phosphorylation was insensitive to such procedures in both cell lines. Larger increases in HCEC KCC1 membrane protein content correlate with their ability to undergo faster and more complete RVD. Furthermore, pharmacological activation of KCC increased p44/42MAPK phosphorylation in HCEC but not in RCEC, presumably a reflection of low KCC membrane expression in RCEC. These findings suggest that KCC1 plays a role in (i) maintaining isotonic steady-state cell volume homeostasis, (ii) recovery of isotonic cell volume after a hypotonic challenge through RVD, and (iii) regulating hypotonicity-induced activation of the p44/42MAPK signaling pathway required for cell proliferation.

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Keywords

corneal epithelial cells; regulatory volume decrease; hypotonic challenge; KCC; p38MAPK; p44/42MAPK; cell proliferation

1. Introduction

The refractive property of the cornea is dependent on its ability to overcome anisotonic-induced cell swelling or shrinkage. In-vitro, it has been shown that overcoming these effects is achieved through the concerted activation of specific ion channels and transporters. Specifically, hypertonic stress triggers corneal epithelial cells (CEC) to accumulate osmolytes, leading to a subsequent influx of water, also known as regulatory volume increase (RVI) (Bildin et al., 2003; Bildin et al., 2000; Bildin et al., 1998; Capó-Aponte et al., 2005). On the other hand, exposure to a hypotonic environment induces cell swelling, which in turn, initiates extrusion of ions and osmotically acquired water, resulting in regulatory volume decrease (RVD) (Al-Nakkash et al., 2004; Al-Nakkash and Reinach, 2001; Capó-Aponte et al., 2005; Farrugia and Rae, 1993; Wu et al., 1997). Several studies of primary rabbit CEC, SV40-immortalized human CEC (HCEC), and SV40-immortalized rabbit CEC (RCEC) have demonstrated that activation of separate Cl^- and K^+ channels contributes substantially to the RVD response (Al-Nakkash and Reinach, 2001; Bonanno et al., 1989; Capó-Aponte et al., 2005; Farrugia and Rae, 1993; Wu et al., 1997; Yang et al., 2000).

Potassium-chloride cotransporter (KCC) has been recognized in many tissues for its contribution to the electroneutral efflux of K^+ coupled with Cl^- . During the RVD response by HCEC, KCC activation parallels increases in K^+ and Cl^- conductance (Capó-Aponte et al., 2005). However, the mechanism of KCC activation and the selective roles of KCC isoform involvement in mediating the RVD response in the aforementioned cell lines have not been described.

KCC is a [(dihydroindenyl)oxy] alkanolic acid (DIOA)-sensitive member of the large cation-chloride cotransporter gene family, which includes the thiazide-sensitive $\text{Na}^+\text{-Cl}^-$ cotransporter and the bumetanide-sensitive $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter (NKCC) (Adragna et al., 2004; Delpire et al., 1994; Gamba et al., 1993; Gillen et al., 1996; Mercado et al., 2000). An important characteristic of KCC is its ability to be activated by thiol modifications with *N*-ethylmaleimide (*NEM*) and by cell swelling (Kramhoft et al., 1986; Lauf et al., 1992; Lauf and Theg, 1980). *NEM* selectively stimulates KCC in multiple cell systems (Adragna et al., 2004) and may be used as a diagnostic tool to detect the functional presence of KCC as well as hypotonicity-induced cell swelling. Four isoforms of KCC (KCC1-4) have been identified (see review by Adragna et al. (Adragna et al., 2006)). KCC1 is an ubiquitously expressed “house-keeping” transporter involved in maintenance of cell volume and ionic homeostasis (Adragna et al., 2006; Gillen et al., 1996). Neuron-specific KCC2 is present in the retina and central nervous system, where it plays a crucial role in maintaining low intracellular Cl^- and GABAergic synaptic inhibition (Payne et al., 1996; Rivera et al., 1999; Vardi et al., 2000; Vu et al., 2000). KCC3 shares a 77% overall amino acid identity with human KCC1, and is expressed at highest levels in skeletal muscle, heart, kidney, brain, and lens (Chee et al., 2006; Hiki et al., 1999; Lee et al., 2003; Mount et al., 1999; Pearson et al., 2001; Race et al., 1999). Human KCC4 shares a 69% amino acid identity with KCC2, and is expressed in muscle, heart, kidney, brain, lung, and lens (Chee et al., 2006; Lee et al., 2003; Mercado et al., 2000; Mount et al., 1999).

In addition to the well-known role of KCC in ionic and osmotic homeostasis, several studies have shown KCC involvement in regulating cell proliferation and invasion (Shen et al.,

2003; Shen et al., 2001; Shen et al., 2004). In cervical cancer cells, expression of KCC1, -3, and -4 is regulated during the cell cycle and is greater than in normal cells. KCC activity correlates with the proliferation, growth, and spread of the cancer, reflecting the role of these transporters in volume regulation during cell growth and division. Similarly, expressed KCC3 in NIH/3T3 fibroblasts has a regulatory role in enhancing cell growth (Shen et al., 2001). KCC activity is also stimulated by certain growth factors, particularly insulin-like growth factor-1, acting via certain kinases—phosphoinositide 3-kinase (PI3-K)/Akt and p44/42 mitogen-activated protein kinase (MAPK)—to promote gene transcription and synthesis of KCC. All of these facts suggest that KCC plays an important role in hypotonicity-induced activation of MAPK pathways in CEC.

