J. Membrane Biol. 193, 1–13 (2003)
DOI: 10.1007/s00232-002-2002-8

The Journal of
Membrane
Biology

Hypertonicity-induced p38MAPK Activation Elicits Recovery of Corneal Epithelial Cell Volume and Layer Integrity

V.N. Bildin[1], Z. Wang[1], P. Iserovich[2], P.S. Reinach[1]
[1]Department of Biological Sciences, College of Optometry, State University of New York, 33 West 42nd St., New York, NY 10036, USA
[2]Department of Ophthalmology, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA

Received: 25 September 2002/Revised: 6 January 2003

Abstract. In hypertonicity-stressed (i.e., 600 mOsm) SV40-immortalized rabbit and human corneal epithelial cell layers (RCEC and HCEC, respectively), we characterized the relationship between time-dependent changes in translayer resistance, relative cell volume and modulation of MAPK superfamily activities. Sulforhodamine B permeability initially increased by 1.4- and 2-fold in RCEC and HCEC, respectively. Subsequently, recovery to its isotonic level only occurred in RCEC. Light scattering revealed that in RCEC 1) regulatory volume increase (RVI) extent was 20% greater; 2) RVI half-time was 2.5-fold shorter. However, inhibition of Na-K-2Cl cotransporter and Na/K-ATPase activity suppressed the RVI response more in HCEC. MAPK activity changes were as follows: 1) p38 was wave-like and faster as well as larger in RCEC than in HCEC (90- and 18-fold, respectively); 2) increases in SAPK/JNK activity were negligible in comparison to those of p38; 3) Erk1/2 activity declined to 30–40% of their basal values. SB203580, a specific p38 inhibitor, dose dependently suppressed the RVI responses in both cell lines. However, neither U0126, which inhibits MEK, the kinase upstream of Erk, nor SP600125, inhibitor of SAPK/JNK, had any effect on this response. Taken together, sufficient activation of the p38 limb of the MAPK superfamily during a hypertonic challenge is essential for maintaining epithelial cell volume and translayer resistance. On the other hand, Erk1/2 activity restoration seems to be dependent on cell volume recovery.

Correspondence to: V.N. Bildin; email: vbildin@sunyopt.edu

Key words: Hypertonicity — MAPKs activity — Volume regulation — Dye penetration — Transepithelial resistance — Cornea

Introduction

Even though regulatory volume responses have been extensively characterized in different cell culture systems, their functional significance to tissue homeostasis is not always apparent. Corneal epithelial layers in culture are a convenient and a relevant model to make such a determination. In these layers, it is possible to conveniently evaluate properties that are reflective of their ability in situ to act as a physical barrier against noxious agents and larger molecular weight hydrophilic solutes (Lu, Reinach & Kao, 2001). A challenge to maintenance of barrier function in situ includes hypertonic stress, which results in cell shrinkage unless regulatory volume increase (RVI) is activated to restore cell volume toward its isotonic condition. Even though there is some evidence that these cells elicit changes in membrane ion transport parameters during exposure to an osmotic stress (Reinach, Ganapathy & Torres-Zamorano, 1994; Candia, Patarca & Alvarez, 1998) and that they activate RVI (Bildin et al., 1998), the role was not determined of regulatory volume behavior in the prevention of breakdown of barrier function. Furthermore, in this tissue there is no information regarding the role of activation of osmosensitive cell signaling pathways in preservation of this function.

In a wide variety of tissues, changes in cell-layer barrier function produced by hypertonic stress can be compensated for by restoring cell volume to its isotonic condition (Hallows & Knauf, 1994). This occurs

through the activation of RVI. Hypertonicity-induced RVI occurs as a result of stimulation of solute influx mechanisms followed by increases in intracellular osmolyte levels (O'Neill, 1999). The membrane ion transporters involved in a RVI response in different tissues can include: 1) Na/K-ATPase and Na-K-2Cl cotransporter (NKCC); 2) Na/H exchanger (NHE) and Cl/HCO_3 exchanger; and 3) organic osmolyte uptake.

The cell signaling pathways that mediate membrane ion transport activation are very diverse in different tissues and only partially characterized. In numerous different cell types, cell signaling events initially responding to a hypertonic stress involve increases in the activity of different branches of the mitogen-activated protein kinase (MAPK) superfamily: ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase), and p38MAPK. In Madin-Darby canine kidney epithelial cells hyperosmotic stress causes sequential stimulation of Raf-1 kinase followed by the Erk1/2 limb, and in parallel S6 kinase, via predominately protein kinase C-dependent and tyrosine kinase-independent pathways (Terada et al., 1994). In contrast, in C6 glioma cells, Erk1/2 activity was reduced under hyperosmotic (405 mOsm) conditions, compared to the isotonic control (Sinning et al., 1997). A detailed study in NIH/3T3 cells showed that the hypertonicity-induced signaling pathway involved sequential PLC activation, DAG release followed by PKCalpha, PKCdelta, and PKCepsilon translocation from the cytosol to the membrane, resulting in Erk1/2 activation (Zhuang, Hirai & Ohno, 2000). At the same time, activity of stress-activated protein kinase JNK was not affected. In bovine aortic endothelial cells, NaCl (350 mOsm) significantly increased first the level of expression of p38 within 2 minutes, Erk1/2 and JNK after 10 minutes, with peak activation occurring between 30 and 60 minutes, followed by return to baseline levels within 2 h (Duzgun et al., 2000). In the rat medullary thick ascending limb of Henle, hyperosmolality induced activation of p38 MAPK and Erk1/2 with no evidence for involvement of JNK (Watts et al., 1998). Even though osmolality-induced modulation of the MAPK cascade component activity was described, there is limited understanding about their specific roles in eliciting RVI behavior during such challenges.

