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# Adaptation by Corneal Epithelial Cells to Chronic Hypertonic Stress Depends on Upregulation of Na:K:2Cl Cotransporter Gene and Protein Expression and Ion Transport Activity

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**Abstract.** We examined the ability of SV40-immortalized human and rabbit corneal epithelial cells (HCEC and RCEC, respectively) to adapt to chronic hypertonic stress. Under isotonic conditions, in the presence of 50 μM bumetanide, proliferation measured as $^{3}$H-thymidine incorporation declined in RCEC and HCEC by 8 and 35%, respectively. After 48 hr exposure to 375 mOsm medium, RCEC proliferation fell by 19% whereas in HCEC it declined by 45%. Light scattering behavior demonstrated that both cell lines mediate nearly complete regulatory volume increase (RVI) responses to an acute hypertonic (375 mOsm) challenge, which in part depend on bumetanide-sensitive Na-K-2Cl cotransporter (NKCC) activity. Following exposing RCEC for 48 hr to 375 mOsm medium, their RVI response to an acute hypertonic challenge was inhibited by 17%. However, in HCEC this response declined by 68%. During exposure to 375 mOsm medium for up to 24 hr, only RCEC upregulated NKCC gene and protein expression as well as bumetanide-sensitive $^{86}$Rb influx. These increases are consistent with the smaller declines in RVI and proliferation capacity occurring during this period in RCEC than in HCEC. Therefore, adaptation by RCEC to chronic hypertonic stress is dependent on stimulation of NKCC gene and protein expression and functional activity. On the other hand, under isotonic conditions, HCEC RVI and proliferation are more dependent on NKCC activity than they are in RCEC.

**Key words:** Hypertonicity — Cell proliferation — Volume regulation — Potassium influx — Na-K-2Cl cotransporter mRNA and protein expression — Cornea

## Introduction

The maintenance of corneal transparency is dependent on the ability of the corneal epithelium to act as a barrier against noxious agents, infection and also secrete osmolytes along with fluid from the stroma towards the tears. A possible challenge to these functions could include increases in tear and stromal osmolarity, which is frequently seen in dry eye patients (Farris, 1994). Some of their symptomology may be a consequence of hypertonicity-induced epithelial cell shrinkage and increases in tight junctional permeability. For example, increases in corneal epithelial erosion and losses in corneal transparency could initially result from osmotically induced epithelial cell shrinkage. One approach to determine whether increases in tear film tonicity could impact on cell function is to evaluate in a tissue culture system the effects of chronic hypertonic stress on cell proliferation, RVI maintenance capacity and levels of NKCC gene and protein expression. However, there is only limited information about the impact of chronic hypertonic stress on these cell functions (Bildin et al., 1998).

It is known that hypertonicity-induced cell shrinkage can be reversed in many cells through a regulatory volume response termed a regulatory volume increase (RVI), which mediates increases in intracellular osmolytes levels through the stimulation of solute influx mechanisms (O'Neill, 1999). The membrane ion transporters involved in the RVI response include: (i) Na/K

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pump and NKCC; (ii) Na/H exchanger; (iii) $Cl/HCO_3$ exchanger. In human and rabbit corneal epithelial cells (HCEC and RCEC, respectively), we found that in response to an acute hypertonic challenge these cells mediate RVI through increases in NKCC activity (Bildin et al., 1998). However, there is no information on whether these cells can sustain RVI behavior subsequent to chronic hypertonic stress. This information is relevant for characterizing their adaptive capacity to such stress.

It is known in fibroblasts and endothelial cells that proliferation is dependent on NKCC activity since inhibition of NKCC activity suppresses cell growth (Panet et al., 1991; 1994). However, the role of NKCC activity in the maintenance of corneal epithelial growth has not been described. Nevertheless, it is conceivable that NKCC activity may play a role in the control of corneal epithelial growth based on some of the symptomology described in dry eye patients. These individuals can be chronically exposed to hypertonic tears and experience losses in epithelium barrier function as well as declines in the rate of wound healing. It is possible that these effects could be associated with decreases in NKCC activity. Such decreases could cause cell shrinkage and barrier function breakdown due to inadequate RVI capacity. Similarly impaired would healing could be the result of NKCC inhibition. On the other hand, corneal epithelial cells may be able to adapt to such a challenge. This response could be related to the ability of these cells to upregulate NKCC gene and protein expression and activity. To test this possibility, we evaluated in HCEC and RCEC whether exposure to hypertonic media ranging from 325 to 600 mOsm for up to 48 hr affects the level of expression of NKCC.

We report here that chronic hypertonic stress causes the RCEC to upregulate NKCC mRNA and protein expression as well as ion transport activity, while HCEC do not exhibit such compensatory responses.

## Materials and Methods

SV40-immortalized HCEC and RCEC, a generous gift from Dr. Araki-Sasaki (Kinki University, Hyogo, Japan), were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 10 ng/ml EGF, 1 μg/ml insulin and an antibiotic mixture containing penicillin and streptomycin (Araki-Sasaki et al., 1993, 1995). The cells were grown in an atmosphere of 5% $CO_2$, 95% ambient air at 37°C. Medium hypertonicity was achieved by the addition of sucrose.

### Cell Proliferation Assay

Logarithmic phase cells (second day after passage) grown on 24-well MULTIWELL Tissue Culture Plates (FALCON, Lincoln Park, NJ) were incubated in isotonic or in hypertonic (up to 600 mOsm) medium for up to 48 hr. Cell proliferation was determined based on measurements of $^3$H-thymidine incorporation. Briefly, after incubation for 1 hr at 37°C in iso/hypertonic medium containing 10 μCi/ml (methyl) $^3$H-thymidine (NEN, Boston, MA), the cells were washed twice with ice-cold PBS, 5% trichloroacetic acid (4 times) and fixed following two washings with 70% ethanol. Then the cells were lysed with 0.5 ml of a solution containing 0.5N NaOH and 5% SDS. Radioactivity was quantified by scintillation counting (LKB model Rack Beta 1219). Protein content per well was determined with the Lowry (1951) method.