Activation of regulatory volume mechanisms requires the cell to sense (i) changes in volume, (ii) transduction of the signal to the specific pathway for ion movement, and (iii) activation of the ion channels and/or transporters. However, both the sensing mechanism and the signaling pathway that modulate recovery of cell volume are not well understood in CEC. Several studies have shown that changes in external osmolarity elicit phosphorylation of tyrosine in a number of different types of tyrosine kinase receptors. MAPK pathways are activated by tyrosine phosphorylation induced by diverse extracellular stimuli, e.g., anisotonic challenge, mechanical stress, and growth factors. Hypotonicity-induced swelling is accompanied by a rapid and transient increase of tyrosine phosphorylation of multiple proteins, including p44/42 (i.e., Erk1/2) and p38MAPK (Noe et al., 1996; Sadoshima et al., 1996; Schliess et al., 1995; Schliess et al., 1996; Sinning et al., 1997; Tilly et al., 1996a; Tilly et al., 1996b; Zhang et al., 1998). Moreover, increased tyrosine phosphorylation was found to be a critical step in activating the RVD process (Tilly et al., 1996a; Tilly et al., 1993).

Activation of p44/42 and p38MAPK signaling cascades in CEC is recognized as the key event leading to increased cell proliferation and migration, respectively. One of the pathways well-studied in CEC is the signaling cascade of epidermal growth factor (EGF)-induced p44/42 and p38MAPK activation (Kang et al., 2000; Kang et al., 2001; Lu et al., 2001; Mergler et al., 2005; Roderick et al., 2003; Tao et al., 1995; Yang et al., 2005; Yang et al., 2003; Yang et al., 2001). It has also been shown that membrane stretch, induced by hypotonic swelling, is capable of activating tyrosine kinase receptors, which, in turn, activate the EGF receptor (Franco et al., 2004).

In the present study, we characterized, for the first time, the diverse KCC isoforms present in CEC. We examined (i) the effects of acute hypotonic challenge in HCEC and RCEC, (ii) the role of KCC in mediating a subsequent RVD response, (iii) the effects of pharmacological manipulation of KCC in maintaining steady-state cell volume, (iv) the effects of hypotonic stress on inducing changes membrane content of specific KCC isoforms, and (v) the role of KCC in hypotonicity-induced activation of the p44/42 and p38MAPK signaling pathways.

Material and methods

2.1 Reagents and experimental solutions

Calcein-AM was purchased from Molecular Probes (Eugene, OR, USA). Dimethyl sulfoxide (DMSO), *NEM*, and DIOA were purchased from Sigma-Aldrich (St. Louis, MO, USA). The inhibitors PD98059, U0126, SB203580, and genistein, were purchased from Biomol (Plymouth Meeting, PA, USA). Control (isotonic, 300 mOsm) solution contained (mM): 147.8 NaCl, 4.7 KCl, 0.4 MgCl₂·6H₂O, 5.5 glucose, 1.8 CaCl₂, and 5.3 HEPES Na, pH 7.4. Hypotonic challenges were attained by aqueous dilution. Sham experiments were performed to validate dilution effects on fluorescence output from calcein-loaded CEC. Osmolarity was verified by measurements of freezing point depression (μ Osmette OsmoMeter, Precision System, Natick, MA, USA). All solutions and reagents were freshly prepared prior to

experimentation. All reagents were dissolved in DMSO unless otherwise specified. The final concentration of DMSO was less than 0.05% for all reagents (preliminary experiments showed that DMSO did not significantly alter cell volume at this concentration).

2.2 Cell culture

HCEC and RCEC, a kind donation of Dr. Araki-Sasaki (Kumamoto University Kumamoto, Japan), were cultured in Dulbecco's modified Eagle's medium (DMEM/F12), supplemented with 10% fetal bovine serum (FBS), 5 ng/ml EGF, 5 µg/ml insulin, and 40 µg/ml gentamicin in an atmosphere of 5% CO₂ at 37 °C. Both cell lines exhibit phenotypic and functional properties similar to those of their primary cultured counterparts while permitting continuous growth for >100 passages (Araki et al., 1993; Huhtala et al., 2002). Passages <16 were used in the present study. Primary HCEC (pHCEC) were purchased from Cascade Biologics, Inc. (Portland, OR, USA). These cells were grown and maintained in EpiLife® medium (Cascade Biologics, Inc., Portland, OR, USA), supplemented with human corneal growth supplement (HCGS) containing bovine pituitary extract, bovine insulin, hydrocortisone, bovine transferrin, and mouse EGF in the absence of antibiotics and antimycotics, as recommended by the manufacturer. Second- or third-passage cells were used for volume regulation experiments and immunoblot analyses. CEC were grown to 80–90% confluence and then deprived of serum and growth factors for 24 h prior to experimentation.