In some tissues, the RVI response significantly depends on increases in NKCC activity in hypertonicity-stressed cells (Russell, 2000). However, the cell signaling pathways that mediate NKCC activation are unknown. Recently, we found that hypertonic exposure lasting from 2 to 48 h was less injurious to RCEC than to HCEC. This was evident because in RCEC declines in proliferation and RVI capacity were markedly smaller than in HCEC. RCEC adapted better than HCEC because only RCEC were able to upregulate NKCC gene and protein expression as well as its functional activity (Bildin et al., 2000). These observations suggest there are significant differences in volume regulatory mechanisms as well as cell-signaling pathways activated by hypertonic stress in these cell lines.

Therefore, we compare here in RCEC and HCEC the short-term (i.e., acute) effects of hypertonic (600 mOsm) challenge on the cell layers' barrier function, which was evaluated based on measurements of: 1) sulforhodamine B dye penetration and 2) translayer electrical resistance (R_t). The time dependence of changes in these parameters was compared with RVI responses during exposure to such stress. In parallel experiments, we analyzed the kinetics and magnitude of Erk1/2, JNK and p38 MAPK activation. In addition, we investigated the effects of specific inhibitors of these cell-signaling pathways on RVI kinetics in these cell lines. Our results indicate that RVI and recovery of barrier properties are faster and more complete in RCEC than in HCEC. These contrasting effects are associated with differences in rapidity and magnitude of MAPK superfamily limb activation.

Materials and Methods

Cell Culture

SV40-adenovirus immortalized HCEC and RCEC, a generous gift from Dr. Araki-Sasaki (Kinki Univ., 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 et al., 1993). The cells were grown in an atmosphere of 5% CO_2, 95% ambient air at 37°C. Medium hypertonicity was increased by adding NaCl to standard Krebs-Ringer buffer (in mM): NaCl 122.9; $NaHCO_3$ 25; KCl 5; $NaH_2PO_4(H_2O)$ 1; $MgSO_4$ 1; $CaCl_2$ 1.7; glucose 5.5; HEPES 5.3.

Monitoring of Relative Cell Volume

Hypertonicity-induced changes in cell volume were evaluated based on a modified light scattering technique first described by Fischbarg (Fischbarg et al., 1993; Bildin et al., 2000). Analyses were performed using paired-data statistics: Student's *t*-test for independent means. Cell signal transduction pathway inhibitors SB203580 (p38MAPK), U0126 (MEK) and SP600125 (SAPK/JNK), were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA).

Monitoring of Transepithelial Dye Penetration

Confluent RCEC and HCEC layers grown on semipermeable polycarbonate Transwell inserts (24 mm diameter and 0.4 μm pore size; Costar, Cambridge, MA) were placed in a bicameral chamber and initially bathed on both sides with 300 mOsm isotonic Krebs-Ringer buffer. Hyperosmotic challenge was performed in both chambers at the same time. The chambers were stirred continu-

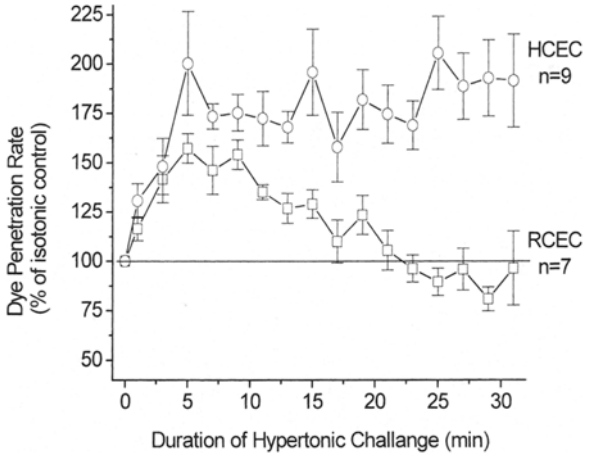

Fig. 1. Comparison of time-dependent effects of acute exposure to a hypertonic stress on RCEC and HCEC translayer dye penetration rate. Controls were incubated in isotonic medium (300 mOsm), whereas the challenged layers were bathed in 600 mOsm medium. Both media contained 25–50 μg/ml sulforhodamine B. Changes in dye penetration rate were characterized based on measurements of light absorption at 565 nm in samples collected every 2 minutes and normalized to their isotonic value. The individual responses for the RCEC and HCEC are shown as open squares and circles, respectively. Data are means ± SEM.