### Cell Volume Monitoring

A light-scattering technique as developed by Fischbarg et al. (1993) was used to monitor relative cell volume. Briefly, layers of HCEC and RCEC grown on 11 × 22 mm rectangular glass coverslips were incubated for up to 48 hr until they reached 70–80% confluence in iso/hypertonic medium. For the measurements, cells were illuminated with a rectangular-profile light beam obtained through an expander and lenses from a 5 mW helium-neon laser. Low-angle forward-scattered light intensity was converted to relative intracellular volume. Data analysis following its computer acquisition included first its deconvolution by performing a four parameter-fit of the experimental data to an Exponential Associate with ORIGIN™ software (MicroCal, Northhampton, MA) (Wu et al., 1997). The extent of RVI and half-time were calculated as described before (Iserovich et al., 1998).

### $^{86}$Rb Uptake Measurement

The congener for potassium, $^{86}$Rb, was used to measure potassium influx. HCEC and RCEC were grown to confluence in multiwell plates and then exposed to either isotonic or hypertonic medium for up to 48 hr. Following preincubation for 10 min at 37°C with or without 50 μM bumetanide (dissolved in 70% ethanol) uptake (10 min at 37°C) was initiated by the addition of iso/hypertonic medium containing 2 μCi/ml $^{86}$Rb (NEN). To stop influx and remove any unincorporated isotope, the cells were washed four times with ice-cold 0.5 M $MgCl_2$. Cells were lysed with 0.5 ml of 5 M guanidine thiocyanate/0.1 M EDTA and radioactivity was quantified by scintillation counting. Influx data was normalized to cellular protein determined by Coomassie Protein Assay (Pierce, Rockford, IL).

### DNA Probe Generation

$^{32}$P-labeled single-stranded DNA probes were generated for NKCC cotransporter mRNA with specific activity of ~$4 \times 10^9$ cpm/μg by asymmetric PCR utilizing a specific antisense primer on corresponding homologous human and rabbit 529-nt DNA fragments. Fragments were isolated as PCR products using the total human and rabbit DNA as the template and specific primers: sense: 1650 TTTCAGGTCATGAGCATGGTGTC 1672, and antisense: 2155 CCCCGAGGAGGTGTGTCCGAGAC 2177. Unreacted nucleotides were removed by spin chromatography through Sephadex G-50. The probes were purified by 1% agarose gel electrophoresis. After identification of the radioactive band location by autoradiography, probes were extracted from agarose slices with QIAEXII Agarose Gel Extraction Kit (QIAGEN).

### Nuclease Protection Assay

RCEC and HCEC grown in 25 $cm^2$ culture flasks were exposed to either isotonic or hypertonic medium for up to 48 hr and lysed in 1 ml of 5 M guanidine thiocyanate (GuSCN)/0.1 M EDTA. Cell lysates were immediately analyzed or stored at −70°C. Twenty microliters of cell lysate (~100–200 K cells) were mixed with 5 μl of probe contained in 2 × standard saline citrate (SSC)/0.1 M EDTA and incubated at 30°C

for 20 hr. After hybridization, 2.5 μl of 10 mg/ml proteinase-K (Sigma) and 2.5 μl of 10% sodium dodecyl sulfate (SDS) were added. The mixture was incubated at 30°C for 30 min. Nucleic acids were extracted with 30 μl of a 1:1 mixture of buffer-saturated phenol and chloroform and isolated by spin chromatography through Sephadex G-50. An equivalent volume was added of S1 nuclease buffer containing up to 20 μg denaturated salmon sperm DNA and 2000 U/ml of S1 nuclease. This mixture was incubated at 45°C for 30 min. Following digestion and then electrophoresis in a 2% agarose gel, the gels were dried, and the position of the RNA-DNA duplexes was determined by autoradiography at −70°C, using REFLECTION Film and REFLECTION Intensifying Screens (DuPont NEN). Excised band radioactivity associated with full-length RNA-DNA duplexes was quantified with scintillation counting. The number of mRNA molecules per cell was calculated as described by Haines and Gillespie (1992) and normalized to a sample's DNA contents (measured by Southern dot hybridization with random primer extension of a $^{32}$P-labeled probe to total rabbit and human DNA). Protein content was determiend by Coomassie Protein Assay (Pierce).

### Western Blot Analysis

After different periods of exposure to hypertonicity, cells were washed twice with PBS and then scraped with a rubber policeman into 0.5 ml of homogenization buffer (HB) containing: 50 mM β-glycerophosphate, pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 0.1 mM Na-vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 mM benzimidine and 10 μg/ml aprotinin. Then the cells were sonicated (three times, 10 sec/ea) on ice with a tip sonicator and centrifuged at 500 × *g* for 15 min. The resulting supernatant was centrifuged for 100,000 × *g* for 30 min at 4°C. Protein pellet contents were determined with the Lowry (1951) method. To characterize the level of NKCC expression, 5 μg of 100,000 × *g* pellet and prestained molecular mass markers (Bio-Rad, Hercules, CA) were denatured in 10 μl of Laemmli sample buffer (2% SDS, 25% glycerol, 0.01% bromophenol blue in 62.5 mM Tris/HCl, pH 6.8) with 5% β-mercaptoethanol. After boiling for 5 min, the samples were electrophoresed on 7.5% polyacrylamide SDS minigels (Bio-Rad, Hercules, CA) at 30 mA per gel. Resolved proteins were electrotransferred overnight to Hybond-C Extra nitrocellulose membranes (Amersham, Arlington Heights, IL) at 30 V. The blots were blocked with 5% nonfat dry milk in PBS containing 0.1% Tween-20 (PBS-T) and then exposed for 1 hr at room temperature to a 1:10,000 dilution of T4 monoclonal antibody (DSHB, Iowa City, IA). After several washes with PBS-T, the blots were incubated with 1:2,500 rabbit antimouse HRP labeled IgG (Amersham) for 1 hr. The immunoreactive bands were detected with ECL + Plus kit (Amersham). Films were scanned and the density of ~185 kD bands was quantified using Sigmagel software (Jandel Scientific, San Rafael, CA).