2.3 Cell lysate preparation

Cell lysates were prepared using a modification of a method developed by Bildin et al. (Bildin et al., 2000). In brief, HCEC, RCEC, and pHCEC were grown in 100-mm culture plates (Fisher Scientific, Pittsburgh, PA, USA) to 80–90% confluence, and deprived of serum and growth factors for 24 h. Cells were washed twice with ice-cold phosphate-buffered saline (PBS), then homogenized on ice with .8 ml of homogenization buffer—20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1.5 mM EGTA, 2.5 mM sodium pyrophosphate, 10 mM β-glycerolphosphate, and 1 mM Na₃VO₄—supplemented with a protease inhibitor mixture—1 mM benzamidine, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were harvested by scraping with a rubber policeman. Bovine eyes obtained from a local slaughterhouse were placed on ice and washed with PBS. Bovine CEC (BCEC) were harvested directly from bovine eyes by first gently everting the corneas, placing them endothelial side down on watch glasses, and exposing them for 2 min to 200 µl of homogenization buffer. Homogenates from HCEC, RCEC, pHCEC, and BCEC were passed through a 21-gauge needle fitted to a 3 ml syringe at least four times, and thereafter through a 26-gauge needle until easy flow was achieved. This was followed by sonication (three times at 40 mV for 10 sec/each) on ice with a tip sonicator. Homogenates were spun first at 3000 rpm for 5 min at 4°C (Ependorff 5417C, Brinkmann Instrument, Westbury, NY, USA) and the resulting supernatants centrifuged at 12 000 rpm for 15 min at 4°C to pellet the cellular debris. Plasma membrane-enriched fractions were obtained by supernatant centrifugation at 32 000 rpm for 30 min at 4°C (SW50.1 rotor and Beckman L5-50 Ultracentrifuge, Palo Alto, CA, USA) (Reinach and Holmberg, 1987). The resulting plasma membrane enriched pellets were resuspended in 100 µl of 2% sodium dodecylsulphate (SDS). Total protein concentration of the resulting whole cell and membrane-enriched fraction lysates was measured using a bichinchoninic acid assay (Micro BCA protein assay kit; Pierce Biotechnology, Rockford, IL, USA). Lysates were dissolved in equal volumes of Laemmli sample buffer—12.5 mM Tris/HCl (pH 6.8), 4% SDS, 20% glycerol, 2.5% β-mercaptoethanol, and 0.1% bromophenol blue. Mouse brain, rat brain, and kidney extract served as positive controls to confirm identity of the obtained KCC bands (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.4 Western blot analysis

After boiling the samples for 5 min, equal amounts of total protein were electrophoresed on 7.5 (KCC experiments) or 10% (MAPK experiments) Tris-HCl Ready Gel (Bio-Rad Laboratories, Hercules, CA, USA) at 160V for 1 h. Resolved protein was transferred from unstained gels to polyvinylidene difluoride (PVDF) membranes (Immun-Blot™, Bio-Rad Laboratories) in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3, and 10% methanol v/v) for 75 min at 50V using a semidry transfer apparatus (Trans-Blot SD, Bio-Rad Laboratories). Blots were exposed to blocking solution—5% nonfat dry milk in PBS containing 0.1% Tween-20 (PBS-T), pH 7.4—for 1 h at room temperature. Incubation of the specific affinity-purified goat polyclonal antibody raised against a peptide mapping at the *N*-terminus of the four KCC isoforms of human origin (Santa Cruz Biotechnology) was performed with gentle agitation overnight at 4°C at 1:500 dilution in blocking solution. The antibodies used to evaluate the activation of MAPK pathways were anti-phospho-p44/42 (1:1000), anti-phospho-p38 (1:1000) (Cell Signaling, Richmond, VA, USA), anti-p44/42 (1:2000), and anti-p38 (1:1000) (Santa Cruz Biotechnology) (Wang et al., 2006). After primary antibody incubation, blots were washed four times with blocking solution and then incubated with appropriate secondary antibody (horseradish peroxidase-conjugated) (Santa Cruz Biotechnology) diluted 1:2000 in blocking solution for 1 h at room temperature. Finally, membranes were washed twice in succession with PBS-T, PBS, and distilled water for 10 min each, followed by incubation in enhanced chemiluminescence (ECL) reagent (ECL Plus Western Blotting detection system; Amersham Biosciences, Piscataway, NJ, USA) for 5 min. Western blots probed with specific KCC isoform-specific antibodies were stripped and reprobed with another KCC isoform or β -actin antibody (Santa Cruz Biotechnology) as an internal standard. Protein abundance levels were analyzed using SigmaScan Pro 5.0 software (SPSS Inc., Chicago, IL, USA).

2.5 Measurement of relative cell volume

Relative changes in cell volume were monitored using a Fusion™ Universal Microplate Analyzer (Perkin-Elmer, Boston, MA, USA,) (Capo-Aponte et al., 2005). Briefly, HCEC, pHCEC, and RCEC were harvested and grown in 24-well plates (MULTIWELL™ Falcon, Lincoln Park, NJ, USA) for up to 48 h, until they reached 80–90% confluence in supplemented growth medium (described above). Growth medium was replaced with isotonic solution containing 10 μ M calcein-AM, in which cells were incubated for 60 min at 37°C. Cells were then washed twice and incubated with 200 μ L isotonic solution (300 mOsm) per well, with or without drugs, for 30 min prior to each experiment.

Before exposing the cells to a hypotonic challenge, initial fluorescence values under isotonic conditions were recorded in order to later correct for any fluorescence drift. Fluorescence readings were taken every 30 s (200 ms exposure per well, followed by a 10-s delay prior to initiating the next 24-well reading cycle). Recordings were set to high sensitivity and an illumination intensity level of 5, in order to achieve an optimal signal without inducing appreciable photodynamic damage or dye bleaching. The bathing osmolarity of each well was quickly adjusted to the desired osmolarity by addition of pre-warmed distilled water using a multi-channel pipettor. Attainment of the desired osmolarity and pH was validated in sham procedures.

2.6 Relative volume determination

Conversion of fluorescence values to relative cell volume was achieved by determination of the relationship between normalized drift-corrected fluorescence (F_t/F_0) during steady-state volume and the reciprocal of the relative osmotic pressure of the external solution (π_0/π_t) (Capo-Aponte et al., 2005; Hamann, 2002). The resultant linear relationship indicates the correspondence between relative changes in fluorescence and changes in cell volume. Conversion of fluorescence to relative cell volume was done by applying the equation: $V_t/$

$V_0 = [(F_t/F_0) - f_b]/(1 - f_b)$, where V_t is the cell water volume at time t , V_0 is V_t at $t = 0$, F_0 is the fluorescence of cells in isotonic solution having osmotic pressure π_0 , and F_t is the fluorescence having osmotic pressure π_t . The y intercept (f_b) is the background fluorescence, which indicates the proportion of intracellular calcein insensitive to external osmolarity.