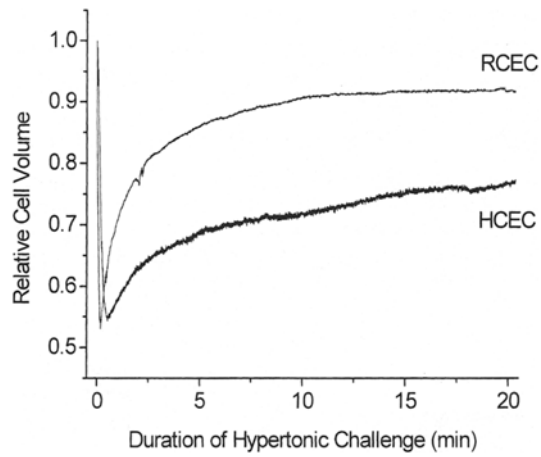

Fig. 2. Hypertonicity-induced RVI responses by RCEC and HCEC. Subconfluent layers grown on 11 × 22 mm rectangular glass coverslips were illuminated with a rectangular cross-section light beam (5 mW helium-neon laser plus expander). The intensity of light scattered by the cells was converted to relative intracellular volume. Representative experiments are shown.

ously with a Teflon stir bar at ~250 rpm. The receiver- and donor-side volumes were ~45 ml and 2 ml, respectively. The donor side contained 25–50 μg/ml sulforhodamine B, M_r = 559, (Sigma, St. Louis, MO). Dye penetration was measured by sample collection every 2 minutes from the receiver side, which was replaced at a rate of 0.5 ml/min. Dye absorption at 565 nm was measured in a spectrophotometer. Dye penetration rate measured during exposure to a 600 mOsm challenge was normalized to its isotonic value.

Measurements of R_t

R_t measurements were used as an index of epithelial layer barrier function. The cells were seeded onto semipermeable polycarbonate Transwell inserts (1.13 cm^2 and 0.4 μm pore size) and allowed to grow to confluence. Inserts were placed into modified Ussing chambers and specific R_t was measured with a DVC 1000 voltage/current clamp by sending 50 μA and measuring amplitude of the peak voltage deflection every 20 seconds. Both devices were manufactured by World Precision Instruments, Sarasota, FL. Solution challenges were performed without disassembling. Simultaneous draining/refilling of the chamber took about 12 seconds. Hydrostatic pressure on both sides of the insert was kept equal. The final R_t values were calculated by subtracting the resistance of bare polycarbonate filters exposed to isotonic or 600 mOsm solutions from the total resistance of the epithelial cell layers. The results are expressed as $\Omega \cdot cm^2$.

Kinase Activity Assay

A period of up to 28 minutes was used to characterize the effect of an acute hypertonic challenge. Cells were homogenized by sonication and centrifuged at 500 × g for 15 minutes. Activity of JNK, p38MAPK and Erk1/2 kinases in supernatants was evaluated with kinase assay kits (Cell Signaling Technology, Inc., Beverly, MA). Briefly, in each case the phospho form of kinase was immunoprecipitated with a specific antibody to measure its activity. Active p38MAPK was obtained through "pull down" from cell lysates with immobilized monoclonal phospho-p38MAPK (Thr180/Tyr182) antibody and thereafter incubated with ATF-2 fusion protein, a substrate for active p38, in the presence of ATP and kinase buffer. JNK was immunoprecipitated with c-Jun (1-89) fusion protein containing a high-affinity binding site for JNK, and then the kinase reaction leading to c-Jun phosphorylation was carried out in the presence of cold ATP. A monoclonal phospho-Erk1/2 (Thr202 and Tyr204) antibody was used to selectively precipitate active Erk1/2 from lysates. The resulting complex was then incubated with Elk-1 fusion protein in the presence of ATP and kinase buffer. Denatured samples were electrophoresed on 7.5% polyacrylamide SDS minigels. Resolved proteins were electrotransferred overnight to PVDF membranes. After blocking with nonfat dry milk, the blots were exposed for 2 h at room temperature to an appropriate dilution of phospho-kinase monoclonal antibodies: 1) ATF-2 (Thr71) to evaluate p38 activity; 2) c-Jun (Ser63) and 3) Elk-1 (Ser383). Thereafter membranes were exposed to a 1:2000 dilution of rabbit antimouse HRP-labeled IgG. The immunoreactive bands were detected with ECL + Plus kit (Amersham Biosciences, Piscataway, NJ). Films were scanned and band density was quantified using Sigmagel software (Jandel Scientific, San Rafael, CA). Exposed membranes were stained with Colloidal Gold (BioRad Laboratories, Hercules, CA) to verify that each lane contained similar amount of material.

Results

Changes in Translayer Dye-Penetration Rate, Resistance and Cell Volume Regulation

To evaluate the effects of an acute hypertonic challenge on barrier function in confluent HCEC and RCEC layers, we compared sulforhodamine B penetration rates across these layers under isotonic and 600 mOsm hyperosmotic conditions. The results shown in Fig. 1 reveal that in HCEC during the first 5 minutes of exposure to this challenge, dye penetration

Table 1. Differential RVI behavior during 20 min exposure to 600 mOsm challenge

	RCEC		HCEC	
	RVI (%)	τ (sec)	RVI (%)	τ (sec)
No inhibitor	84.1 ± 0.8	71.5 ± 4.8	65.5 ± 2.4	170.3 ± 4.7
Bumetanide	70.1 ± 2.7	72.0 ± 5.6	48.4 ± 5.3	369.2 ± 16.2
Ouabain	70.0 ± 1.8	115.4 ± 4.2	(50.1 ± 10.1)*	465.1 ± 45.3

Values represent the mean of 6 to 9 independent experiments (±SEM). Bumetamide and Ouabain concentration, 50 μM.
* In this case, parameters were evaluated after 15 min.