### Statistical Analysis

All experimental data are expressed as mean ± SEM. The Student's *t*-test was employed throughout to evaluate the level of significance (i.e., $P < 0.05$).

## Results

### Chronic Hypertonic Stress Differentially Suppresses HCEC and RCEC Proliferation

Figure 1*A* (HCEC) and *B* (RCEC) shows the comparative time-dependent effects on proliferation of culturing these cell lines in isotonic and 375 mOsm media. The latter condition was chosen since such a level of hypertonicity was measured in the tears of some dry eye patients. After 24 hr, in both cell lines proliferation relative to the value measured in isotonic medium (i.e., 300 mOsm) was similarly inhibited by 11%. On the other hand, after 48 hr HCEC proliferation was significantly inhibited by 51% relative to HCEC cultured for the same length time of time in isotonic medium whereas in RCEC the decline was only 22%. Therefore, the proliferation capacity of RCEC appears to be less sensitive to chronic hypertonic stress than HCEC.

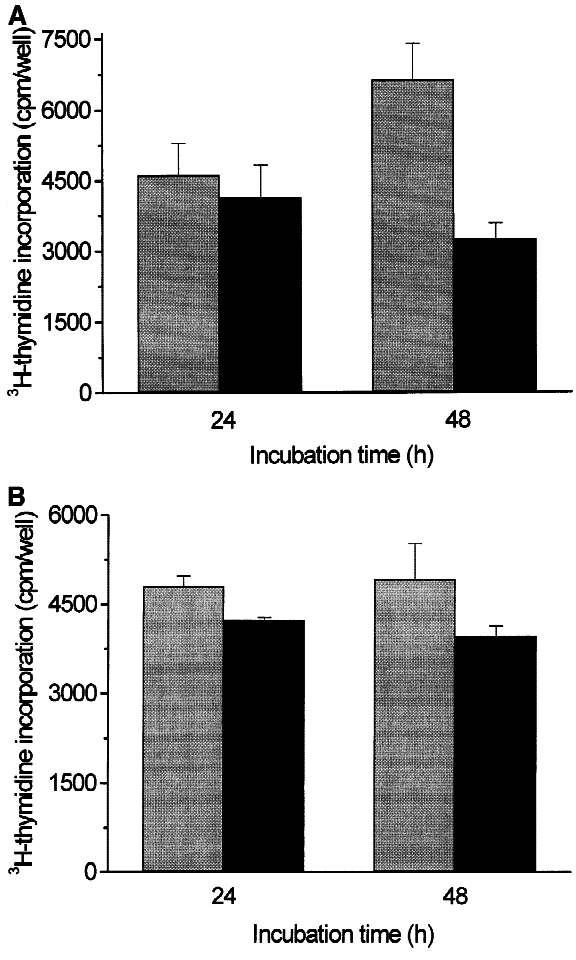


**Fig. 1.** Effect of chronic hypertonic stress on proliferation activity of HCEC (1A) and RCEC (1B) as determined by $^3$H-thymidine incorporation per well. Exponentially growing cells were exposed for 24–48 hr to hypertonic (375 mOsm) medium. Lightly shaded bars are the isotonic condition. Solid bars are the hypertonic condition. In all cases, $n = 4$.

Figure 2 shows the comparative effects on RCEC and HCEC proliferation of exposure for 24 hr to variations in medium osmolarity from 300 to 600 mOsm. Over the range between 300 and 350 mOsm, HCEC proliferation was significantly stimulated whereas from 350

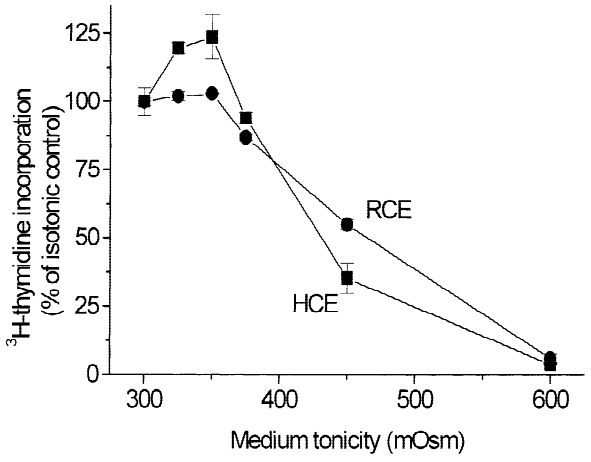


**Fig. 2.** Effect of increases in medium tonicity on proliferation of HCEC and RCEC. Cell proliferation activity was determined as a 1-hr $^3$H-thymidine incorporation performed after a 24 hr period of exposure to hypertonic media of varying tonicity ($n = 4$).

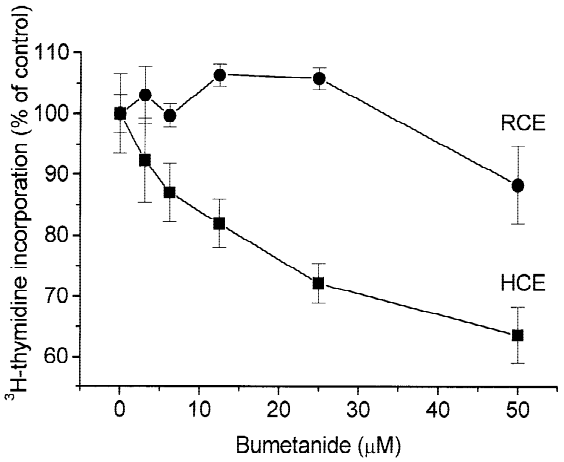


**Fig. 3.** Effect of Na-K-2Cl cotransporter inhibition on cell proliferation. Bumetanide in various concentrations was added to the culture medium 24 hr before one-hour $^3$H-thymidine incorporation measurements ($n = 4$).

to 600 mOsm it was progressively suppressed. On the other hand, RCEC proliferation was unchanged during exposure to media up to 350 mOsm. Interestingly, at 375 mOsm, proliferation was slightly more inhibited in RCEC than in HCEC whereas after 48 hr proliferation declined much more in HCEC than in RCEC (*cf.* Fig. 1*A* and *B*).