2.7 Data analysis and statistics

Raw data were corrected for fluorescence drift independent of cell volume changes, as previously described (Capó-Aponte et al., 2005). Plots are V_t/V_0 , as a function of time, where V_t is the cell volume at time t and V_0 is the initial cell volume. The percentage (%) of volume recovery was calculated as $(V_{\max} - V_f)/(V_{\max} - V_0) \times 100$, where $V_{\max}$ is the maximum volume change during the challenge and V_f is the final volume. Data were analyzed using ORIGIN™ 6.1 software. Using the Student's two-tailed t -test, differences were considered statistically significant when $p < 0.05$. Data are shown as mean value $\pm$ standard error of the mean (SEM).

3. Results

3.1 KCC is involved in RVD and maintenance of steady-state cell volume

Under anisomotic stress, isotonic cell volume restoration depends on selective ion membrane transporter and channel activation. In HCEC, a hypotonic-induced RVD response involves KCC activation in parallel with separate increases in K^+ and Cl^- channel conductance (Capó-Aponte et al., 2005). This notion is based on RVD inhibition induced by the KCC inhibitor, DIOA and cell shrinkage induced by the KCC activator, *NEM*, under isotonic conditions. Additionally, since most of our current knowledge regarding CEC volume regulation derives from studies of RCEC using light scattering techniques and, since the RVD time course reported with this methodology is slower than the RVD response previously obtained in HCEC using the fluorescence microplate analyzer, we employed the latter technique to evaluate both HCEC and RCEC hypotonic-induced relative cell volume responses (Al-Nakkash et al., 2004; Wu et al., 1997). Consistent with previous reports, HCEC are able to undergo a complete and relatively rapid RVD ($\tau_c = 2.5$ min) subsequent to a 150 mOsm hypotonicity-induced swelling. This RVD was inhibited by 35% in the presence of 100 μ M DIOA (Figs. 1A and C). In contrast, RCEC displayed an incomplete and slower RVD ($\tau_c = 10$ min) induced by a similar challenge. In RCEC, DIOA had no significant effect on RVD when compared to the untreated counterpart (Figs. 1B and C). These results show that HCEC have a higher RVD activation capacity than do RCEC. Furthermore, DIOA only inhibited RVD in HCEC suggesting that KCC activity could account for the larger RVD response in HCEC.

We further validated the use of the SV40-immortalized HCEC line for cell volume regulation studies by exposing pHCEC to similar challenges. In the presence of a 50% hypotonic challenge, pHCEC underwent a rapid and complete RVD ($\tau_c = 3$ min). Furthermore, DIOA blocked the RVD by 43%, which was not different from the volume recovery observed in HCEC (Fig. 1A).

To further elucidate the functional roles of KCC, we evaluated its involvement in the maintenance of steady-state cell volume by assessing whether inhibition or stimulation of KCC produces alterations in cell volume under isotonic conditions in this cell lines. In HCEC, addition of DIOA (100 μ M) under isotonic conditions induced a 31% swelling, whereas treatment with *NEM* (1 mM) induced 47% cell shrinkage (Figs. 2A and C). Figures 2B and D show in RCEC that DIOA only induced a 9% cell swelling, whereas *NEM* induced 15% cell shrinkage. Although small, both events were significant when compared to untreated RCEC in isotonic solution. The specificity of *NEM* for inducing stimulation of KCC was verified by pre-incubating cells in DIOA (100 μ M) for 30 min before stimulation with *NEM*. This manipulation significantly decreased the *NEM*-induced cell shrinkage in both cell lines (Figs.

2A and B). The significant difference in relative volume recovery induced by a hypotonic challenge and the cellular responses to these reagents under isotonic conditions suggest that the patterns of KCC isoform expression are not the same in HCEC and RCEC. Alternatively, the delayed RVD in RCEC is explained by lower KCC expression levels in RCEC than HCEC.

3.2 Corneal epithelial cells express multiple KCC isoforms

KCC1, KCC3, and KCC4 isoforms were revealed in HCEC, RCEC, and BCEC based on bands at ~135, 150, and 170 kDa, respectively (Figs. 3A–C, upper panel). These bands were no longer detectable when the blot was incubated with the antibody in the presence of the respective peptide antigen (5:1 molar ratio) in the abovementioned membrane preparations (data not shown). Interestingly, the relative levels of KCC1 expression in HCEC under isotonic conditions were ~three-fold higher than in RCEC. Conversely, the relative levels of KCC3 were ~two-fold higher in RCEC compared to HCEC (Figs. 3A and B, lower panel). KCC4 expression levels were the same in the different cell lines (Fig. 3C). In contrast, KCC2 was undetectable even though it was detectable in the mouse and rat brain extracts used as positive controls (Fig. 4).

RVD has been previously described for many other cell systems (Adragna et al., 2004; Gillen et al., 1996; Hiki et al., 1999; Mercado et al., 2000). However, the relative roles of the different KCC isoforms in isotonic steady-state cell volume maintenance and RVD are less clear. Since HCEC can undergo faster RVD (Figs. 1A and B) and possess ~three-fold greater KCC1 protein abundance than RCEC (Figs. 3A and B), we suggest that KCC1 is, indeed, the housekeeping transporter responsible for the maintenance of steady-state cell volume.