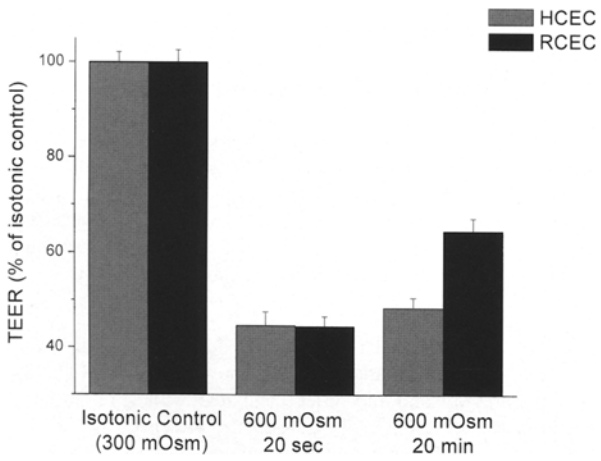

Fig. 3. Hypertonicity-induced changes in transepithelial electrical resistence R_t (TEER) in RCEC and HCEC. The cells were grown to confluence on semipermeable polycarbonate inserts. TEER was measured by sending 50 μA and measuring amplitude of the peak voltage. The final TEER values were calculated by subtracting the resistance of bare polycarbonate inserts exposed to isotonic or 600 mOsm solutions from the total resistance of the epithelial cell layers.

rate increased up to 200%. It then stabilized at a slightly lower value without returning towards its isotonic baseline value ($n = 9$). In contrast, in RCEC, dye penetration rate initially increased during 5 minutes by 150% and then remained stable for 4 minutes. Subsequently, the dye penetration rate gradually declined and after 16 minutes reached its isotonic value despite the continued presence of the hypertonic challenge.

To identify the physiological mechanisms underlying these changes in dye penetration rate, light scattering measurements were used to evaluate RVI responses occurring during exposure to a hypertonic challenge. Figure 2 shows the results of a representative experiment following the substitution of isotonic NaCl Ringer (300 mOsm) medium with its 600 mOsm counterpart. Due to hypertonic stress, there was initially in both cell lines a rapid decrease in relative cell volume followed by RVI responses. RVI responses were evaluated based on: 1) the extent of RVI after 20 minutes, which is expressed as a percent of the initial cell volume; 2) half time of this process (τ). The average responses (Table 1) indicate that in fact the τ and the extent of RVI were markedly different in the two cell lines. RVI in RCEC was about 20% greater and τ was about 2.5-fold shorter than that in HCEC. Taken together, these data suggest that the pattern of changes in dye penetration rate is dependent on the RVI response occurring during a hypertonic challenge.

Another approach towards characterizing barrier-function behavior during exposure to a hypertonic challenge is to measure translayer electrical resistance (TEER; R_t). This measurement reflects tight-junctional integrity, which we hypothesized is dependent on the magnitude and kinetics of RVI responses during a hypertonic challenge. HCEC and RCEC were cultured atop an insert until they reached a R_t of about 450 Ωcm^2 under isotonic conditions. Figure 3 shows that exposure to a 600 mOsm medium rapidly and markedly decreased R_t. After 20 seconds, the R_t values in both cell lines fell by 55% from their initial values. However, after 20 minutes, the R_t in RCEC increased to 64% of its isotonic value, whereas in HCEC it only increased by 3%. During the next 40 min, no additional recovery occurred in either cell line. The magnitude of the RCEC R_t recovery is in qualitative agreement with changes in its dye penetration rate and the RVI response to the same hypertonic challenge shown in Figs. 1 and 2 even though the completion of RVI recovery was much faster than that of R_t. This discrepancy suggested to us that other factors besides cell volume recovery contribute to restoration of the R_t. Therefore, in the following experiments we used dye penetration as an index of barrier restoration during exposure to an imposed challenge. In addition, measurements of dye penetration rate are physiologically relevant for assessing epithelial barrier properties, as they are frequently used to evaluate this parameter in vivo.

Roles of NKCC and Na/K-ATPase in Recovery of Cell Volume and Dye Penetration Rate

In order to assess NKCC and Na/K-ATPase importance for the maintenance of translayer barrier function and RVI responsiveness in HCEC and RCEC, the individual effects were measured of bumetanide and ouabain on these parameters. In