### Association Between Na:K:2Cl Cotransport Activity and Cell Proliferation

To evaluate whether inhibition of NKCC activity affects HCEC and RCEC proliferation, we characterized under isotonic conditions the concentration-dependent effects of bumetanide, a selective inhibitor of NKCC activity, on $^3$H-thymidine incorporation over a 24 hr period. As can be seen in Fig. 3, at concentrations of up to 25 μM bumetanide, $^3$H-thymidine incorporation by RCEC was resistant to NKCC inhibition whereas in HCEC cells proliferation significantly declined by 30%. With 50 μM bumetanide, the declines in RCEC and HCEC reached 13 and 37%, respectively. These inhibitory effects suggest that volume regulation mediated by NKCC activity contributes more to growth control in HCEC than in RCEC. These small declines in proliferation during exposure to bumetanide suggest that under isotonic conditions NKCC activity plays a relatively minor role in the control of cell growth in these two cell lines.

### Dependence of RVI Capacity on NKCC Activity

RVI capacity was characterized based on the extent of recovery towards the initial isotonic cell volume that occurred subsequent to shrinkage induced by acute exposure to a 375 mOsm challenge. Fig. 4*A* and *B* shows the sensitivity of RVI to exposure to 50 μM bumetanide in RCEC and HCEC. Fig. 4*A* shows the extent of cell volume recovery, which is expressed in terms (%) of the relative cell volume in isotonic medium. Fig. 4*B* demonstrates the kinetics for this recovery, which is expressed in terms of $t_{1/2}$. As can be seen in these two figures, the individual cell volume recovery and $t_{1/2}$ values were, respectively: (i) RCEC ($n = 6$) 95 ± 1% and 51 ± 3 sec; (ii) HCEC ($n = 6$) 83 ± 3% and 82 ± 4 sec. With 50 μM bumetanide, these values for RVI were affected to different extents: (i) RCEC ($n = 6$) 75 ± 7% and 65 ± 4 sec ($P < 0.01$); (ii) HCEC ($n = 6$) 33 ± 1% and 227 ± 8 sec ($P < 0.01$). These results suggest that the magnitude of the RVI response during this challenge is more dependent on NKCC activity in HCEC than in RCEC.

### Comparison of Declines in RVI Capacity Subsequent to Chronic Hypertonic Stress

Figure 5 shows the time-dependent effects on the RVI response of exposure to 375 mOsm medium for up to 48 hr. This was done at the indicated times by removing the cells from the 375 mOsm medium and incubating them in isotonic medium until the relative cell volume reached a stable value. Subsequently, the cells were reexposed to 375 mOsm medium. As can be seen, after 24 hr of hypertonic exposure, the extent of RCEC RVI was essentially unaltered. However, after the same time HCEC RVI recovery decreased significantly to 24 ± 2% of the control and the difference between RVI in the two cell lines reached a maximal value. This difference persisted up to 48 hr. Therefore, chronic exposure to 375 mOsm

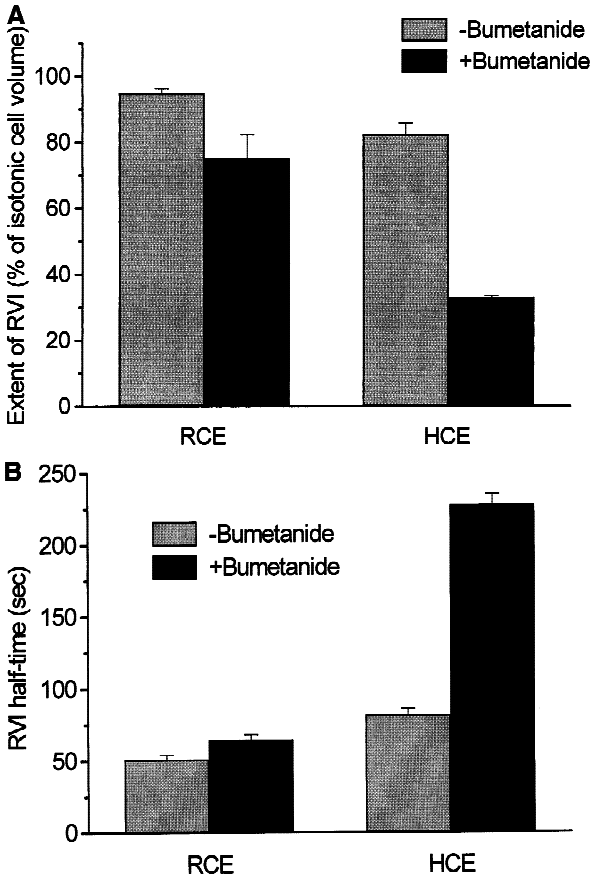


**Fig. 4.** Characteristics of RVI cycles in RCEC and HCEC, and the effects of 50 μM bumetanide in both cases.

medium for up to 48 hr inhibits RVI in HCEC more than in RCEC.