We also explored whether there is a significant difference in KCC expression patterns between non-transformed pHCEC and transformed HCEC lines. There are similar expression levels of KCC1 in pHCEC and HCEC, as well as a comparable overall pattern of KCC1 abundance compared to the KCC3 and KCC4 isoforms (Fig. 5). Similar to the results obtained for HCEC, we were unable to detect KCC2 in pHCEC, but did detect the protein in the mouse and rat brain extracts used as positive controls (data not shown).

3.3 KCC membrane content increases during hypotonic challenge

The membrane content of the various KCC isoforms in HCEC and RCEC during a hypotonic challenge was assessed for the purpose of determining their relative contribution to RVD. In HCEC, there was a 2.8-fold increase in the level of KCC1 membrane content, which peaked at 2.5 min exposure to a 50% hypotonic challenge ($p < 0.001$) (Fig. 6A). In contrast, RCEC only displayed a peak 27% increase of KCC1 content at 15 min exposure to this challenge, albeit significant ($p < 0.05$) (Fig. 6B). Although KCC3 protein content was consistently higher (~two-fold) in RCEC compared to HCEC, in neither cell line did the level of its membrane content increase above isotonic levels in the presence of this hypotonic challenge (Figs. 7A and B). Similarly, the level of KCC4 membrane content was not altered during exposure to such stress (Figs. 8A and B). The higher expression of KCC1 under isotonic conditions and the increased level of membrane content during hypotonic challenge in HCEC are in accordance with these cells' ability to undergo a faster and more complete RVD than RCEC. Furthermore, the more rapid time course for increases in HCEC KCC1 membrane content than in RCEC is in agreement with the faster time constants for cell volume recovery in HCEC (i.e., 2.5 versus 15 min, respectively).

3.4 Time-dependent MAPK activation during hypotonic challenge

Changes in cell volume are imperative for cell proliferation and migration. Accordingly, we examined whether the regulation of volumes in HCEC and RCEC affect the activation of p44/42 and p38MAPK, both of which are required for CEC proliferation and migration,

respectively. A 50% hypotonic challenge markedly activated p44/42MAPK within 1 min and peaked at levels five- and four-fold above baseline at 2.5 and 5 min in HCEC and RCEC, respectively (Figs. 9A and B). Such activation gradually decreased to control levels at 30 and 60 min following challenge initiation.

Similarly, time-dependent phosphorylation of p38MAPK was observed with three- and six-fold increases during exposure to the same challenge, with peak activations at 2.5 and 15 min in HCEC and RCEC, respectively (Figs. 10A and B). Levels of phosphorylated p38MAPK returned to baseline after 30 min in HCEC. In RCEC, however, the phosphorylated level remained ~two-fold higher than control levels, even after 60 min. These results suggest that transient swelling may be an initiator of downstream signaling required for cell proliferation and migration.

3.5 KCC involvement in hypotonicity-induced MAPK activation

Several reports indicate a dependence of MAPK superfamily signaling on KCC activation, which is requisite for control of cell proliferation and invasiveness (Shen et al., 2003; Shen et al., 2001; Shen et al., 2004). Therefore, we evaluated the involvement of KCC in controlling activation of the MAPK signaling pathways responsible for inducing cell proliferation and migration (i.e., p44/42 and p38MAPK, respectively). Interestingly, pre-incubation with DIOA (100 μ M) abrogated hypotonicity-induced activation of p44/42MAPK by 65% ($p < 0.001$) compared to untreated HCEC exposed to the same 50% hypotonic challenge. Conversely, activation of KCC under isotonic conditions by *NEM* (1 mM) resulted in a five-fold increase in p44/42MAPK phosphorylation above that noted for untreated cells in the same isotonic medium ($p < 0.001$) (Fig. 11A). To substantiate the pharmacological specificity of DIOA and *NEM* in eliciting modification of activation of p44/42MAPK in CEC, we used specific inhibitors for p44/42MAPK (PD98059 and U0126), p38MAPK (SB203580), and protein tyrosine kinase (genistein) at concentrations previously described as being effective in these cell lines (Bildin et al., 2003; Liang et al., 1998; Wang et al., 2006). PD98059 (10 μ M) and U0126 (10 μ M) completely prevented HCEC phosphorylation of p44/42MAPK. Moreover, pre-treatment with the p38MAPK inhibitor, SB203580 (10 μ M), induced a further activation of p44/42MAPK (i.e., 19%; $p < 0.05$) during the hypotonic challenge. Hypotonicity-induced activation of p44/42MAPK was completely abolished in the presence of the relatively specific protein tyrosine kinase inhibitor genistein (40 μ M).

Parallel studies on RCEC revealed disparate results in terms of p44/42MAPK activation during pharmacological manipulation of KCC. Unlike HCEC, pre-incubation of RCEC with DIOA had no significant effect on the hypotonicity-induced activation of p44/42MAPK. Moreover, treatment with *NEM* did not induce p44/42MAPK phosphorylation under isotonic conditions (Fig. 11B). Pre-incubation with U0126 and PD98059 decreased the hypotonicity-induced activation p44/42MAPK by 62 and 47%, respectively ($p < 0.001$). Similar to HCEC, inhibition of p38MAPK with SB203580 induced an additional activation of p44/42MAPK (i.e., 26%; $p < 0.05$) during hypotonic challenge, compared to the challenge alone (Fig. 11B).