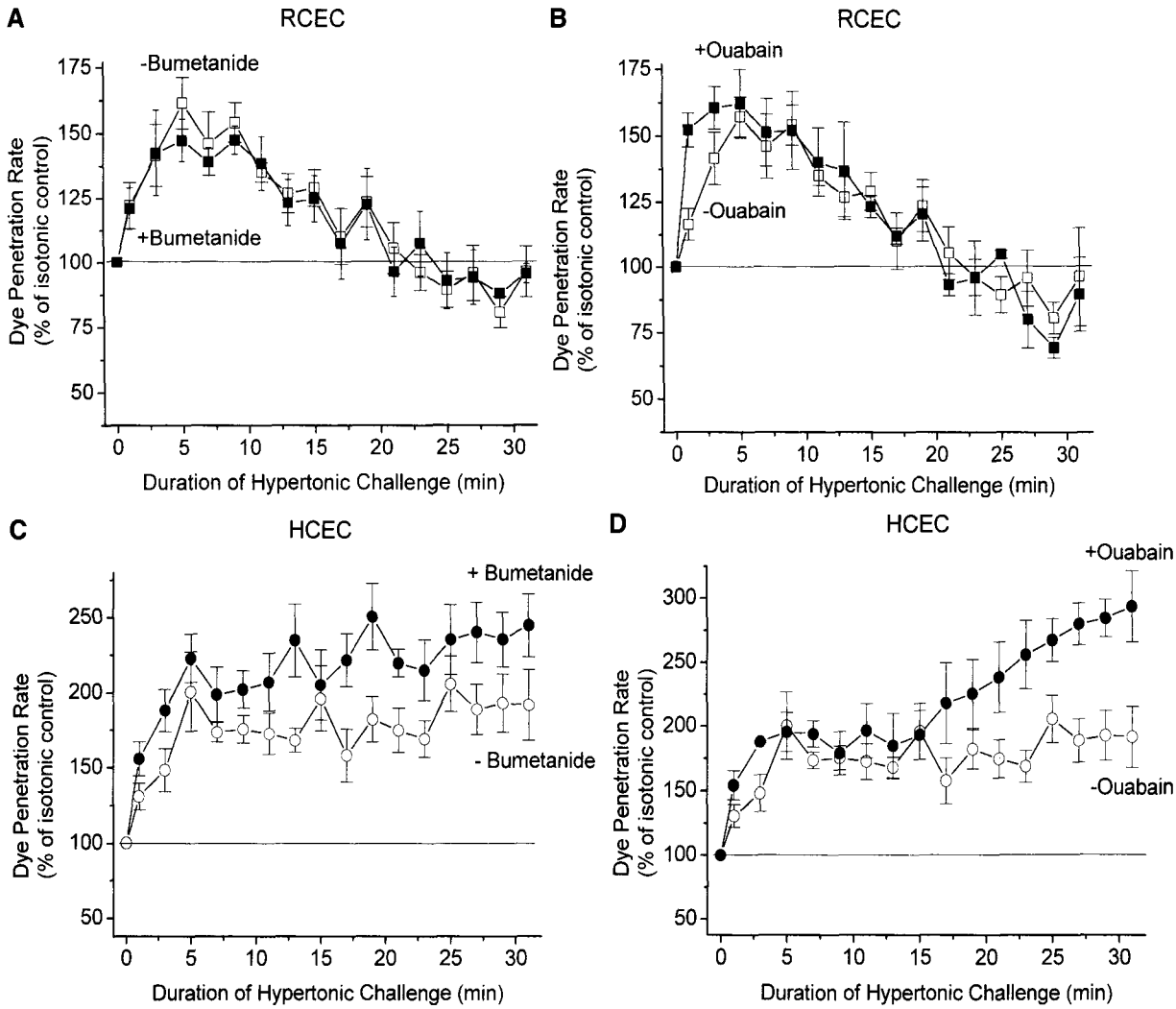

Fig. 4. Effects of NKCC and Na/K-ATPase inhibitors on translayer dye penetration rate in hypertonicity-stressed RCEC and HCEC. Methodology is the same as described in the legend of Fig. 1. The individual responses by RCEC (*A* and *B*) and HCEC (*C* and *D*) are shown as empty squares and circles, respectively, when the layers were incubated without inhibitors and as filled symbols in the presence of 50 μM either bumetanide or ouabain. Data in each case are means ± SEM from 6 different inserts.

RCEC, 50 μM bumetanide had no effect on the dye-penetration transient during a hypertonic challenge (Fig. 4*A*). In parallel studies with RCEC, bumetanide inhibited RVI about 14%, but without any significant effect on its τ (Fig. 5*A*, Table 1). On the other hand, during the first 3 minutes of the hypertonic challenge in the presence of 50 μM ouabain, a significant increase in the dye penetration rate was observed (Fig. 4*B*). As with bumetanide, ouabain inhibited RVI in RCEC by 14% and nearly doubled the τ (Fig. 5*B*, Table 1). However, in HCEC, bumetanide had a more profound effect on the rate of dye penetration: it significantly increased its rate during hypertonic exposure (Fig. 4*C*). In parallel experiments, bumetanide inhibited RVI of cell volume by about 17% and increased τ about 2.2-fold, which was not observed in RCEC (Fig. 5*C*, Table 1). In HCEC, exposure to ouabain caused an initial increase in the rate of dye penetration, followed by quasi stabilization for 10 min and then a further increase in a linear fashion (Fig. 4*D*). Incubation with ouabain also completely altered the kinetics of RVI. The relative cell volume increased during the first 15 minutes to a value that was 50% of the control cell volume and then subsequently declined towards the initial values obtained at the beginning of the stress period (Fig. 5*D*).