### Contribution of NKCC Activity to Total $^{86}$Rb Influx Under Isotonic and Chronic Hypertonic Conditions

We characterized the dose dependent inhibitory effects of bumetanide on $^{86}$Rb uptake in both RCEC and HCEC under isotonic conditions. As can be seen in Fig. 6, $^{86}$Rb uptake by HCEC was more sensitive to bumetanide inhibition than that in RCEC. From the fitted inhibitory functions, NKCC activity accounts for 34 and 53% of $^{86}$Rb influx in RCEC and HCEC, respectively. The *k* value is smaller for HCEC (2.5 *vs.* 5.3 μM); with the paucity of data in the initial segment of the curve, that difference is not significant, but a difference in affinity for bumetanide between the isoforms expressed in the two species cannot be ruled out. Figure 7 compares the time dependent effects of exposure to 375 mOsm medium on bumetanide-sensitive ([bumetanide] = 50 μM) $^{86}$Rb uptake in HCEC and RCEC. As can be seen, this

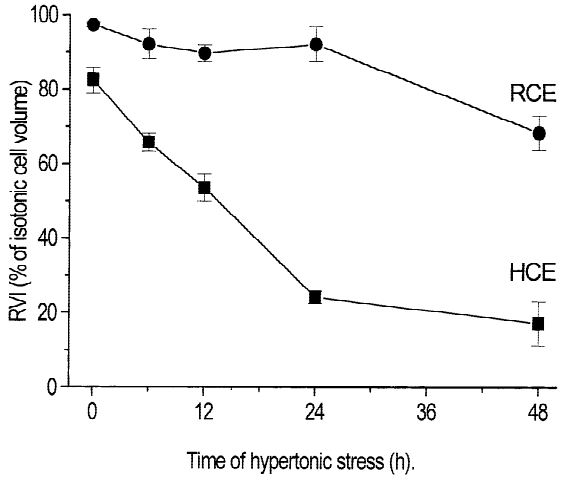


**Fig. 5.** Time-dependent effect of chronic hypertonic exposure on the capability of HCEC and RCEC to undergo regulatory volume increase. After exposure of cell monolayers up to 48 hr to hypertonic (375 mOsm) medium, they were incubated in isotonic medium until the relative cell volume reached a stable value. Subsequently, the cells were reexposed to 375 mOsm medium. The individual responses for the HCEC and RCEC are shown as solid squares and circles, respectively. Data are means ± SE from 6 different coverslips.

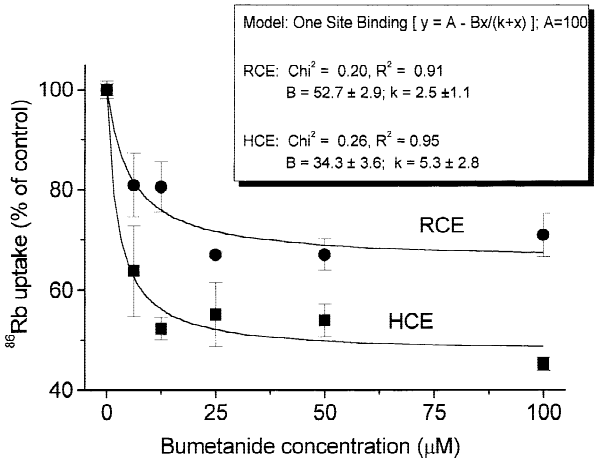


**Fig. 6.** Sensitivity to bumetanide of the $^{86}$Rb uptake by RCEC and HCEC. Symbols are the means, and deviations indicate the range ($n$ = 2 wells).

hypertonic stress produced a significant increase in bumetanide-sensitive $^{86}$Rb uptake in RCEC, whereas in HCEC the level of sensitivity either remained unchanged or decreased.

### Temporal Relationships Among NKCC Gene Expression and Bumetanide-Sensitive $^{86}$Rb Influx in Response to Hypertonic Stress

To evaluate NKCC gene expression, we used a modified S1 nuclease protection assay. Figure 8 shows the results

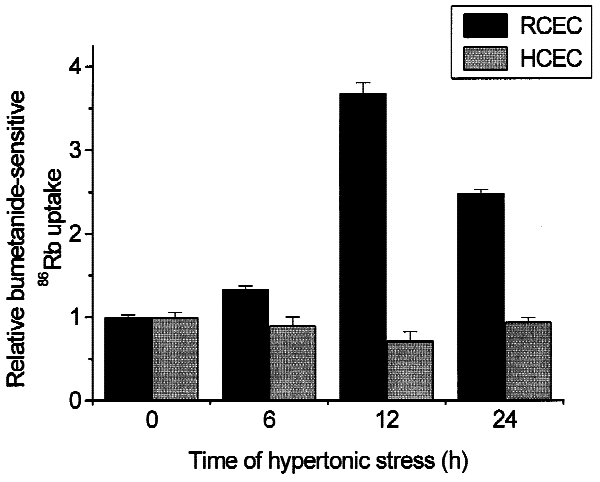


**Fig. 7.** Time-dependent effects of exposure to hypertonic medium (375 mOsm) on the bumetanide-sensitive ([bumetanide] = 50 μM) component of $^{86}$Rb uptake in RCEC and HCEC.