We also evaluated whether KCC has any effect on p38MAPK activation induced by hypotonic stress. Pre-treatment of both cell lines with DIOA had no effect on hypotonicity-induced activation of p38MAPK (Figs. 12A and B). Furthermore, stimulation of KCC with *NEM* under isotonic conditions did not induce any discernible effect on p38MAPK phosphorylation in either cell line. Consistent with the crosstalk paradigm, inhibition of the HCEC p44/42MAPK pathway with U0126 and PD98059 induced a 23 and 18% increase of p38MAPK activation, respectively, above the activation observed with hypotonic challenge alone ($p < 0.05$). Similar results were obtained when RCEC p44/42MAPK was inhibited with U0126 and PD98059 (40 and 32%, respectively) ($p < 0.001$). Pre-treatment with SB203580 and genistein completely prevented hypotonicity-induced p38MAPK phosphorylation in both cell lines.

4. Discussion

CEC are capable of adapting to hypotonic stress by restoring their volumes to isotonic levels through activation of specific ion transport mechanisms mediating net ion efflux. We previously suggested that KCC—in parallel with K^+ and Cl^- channel-coupled conductance—is a mechanism contributing to ion efflux in HCEC. However, KCC protein expression in HCEC, or the molecular and functional activity were not evaluated in the RCEC used in the present study (Capó-Aponte et al., 2005). Since most earlier studies employed RCEC rather than HCEC, (Al-Nakkash et al., 2004; Al-Nakkash and Reinach, 2001; Farrugia and Rae, 1993; Wu et al., 1997; Yang et al., 2000), we compared here RCEC behavior with that of HCEC during a hypotonic challenge. We demonstrate that HCEC have a greater RVD capacity than do RCEC (Fig. 1). Pharmacological manipulation of KCC with DIOA and *NEM* established KCC's functional role in the RVD response and in HCEC maintenance of steady-state volume; however similar manipulations did not affect isotonic volume homeostasis or the regulatory volume responses induced by hypotonic challenge in RCEC (Figs. 2). The slower control RCEC RVD time course (obtained with the fluorescence microplate analyzer) observed compared to its HCEC counterpart is in agreement with the RVD time course (obtained using light scattering techniques) previously described for RCEC (Al-Nakkash et al., 2004; Wu et al., 1997). This similarity is in line with the findings that (i) there is an actual difference in the kinetics and extent of volume regulation between the two species and rather than accounted for by variation in methodology.

Based on both the similar volume regulatory responses to a hypotonic challenge as well as KCC isoform content in primary non-transformed cell line counterparts (Figs. 1A and 5), this study further validates the use of transformed cell lines of HCEC for cell volume regulation studies.

Because of disparate RVD capacity and sensitivity to pharmacological treatment of KCC in RCEC and HCEC, we determined whether or not such differences could be accounted for by variations in KCC isoform expression patterns. Our results demonstrate the presence of KCC1, -3, and -4 isoforms in HCEC, RCEC, pHCEC, and BCEC. Not surprisingly, we found that CEC from different species express different levels of specific KCC isoforms. KCC1 protein level expression is ~three-fold higher in HCEC than in RCEC (Fig. 3A). In contrast in HCEC, KCC3 level of expression was more than double in RCEC, whereas no difference in KCC4 expression was noted between these two species (Figs. 3B and C). In agreement with the present notion that KCC2 is a neuron-specific isoform, we did not detect its presence in CEC, but did detect it in mouse and rat brain extract controls (Fig. 4).

In addition to having higher levels of KCC1 expression under isotonic conditions, we determined in HCEC that a 50% hypotonic challenge induces a significant increase in KCC1 membrane content within 1 min (Fig. 6A). In contrast, only RCEC display modest increases in KCC1 membrane content after 15 min (Fig. 6B). Moreover, KCC3 and KCC4 membrane contents were not altered after exposure to hypotonic stress (Figs. 7 and 8). The more rapid RVD response by HCEC than in RCEC, could be attributed to higher KCC1 expression levels. These results validate that—as in many other cell types (Adragna et al., 2006; Adragna et al., 2004; Gillen et al., 1996; Ubels et al., 2006)—KCC1 is the “house-keeping” isoform, involved in maintenance of steady-state ionic homeostasis as well as CEC volume regulation.

Restitution of isotonicity via RVD is important for maintenance of cell viability and function, e.g., proliferation and migration (Mao et al., 2005; Nilius et al., 1997; Nilius and Wohlrab, 1992; Shen et al., 2003; Shen et al., 2001; Shen et al., 2004). We show here that RVD is accompanied by transient increases in activity of p44/42 and p38MAPK. Activation of p44/42 and p38MAPK are key elements for cell proliferation and migration, which in turn, are essential

for corneal epithelial renewal capacity. Proliferation is also dependent on EGF-induced increases in K^+ and Cl^- conductance, which results in net osmolyte efflux and shrinkage. Previous studies have shown that RVD inhibition—through blockage of K^+ , Cl^- channels and KCC—is capable of hampering cell proliferation and migration (Mao et al., 2005; Nilius et al., 1997; Nilius and Wohlrab, 1992; Shen et al., 2003; Shen et al., 2001; Shen et al., 2004). Therefore, cell proliferation is dependent on coordinated regulation of ion transport pathway activity.