P38MAPK Activation Profiles During the Hypertonic Challenge

There is tissue-specific activation of different members of the MAPK superfamily in response to an acute hypertonic challenge. To determine whether adaptation to such stress in RCEC and HCEC entails selective activation of one or more of these pathways, we measured the time-dependent effects of a 600 mOsm hypertonic challenge on their activation profiles with Western/ECL analysis. Figure 6*A*,*B* com-

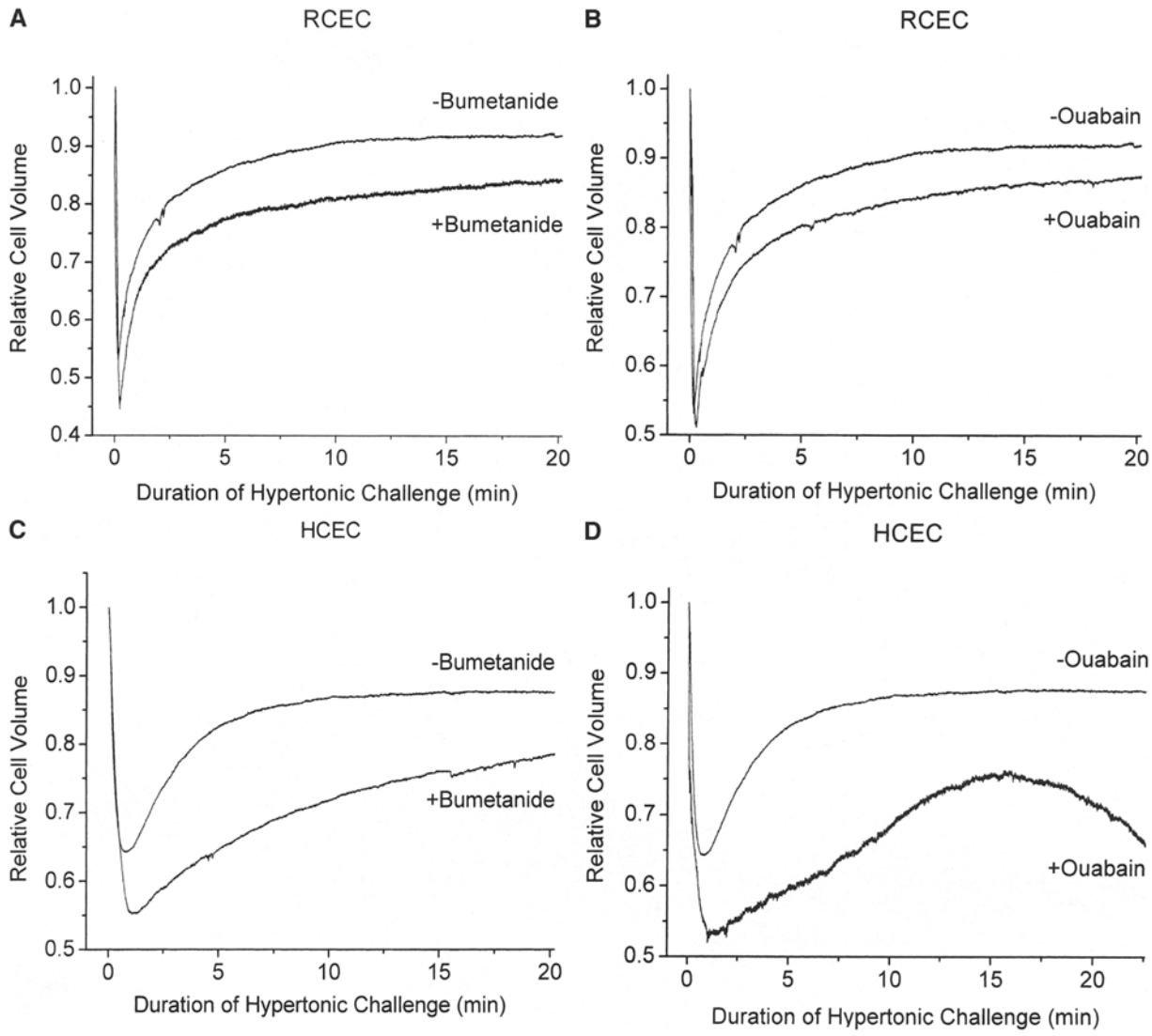

Fig. 5. Effects of NKCC and Na/K-ATPase inhibitors on RVI responses by hypertonicity-stressed RCEC (*A* and *B*) and HCEC (*C* and *D*). Methodology is the same as described in the legend of Fig. 2. In each case, data representative of 6 different experiments are shown.

pares representative experiments in HCEC ($n = 4$) and RCEC ($n = 4$) of the p38 activation profile over a 28 min. As can be seen, p38 kinase activity changed in both cell lines in a wave-like manner relative to the isotonic controls. The results of densitometric analysis of these experiments shown in Fig. 6*C* demonstrate that in HCEC, during the first 8–12 minutes, it increased about 18-fold above the baseline level. After the next 4 minutes, it declined by about 40%. In the subsequent 4 to 8 minutes, p38 kinase activity transiently increased to its maximum value followed by another decline of nearly 50%.

On the other hand, in RCEC, the oscillatory changes in p38 activity were even more exaggerated than those in HCEC. During the first 2 minutes of the hypertonic challenge, p38 activation increased about 90-fold above its baseline level. Subsequently, the activity fell by 60% and continued to oscillate for the next 24 minutes. The main difference between the two cell lines is that the swings in the levels of p38 kinase activity were much faster and greater in RCEC than in HCEC.

SAPK/JNK Kinase Activation Profiles During the Hypertonic Challenge

Figure 7*A*,*B* shows typical results of SAPK/JNK kinase activity measurements in HCEC and RCEC. Densitometric analysis of these bands revealed that in RCEC SAPK/JNK activity was essentially invariant throughout the entire period of exposure to hypertonicity (Fig. 7*C*). However, in HCEC a reproducible ($n = 4$) increase was observed of up to 2-fold during the first 4 to 8 minutes of the challenge.