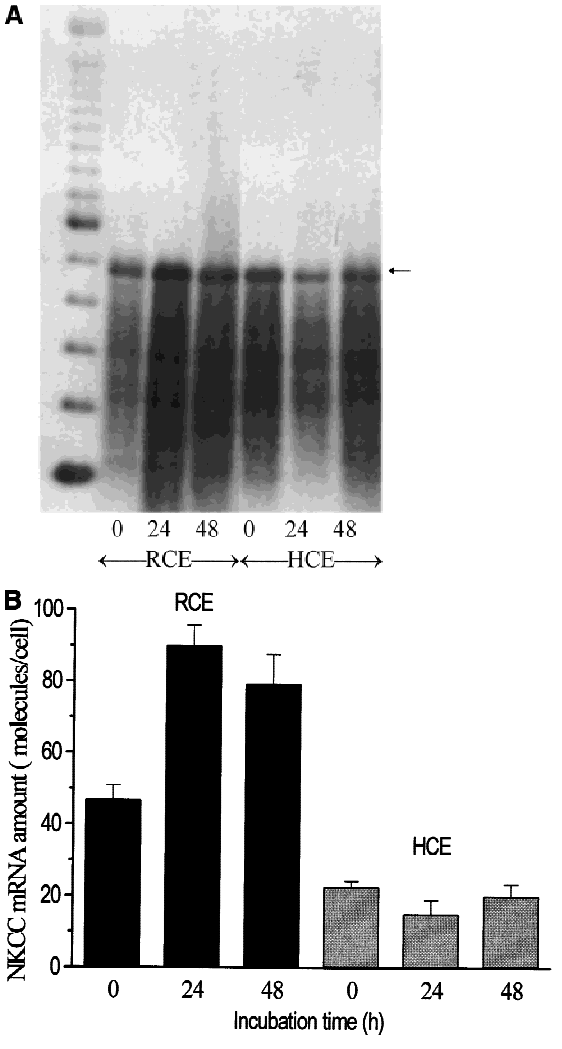


**Fig. 8.** Effect of chronic (24–48 hr) hypertonic exposure of RCEC and HCEC on the amount of their NKCC mRNA. Cells were incubated in hypertonic (375 mOsm) medium, lysed in GuSCN and subjected to lysate nuclease protection analysis. Following digestion and electrophoresis in 2% agarose gels, the position of protected 529 nt RNA-DNA duplexes (arrow) was determined by autoradiography (*A*). Left lane: 100 bp DNA ladder; numbers below other lanes: time of incubation (hr) in hypertonic (375 mOsm) medium. Results were quantified by scintillation counting of excised bands (*B*). Numbers of mRNA molecules per cell in the histograms were calculated using the procedure of Haines and Gillespie (1992).

of a representative experiment, which quantifies the absolute levels of NKCC mRNA expression per cell after 24 and 48 hr of exposure to 375 mOsm hypertonic stress. Figure 8*A* shows an autoradiogram of RCEC and HCEC NKCC RNA/DNA protected duplexes under isotonic conditions (i.e., 0 hr) and after 24 and 48 hr of exposure to 375 mOsm medium. As can be seen, during exposure to hypertonicity, NKCC mRNA expression increased only in RCEC. In Fig. 8*B*, the results from experiments shown in Fig. 8*A* at the above-mentioned times were normalized on the basis of their DNA content. The results indicate that the average steady-state NKCC mRNA level is significantly higher in RCEC than in HCEC (47 *vs.* 23 molecules per cell, respectively). Furthermore, in RCEC, during the initial 24 hr of incubation in hypertonic medium, the mRNA level increased 2-fold compared to its isotonic value whereas the level remained unchanged in HCEC. These results further document that in response to hypertonic stress, increases in NKCC mRNA amount precede rises in NKCC activity, which is associated with the ability of RCEC cells to effectively adapt to a hypertonic stress.

To further ascertain the contribution of NKCC to sustaining RVI behavior during chronic exposure to 375 mOsm, we compared the time-dependent effects of this challenge on HCEC and RCEC NKCC gene expression and transport activity. This was done by measuring in parallel levels of NKCC mRNA expression and bumetanide-sensitive (i.e., 50 μM) $^{86}$Rb uptake. Figure 9 shows that in RCEC there were parallel increases in both the levels of NKCC mRNA expression and $^{86}$Rb influx. The maximal increase in mRNA expression was 2.4-fold, which occurred at 9 hr. On the other hand, the maximal increase in $^{86}$Rb uptake was 3.7-fold at 12 hr. Subsequent to reaching their respective maximal increases, each of these parameters fell to values that were 1.7- and 2.5-fold above their levels measured under isotonic conditions. In marked contrast, HCEC did not elicit any significant increases in either NKCC mRNA expression or cotransport activity under the same conditions. As a matter of fact, slight declines were even seen in both NKCC mRNA expression and transport activity after 12 hr of being exposed to this hypertonic medium. These results further indicate that increases in levels of

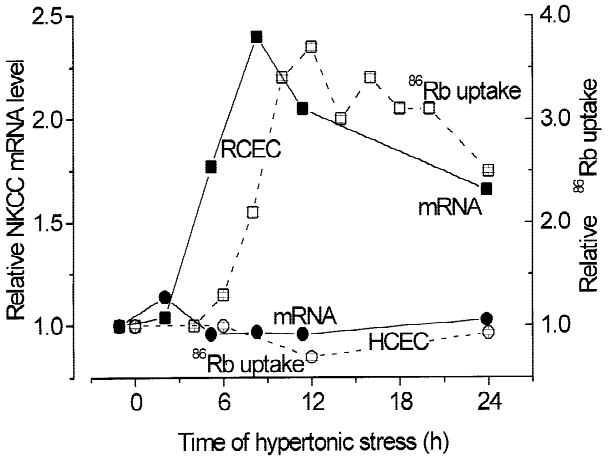


**Fig. 9.** Time-dependent effects of exposure to hypertonic (375 mOsm) medium on RCEC and HCEC NKCC gene expression and transport activity. Solid symbols: levels of NKCC mRNA expression by nuclease protection assay. Open symbols: bumetanide-sensitive (at 50 μM [bumetanide]) $^{86}$Rb uptake. In all cases, values are relative to those at zero time.