Apart from the functional role of KCC in maintaining volume homeostasis and restoring isotonic cell volume after hypotonic stress, we evaluated its functional involvement in the hypotonicity-induced activation of p44/42 and p38MAPK pathways. Only in HCEC did drug-induced alteration of KCC activity modify the hypotonicity-induced activation of p44/42MAPK. Such a difference in reagent sensitivity between HCEC and RCEC could be explained by a difference in KCC1 membrane expression under both isotonic and hypotonic conditions (Figs. 3 and 6). This difference is reflected in the observation that HCEC recover their relative isotonic cell volume more completely and faster (four-fold) than do RCEC during acute hypotonic challenge (Fig. 1). Such differences in extent and rapidity of recovery suggest that HCEC restore cell contacts more rapidly than do RCEC. Restoration of cell contacts, in turn, leads to more rapid p44/42MAPK deactivation (Fig. 9), suppresses proliferation, and maintains the cells in a quiescent state. Thus, KCC—specifically KCC1—appears to be an upstream regulator of hypotonicity-induced p44/42MAPK activation in HCEC. The functional roles of KCC3 and KCC4 isoforms in CEC remain to be elucidated.

p38MAPK phosphorylation was maximized at 2.5- and 15-min exposures to hypotonic challenge in HCEC and RCEC, respectively (Fig. 10), but its activation was insensitive to DIOA and *NEM* in either cell line (Fig. 12). This suggests that hypotonicity-induced activation of p38MAPK in CEC is KCC-independent. Another interesting finding is that peak activations of p44/42 and p38MAPK in HCEC displayed similar time courses (i.e., 2.5 min), suggesting a concomitant activation of these MAPKs. In contrast, RCEC exhibited differential p44/42 and p38MAPK peak activations at 5 and 15 min, respectively. This disagreement in the time courses of MAPK activation between HCEC and RCEC under hypotonic stress suggests that, although these cell lines share some signaling pathways triggered by tyrosine kinase receptor activation, they have different sets of upstream MAPK regulators. Such variations may be species-specific.

Our data also substantiate the present notion that tyrosine phosphorylation of p44/42 and p38MAPK during hypotonic stress is essential for RVD activation (Tilly et al., 1996b; Tilly et al., 1993). Pre-treatment of HCEC and RCEC with genistein prevented the hypotonicity-induced phosphorylation of these MAPKs, thus indicating that this response is mediated by a tyrosine kinase-dependent mechanism in CEC (Figs. 11 and 12). Tyrosine kinase receptors can be activated by membrane stretch induced by hypotonic swelling, which in turn, activates the EGF receptor (Franco et al., 2004). Such a rationale could explain how, in CEC, both hypotonic swelling and EGF receptor stimulation produce the same effect, i.e., MAPK pathway activation. However, the fact that inhibition of KCC by DIOA prevents the hypotonicity-induced activation of p44/42MAPK, but not that of p38, suggests that KCC is an upstream component of the signaling pathway inducing p44/42MAPK activation. In contrast, the inability of DIOA to block the hypotonicity-induced activation of p38MAPK or to activate p38MAPK with *NEM* under isotonic conditions suggests that KCC is not part of such activation, or downstream from p38MAPK.

Finally, the additional increase in hypotonicity-induced p44/42 and p38MAPK activation observed after selective inhibition of these parallel pathways is consistent with the growth factor-induced, crosstalk-mediated activation of MAPK pathways in CEC (Sharma et al., 2003; Wang et al., 2006). This supports the finding that the pattern of hypotonicity-induced

stimulation of each pathway is dependent on the extent of crosstalk between p44/42 and p38MAPK.

In summary, CEC express KCC1, KCC3, and KCC4 isoforms. KCC1 is the isoform involved in isotonic steady-state volume homeostasis and RVD during hypotonic challenge, as demonstrated by pharmacological manipulation and increase of KCC1 membrane content during this challenge. Hypotonicity-induced activation of p44/42MAPK in HCEC is dependent on functional KCC1 expression, as demonstrated by (i) the higher content of this isoform during hypotonic stress, (ii) inhibition of hypotonicity-induced phosphorylation of p44/42MAPK by DIOA, (iii) increased phosphorylation of p44/42MAPK activation during isotonic conditions induced by *NEM*. In RCEC, such pharmacological manipulation had no effect. This negative result is in accord with the low KCC1 expression level in this cell line. Pharmacological inhibition or stimulation of KCC did not appreciably affect hypotonicity-induced p38MAPK phosphorylation, suggesting that this signaling pathway is largely independent of KCC1. Based on these findings, we suggest that KCC may be the sensor for detecting changes in external osmolarity or functions as a signal transduction receptor, upstream of p44/42MAPK. Our results further suggest that modulation of KCC expression and activity could be the basis for a novel therapeutic approach for hastening corneal wound healing. To our knowledge, this is the first report elucidating the molecular expression and functional roles of KCC in CEC.

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Fig. 1. KCC contributes to RVD. After initial exposure to 300 mOsm isotonic solution (A) HCEC, pHCEC, and (B) RCEC were exposed to 150 mOsm hypotonic challenge in the presence or absence of the KCC inhibitor DIOA (100 μ M). Data are presented as mean $\pm$ SEM ($n = 6$). Error bars are not shown when smaller than the symbols. C: Summary of the volume recovery (%) of HCEC and RCEC. * $p < 0.001$ versus untreated cells during hypotonic challenge.

Fig. 2.

KCC participates in steady-state volume homeostasis. After initial exposure to isotonic conditions, (A) HCEC and (B) RCEC were exposed to the following reagents added in isotonic solution: 100 μ M DIOA, 1 mM NEM, and NEM after 30 min pre-treatment with DIOA.

Untreated cells in isotonic solution are shown for comparison. Summary of the maximum volume change (%) in (C) HCEC and (D) RCEC induced by above reagents. Data are presented as mean $\pm$ SEM ($n = 7$). Error bars are not shown when smaller than the symbols. * $p < 0.001$; ** $p < 0.05$ versus untreated isotonic controls.

Fig. 3.