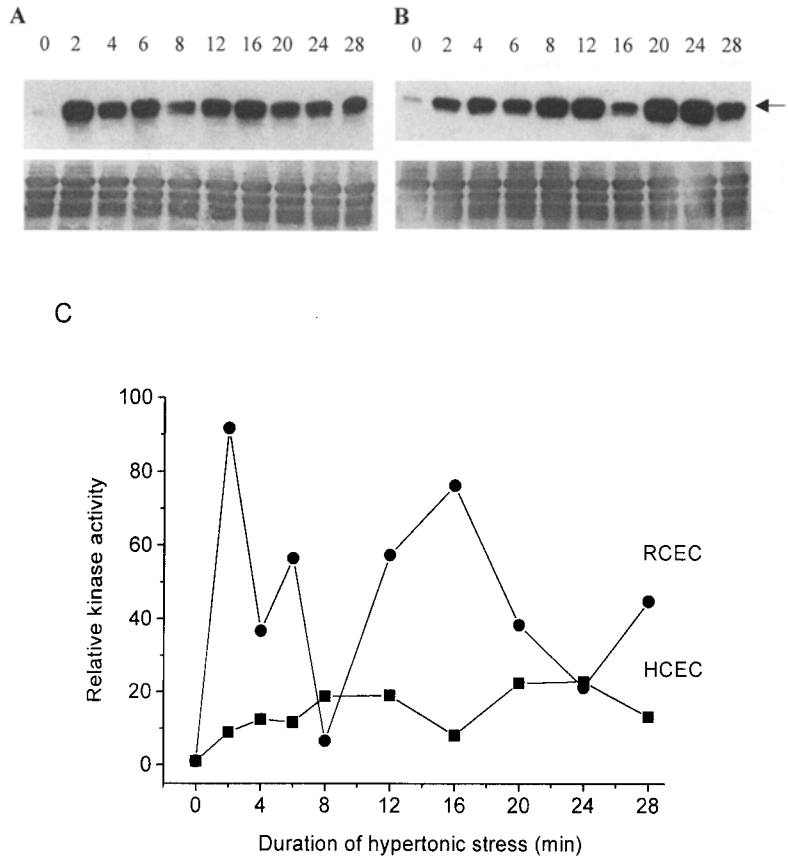

Fig. 6. Hypertonicity-induced changes in RCEC and HCEC p38MAPK activity. The top rows in panels *A* and *B* show quantitative immunoblotting with phospo-ATF-2 (Thr71) antibody in RCEC and HCEC, respectively. Cell extracts were incubated overnight with 20 μl of immobilized p38MAPK (Thr180/Tyr182) monoclonal antibody. After extensive washing, the kinase reaction was performed in the presence of 100 μM of cold ATP and 2 μg of ATF-2 fusion protein. Phosphorylation of ATF-2 at Thr71 was measured by Western blot/ECL using phospho-ATF-2 (Thr71) antibody, as indicated by the arrow. The bottom rows of these panels reveal uniform staining of exposed membranes with colloidal gold. (*C*) The results shown in panels *A* and *B* were quantified by densitometry and normalized to their corresponding value at zero time (control).

Erk1/2 Activation Profiles During the Hypertonic Challenge

Figure 8*A*,*B* shows representative changes in Erk1/2 activity in hypertonicity-stressed HCEC and RCEC (n = 4). Normalized densitometric data (Fig. 8*C*) shows that Erk1/2 activity in both cell lines continuously declined relative to its isotonic value during exposure to the 600 mOsm hypertonic challenge. After 12 minutes, its activity reached a nadir that was 30–40% of its isotonic level. Subsequently there were insignificant changes in HCEC Erk1/2 activity for the next 16 minutes, but in RCEC the initial decline was followed by a recovery up to 90% of its control level during the next 8 minutes.

Role of MAPK Limbs in Mediating RVI During the Hypertonic Challenge

The contributions by each of the three limbs of the MAPK superfamily to RVI were evaluated based on the effects of specific inhibitors of the ERK, p38 and JNK pathways. Table 2 shows that the inhibitor of p38 activation, SB203580, dose dependently depressed the magnitude of RVI in both cell lines, whereas τ was only prolonged in HCEC. It is noteworthy that neither inhibition of the ERK nor JNK limbs had any significant effect on either of the RVI parameters.

Discussion

The goal of this study was to determine the cell signaling cascades that mediate volume regulatory responses in corneal epithelial cell layers needed for the maintenance of their barrier function during an acute hypertonic exposure. This was done in both HCEC and RCEC because during chronic hypertonic stress (i.e., up to 48 h) the cells' capacities to adapt were markedly different from one another. RCEC could better maintain their RVI capacity than HCEC (Bildin et al., 2000). This resilience in RCEC was associated with upregulation of NKCC1 gene and protein expression as well as with NKCC1 functional activity. Given this background, we hypothesized that RCEC layers could preserve their barrier properties better than HCEC layers can during an acute 600 mOsm challenge. The testing of this hypothesis is a relevant issue because in daily living the corneal epithelial barrier properties may be repeatedly challenged by variation in the osmolality of the overlying tear film. In the face of these changes, the maintenance of the corneal barrier optical properties may be dependent on the ability of the epithelial layer to manifest regulatory volume responses that sustain corneal transparency. Therefore, we evaluated the hypertonicity-induced changes in barrier properties based on measurements of 1) dye penetration rate; 2) R_t; 3) light scattering behavior. To unravel the roles of specific