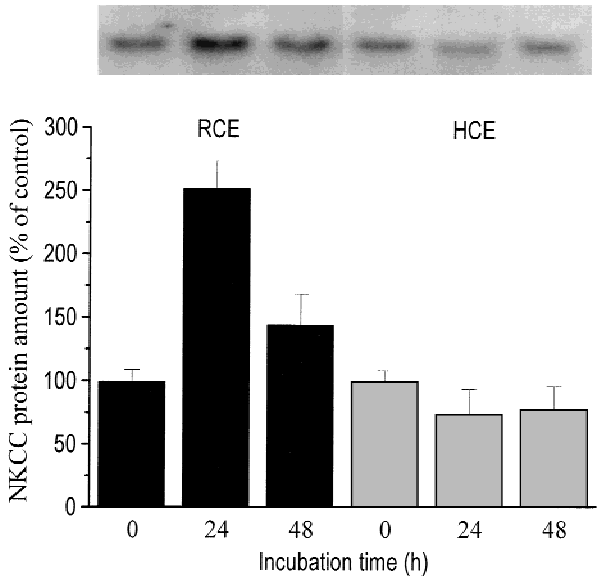


**Fig. 10.** Effect of chronic (24–48 hr) hypertonic exposure of RCEC and HCEC on the levels of NKCC protein expressed in their cellular membranes. The top panel represents immunoblots with T4 antibody to NKCC. The imaging films were exposed for 10 min (RCEC) and 20 min (HCEC). The columns below each band indicate the respective density of the band compared to the corresponding determination at zero time (control).

NKCC mRNA and transport activity are associated with the ability of RCEC to adapt to chronic hypertonic stress.

### Differential Hypertonic Induced Increases in NKCC Membrane Protein Abundance

We investigated whether changes that occur in NKCC protein expression are in accord with those occurring in functional NKCC activity (bumetanide-sensitive $^{86}$Rb influx). Western blot analysis was performed of NKCC protein expression after 24 and 48 hr of exposure to 375 mOsm medium. Figure 10 (top panel) provides representative data of HCEC and RCEC blots probed with the mouse anti-NKCC T4 antibody. Densitometry of the band (bottom panel) indicates that (i) under physiological conditions, the average NKCC protein level is about 2-fold higher in RCEC than in HCEC; (ii) NKCC amount in RCEC significantly increased 2.4- and 1.4-fold after 24 hr and 48 hr incubation in 375 mOsm medium, respectively; (iii) the changes of NKCC expression in hypertonic stressed HCEC were not significant. These results show that increases in NKCC functional activity and protein expression are associated with the ability of RCEC to adapt to hypertonic stress.

## Discussion

The basis for this study was that in dry eye patients tear film hypertonicity occurs (Farris, 1994). To gain insight into whether such stress contributes to the symptomology of this complex disease, we characterized in vitro the effects of chronic hypertonic stress on RVI and proliferation/survival in HCEC and RCEC. This comparison was made since we hypothesized that these functions in RCEC are more resistant to hypertonic stress than in HCEC. The basis for this hypothesis is that tear film renewal occurs more often in humans because blink frequency is higher in humans than in rabbits. This suggests that in rabbits tear film tonicity could rise to higher levels than in humans without affecting their corneal epithelial functions. Should functional changes occur, they could include compromise of corneal epithelial barrier properties because its maintenance may depend on RVI and cell proliferation. Some evidence for this association is that increases in the rate of corneal epithelial erosion (Gobbels, 1992; Xu, 1996), infection (Fleiszig, Zaidi & Pier, 1994) and resulting declines in corneal transparency (Imanishi et al., 2000) are frequently observed in dry eye patients.

We found that in both cell lines after 24 hr exposure to tonicity above 350 mOsm, osmolarity-dependent declines occurred in proliferation/survival indicating that cell adaptation to such stress was inappropriate (Fig. 1). These results are consistent with reports that in dry eye healing can be impaired and may help explain the observation of corneal epithelial barrier breakdown in this disease. Such breakdown could be associated with hypertonic stress-induced decreases in basal cell proliferation and/or cell shrinkage.

The fact that exposure of HCEC to media of tonicity

up to 350 mOsm significantly stimulated cell proliferation (Fig. 2) suggests that activation of mitogen activated protein kinases (MAPK) occurred due to sustained shrinkage of these cells (our *unpublished data*). Such increases in MAPK activity have been reported in a variety of cells and lead to the stimulation of downstream events resulting in increases in proliferation (Force, 1998; Dikic, 1999; Fanger, 1999). That this effect was limited to HCEC could mean that only in these cells the shrinkage resulting from hypertonic stress was long enough in duration to further stimulate MAPK above its isotonic level.

Our results indicate that RCEC adapt better than HCEC to chronic hypertonic stress that lasts up to 48 hr. This assessment is based on our findings that the RVI (Fig. 5) and proliferation capacity (Fig. 1) in RCEC are better maintained than in HCEC. Moreover, since RVI responses could be only partially inhibited by bumetanide (Fig. 4), we can conclude that RVI capacity is dependent to different degrees on NKCC activity in these cell lines. In response to an acute hypertonic challenge, bumetanide had a larger inhibitory effect on RVI in HCEC and RCEC, which indicates that NKCC activity contributes more to RVI in HCEC than in RCEC. However, it is interesting to note that adaptation to chronic hypertonic stress by RCEC was associated with upregulation of NKCC gene and protein expression (Fig. 8 and Fig. 10) as well as its functional activity (Fig. 7) whereas these responses were absent in HCEC. Additional suggestive evidence indicating the importance of upregulation of NKCC gene expression and ion transport activity to adaptation by RCEC to hypertonic stress is that only in RCEC increases in NKCC mRNA expression preceded by 3 hr the rises in $^{86}$Rb uptake (Fig. 9). On the other hand, in HCEC no such increases were observed. As a matter of fact, after 6 hr, significant declines occurred in NKCC mRNA level and ion transport activity of 10 and 30%, respectively. Currently, it is not yet apparent which mechanisms allow RCEC to adapt to chronic hypertonic stress through upregulation of NKCC expression and ion transport activity. The possibilities include species specific NKCC isoform expression and differences in hypertonicity-linked cell signaling control of NKCC function. We have recently obtained preliminary data indicating that this challenge induces increases in mitogen activated protein kinase (MAPK) phosphorylation that are associated with control of NKCC activity. Interestingly, we found that exposure to a 375 mOsm challenge more markedly and rapidly increased in RCEC than in HCEC the activity of a MAPK superfamily component, p38. Taken together our findings suggest that the RVI response to an acute hypertonic challenge is mediated by a cell signaling pathway that may be different from the one involved in inducing adaptation to chronic hypertonic stress. One difference appears to be that the cell signaling pathway mediating the acute response includes only post-translational events whereas cell volume regulation and proliferation during chronic hypertonic stress are dependent on a different cell signaling pathway leading to increases in NKCC gene expression.