CEC express multiple KCC isoforms. Representative Western blot of HCEC (H), RCEC (R), and BCEC (B) membrane fractions loaded onto a 7.5% SDS gel at 24 or 12 μ g per lane, as noted. The separated proteins were transferred to PVDF membranes and probed with polyclonal antibody (1:500) against (A) KCC1, (B) KCC3, or (C) KCC4. Blots were stripped and reprobed with β -actin antibody to compare protein loading. Summary of the differential expression levels of each KCC isoform in CEC is displayed in the lower panels, expressed as arbitrary units. Error bars are not shown when smaller than the symbols. Data are presented as the mean $\pm$ SEM of three independent experiments. * $p < 0.001$; ** $p < 0.05$.

Fig. 4. KCC2 isoform is not expressed in CEC. Representative blot of three independent Western blot analyses of various brain and corneal epithelium preparations. Fifty μ g of protein per lane of mouse brain (M), rat brain (Rt), HCEC (H), RCEC (R), and BCEC (B) were loaded onto a 7.5% SDS gel, as indicated. Polyclonal antibody against KCC2 (1:500) identified the presence of KCC2 in brain extracts but failed to detect KCC2 in CEC preparations.

Fig. 5. HCEC and pHCEC express similar levels of KCC isoforms. Representative images of three independent Western blots of HCEC and pHCEC membrane fractions loaded onto a 7.5% SDS gel (50 μ g per lane). The separated proteins were transferred to PVDF membranes and probed with polyclonal antibody (1:500) against KCC1, KCC3, or KCC4.

Fig. 6. Time-dependent changes of KCC1 membrane content induced by hypotonic stress. Representative Western blot and summary of hypotonicity-induced changes in KCC1 membrane content of (A) HCEC and (B) RCEC. Cells were exposed to isotonic solution or 50% hypotonic challenge for the indicated times. Membrane content of KCC1 is presented as fold change to isotonic control. Data are presented as mean $\pm$ SEM of three independent experiments. * $p < 0.001$; ** $p < 0.05$.

Fig. 7. Time-dependent changes of KCC3 membrane content induced by hypotonic stress. Representative Western blot and summary of hypotonicity-induced changes in KCC3 membrane content of (A) HCEC and (B) RCEC. Cells were exposed to isotonic solution or 50% hypotonic challenge for the indicated times. Membrane content of KCC3 is presented as fold change to isotonic control. Data are presented as mean $\pm$ SEM of three independent experiments.

Fig. 8. Time-dependent changes of KCC4 membrane content induced by hypotonic stress. Representative Western blot and summary of hypotonicity-induced changes in KCC4 membrane content of (A) HCEC and (B) RCEC. Cells were exposed to isotonic solution or 50% hypotonic challenge for the indicated times. Membrane content of KCC4 is presented as fold change to isotonic control. Data are presented as mean $\pm$ SEM of three independent experiments.

Fig. 9.

Time-dependent changes in p44/42MAPK phosphorylation induced by hypotonic stress. (A) HCEC and (B) RCEC were serum-starved for 24 h at 80–90% confluence and exposed to 50% hypotonic challenge for the indicated times. Samples were loaded onto a 10% SDS gel. Representative Western blots of membranes probed with anti-phospho-p44/42MAPK antibody (upper panels) and summary of time-dependent changes in p44/42MAPK phosphorylation status (lower panels), expressed as fold change to isotonic control. Data shown as mean $\pm$ SEM of three independent experiments.

Fig. 10.

Time-dependent changes in p38MAPK phosphorylation induced by hypotonic stress. (A) HCEC and (B) RCEC were serum-starved for 24 h at 80–90% confluence and exposed to 50% hypotonic challenge for the indicated times. Samples were loaded onto a 10% SDS gel. Representative Western blots of membranes probed with anti-phospho- p38MAPK antibody (upper panels) and summary of time-dependent changes in p38MAPK phosphorylation status (lower panels), expressed as fold change to isotonic control. Data shown as mean $\pm$ SEM of three independent experiments.

Fig. 11.

KCC role on hypotonicity-induced p44/42MAPK phosphorylation. HCEC (A) and (B) RCEC were serum-starved for 24 h and exposed to either 100 μ M DIOA, 10 μ M U0126 (U0), 10 μ M SB203580 (SB), 10 μ M PD98059 (PD), or 30 μ M genistein (Gen) for 30 min. Subsequently, HCEC and RCEC were exposed to 150 mOsm hypotonic challenge for 2.5 and 5 min, respectively, in the continuous presence of the same inhibitors. Control cells were maintained in isotonic solution, whereas KCC activation was achieved by *NEM* (1 mM) addition under isotonic conditions. A representative blot shows the phosphorylation status of p44/42MAPK using a specific monoclonal antibody (upper panels) and summary of p44/42MAPK

phosphorylation status of three independent experiments (lower panel). Data are shown as mean $\pm$ SEM.

Fig. 12.

Role of KCC in hypotonicity-induced p38MAPK phosphorylation. HCEC (A) and (B) RCEC were serum-starved for 24 h and exposed to either 100 μ M DIOA, 10 μ M U0126 (U0), 10 μ M SB203580 (SB), 10 μ M PD98059 (PD), or 30 μ M genistein (Gen) for 30 min. Subsequently, HCEC and RCEC were exposed to 150 mOsm hypotonic challenge for 2.5 and 5 min, respectively, in the continuous presence of the same inhibitors. Control cells were maintained in isotonic solution, whereas KCC activation was achieved by *NEM* (1 mM) addition under isotonic conditions. A representative blot shows the phosphorylation status of p38MAPK using a specific monoclonal antibody (upper panels) and summary of p38MAPK phosphorylation status of three independent experiments (lower panel). Data are shown as mean $\pm$ SEM.