There was a dissociation between the times it took to inhibit RVI capacity and proliferation in RCEC and HCEC (Fig. 5 and Fig. 1). After 24 hr of exposure to 375 mOsm medium, RVI capacity was inhibited by 76% in HCEC but insignificantly in RCEC. At this time, in both cell lines proliferation similarly declined by about 12%. On the other hand, after 48 hr, proliferation in RCEC was only inhibited by 22% whereas it declined by 52% in HCEC. These later effects suggest that declines in cell proliferation are preceded by a decrease in their RVI capacity. On the other hand, cell adaptation to hypertonic stress could be associated with upregulation of a membrane ion transporter(s) which maintains cell volume regulation capacity. A linkage between NKCC activity and cell proliferation has been described in fibroblasts and endothelial cells where specific NKCC inhibitors bumetanide and furosemide inhibited proliferation (Panet et al., 1991, 1994). Another indication of the importance of NKCC activity to cell volume regulation in proliferation control is that in 3T3 fibroblasts transfected with the Ha-*ras* oncogene, an upstream regulator of the MAPK superfamily, NKCC activity was increased approximately 4-fold and associated with this increase there were parallel increases in cell volume and cell proliferation (Meyer et al., 1991). These increases were attributable to NKCC stimulation since they were suppressed by the NKCC inhibitor, furosemide. Another indication of the dependence of cell proliferation on NKCC activity is that in mouse Balb/c 3T3 cells stable transfection with the shark rectal gland NKCC cDNA caused a 10-fold increase in bumetanide-sensitive $^{86}$Rb uptake and lost its contact growth inhibition (Panet, Marcus & Atlan, 2000). Furthermore, these cells were even able to still proliferate in media with low serum concentration. The results of these aforementioned studies are a direct indication that NKCC is involved in the control of cell proliferation. NKCC activation is a downstream event from MAPK superfamily activation resulting from growth factor receptor stimulation, osmotic as well as other stresses. In RCEC we have preliminary evidence that elevations in NKCC activity leads to cell swelling and is a component of the cell signaling system linking stimulation of epidermal growth factor (EGF) receptor to an increase in cell proliferation. Therefore, it is plausible that the maintenance of proliferation during chronic hypertonic stress could be associated with the ability of the cells to upregulate NKCC gene and protein expression as well as functional ion transport activity.

The increases in functional NKCC transport activity

in RCEC associated with rises in NKCC gene expression could be a result of elevations in NKCC protein expression and/or changes in its kinetic parameters (i.e., $V_{max}$ or apparent $K_m$). Accordingly, to determine whether changes in NKCC gene expression are reflected by changes in NKCC protein levels in RCEC and HCEC, we determined the effects of chronic hypertonic stress for up to 48 hr on NKCC protein expression in both cell lines. The results shown in the upper and lower panels of Fig. 10 document that increases in NKCC protein expression occurred only in RCEC. It is evident that there is moderate decrease in the intensity of NKCC band in HCEC whereas after 24 hr the band intensity markedly increased in RCEC and then declined to a level that was still higher than that found in isotonic control. The quantification of the data shown in the lower panel indicate that NKCC protein level was slightly decreased in HCEC. On the other hand, in RCEC, it increased 2.5-fold after 24 hr and declined at 48 hr to a level that was 1.4-fold higher than the control value. This pattern of changes is in qualitative agreement with those seen for RCEC NKCC mRNA contents (Fig. 8) and suggests that the maintenance of RVI capacity during chronic hypertonic stress is associated with the ability of RCEC to increase both NKCC gene expression and membrane protein levels. In addition, there is an absolute quantitative agreement between increases in NKCC ion transport activity (Fig. 9) and RCEC NKCC protein amounts after 24 hr exposure to hypertonic stress. This correlation suggests that during chronic hypertonic stress kinetic parameters of NKCC remain unchanged. The slight subsequent declines in RCEC NKCC mRNA expression and transport activity, which started to occur after 12 hr could be followed by other adaptive mechanisms identified in some other cell systems. They include further accumulation of organic osmolytes (e.g., myo-inositol, sorbitol, glycerophosphorylcholine glycinebetaine (Yamauchi, 1991; Wiese, 1996; Matsuoka, 1999)).

The inhibition of NKCC activity under isotonic conditions had a smaller inhibitory effect on RCEC cell volume regulation (Fig. 4) and cell proliferation (Fig. 3) than in HCEC. On the other hand, during a hypertonic exposure RCEC were better able to adapt than HCEC to such a challenge because only RCEC upregulated NKCC mRNA and protein expression and transport activity. Another indication of adaptation by RCEC was that its proliferation was less inhibited than it was in HCEC. On the other hand, HCEC NKCC gene and protein expression as well as transport activity were unresponsive and even suppressed during hypertonic exposure. Our observation that HCEC could not adapt to hypertonic exposure is consistent with our suggestion that chronic exposure to hypertonic tears in dry patients leads to decreases in NKCC activity, resulting in barrier function disruption and impairment of wound healing.

Our finding that RCEC adapt to chronic hypertonic stress through upregulation of NKCC functions provides us with the means to further dissect how osmotic adaptation occurs. Our first approach will be to identify differences in cell signaling between RCEC and HCEC regulating NKCC activity during hypertonic stress. Should we find a difference at this level, it will be of interest to attempt to endow HCEC with the same adaptive capacity found in RCEC.

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