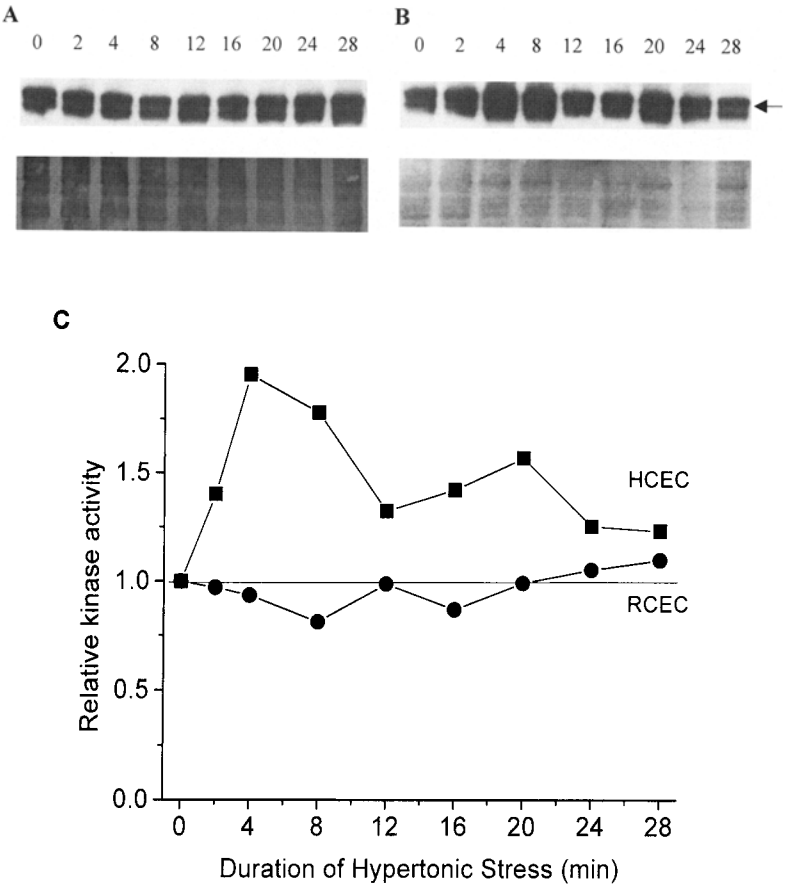

Fig. 7. Hypertonicity-induced changes in RCEC and HCEC JNK activity. The top rows of panels *A* and *B* show quantitative immunoblotting with phospho-c-Jun (ser63) antibody in RCEC and HCEC, respectively. Cell extracts were incubated overnight with 2 μg of immobilized c-Jun (1-89) fusion protein. After kinase reaction, phosphorylation of c-Jun at Ser63 was measured by Western blot/ECL using a phospho-c-Jun (Ser63) antibody as indicated by the arrow. The bottom rows of these panels reveal uniform staining of exposed membranes with colloidal gold. (*C*) The results shown in panels *A* and *B* were quantified with densitometry and normalized to their corresponding value at zero time (control).

cell signaling pathways underlying these responses, we also characterized the hypertonicity-induced time-dependent variations in activity of MAPKs that may be associated with increases in NKCC and Na/K-ATPase activity and needed for recovery of cell volume as well as cell layer barrier function.

We found that RCEC layers have a greater ability than their HCEC counterpart to adapt to acute hypertonic stress. It is noteworthy that in both cases the patterns of recovery of translayer dye penetration rate and R_t correlate with RVI behavior. Furthermore, we demonstrate that these responses are associated with the time dependence and magnitude of changes in p38 activity rather than with those of either JNK or Erk1/2. Activation of p38 appears to be essential for increases in Na/K-ATPase and NKCC activity, which affect rises in osmolyte levels that are needed to counter the hypertonic challenge.

Dependence of Translayer Dye Penetration and Electrical Resistance on Cell Volume Regulation

Sulforhodamine B permeability was previously used as an index to evaluate corneal epithelial barrier properties in the isolated rabbit cornea (Araie & Maurice, 1987). This was done because its transepithelial permeation rate is presumably a measure of the paracellular permeability, which reflects tight junctional and lateral intercellular space resistance. To evaluate the barrier properties of confluent RCEC and HCEC layers, we monitored the rate of sulforhodamine B penetration across these layers under isotonic and 600 mOsm hyperosmotic conditions. We found that during acute hypertonic stress dye permeability across RCEC layers initially increased, whereas it subsequently returned towards its isotonic baseline value (Fig. 1). On the other hand, the dye permeability across HCEC layers increased and remained stable without any recovery towards its isotonic baseline value. The observed difference in RVI responses is consistent with our previous results that during a hypertonic (375 mOsm) challenge cell volume recovery is greater in RCEC than HCEC (Bildin et al., 1998). These profiles are consistent with the observation that the RVI rate and its extent in HCEC were significantly smaller than those in RCEC, and the time dependence for barrier recovery was similar to their RVI response (Fig. 2, Table 1). Furthermore, there was a correspondence between the hypertonicity-induced cell shrinkage and the overall increase in dye penetration rate: both changed by a factor of about two. The correspondence among all of the above mentioned responses suggests that the RVI behavior could account for cell layer resistance recovery. It is noteworthy that the extent of barrier

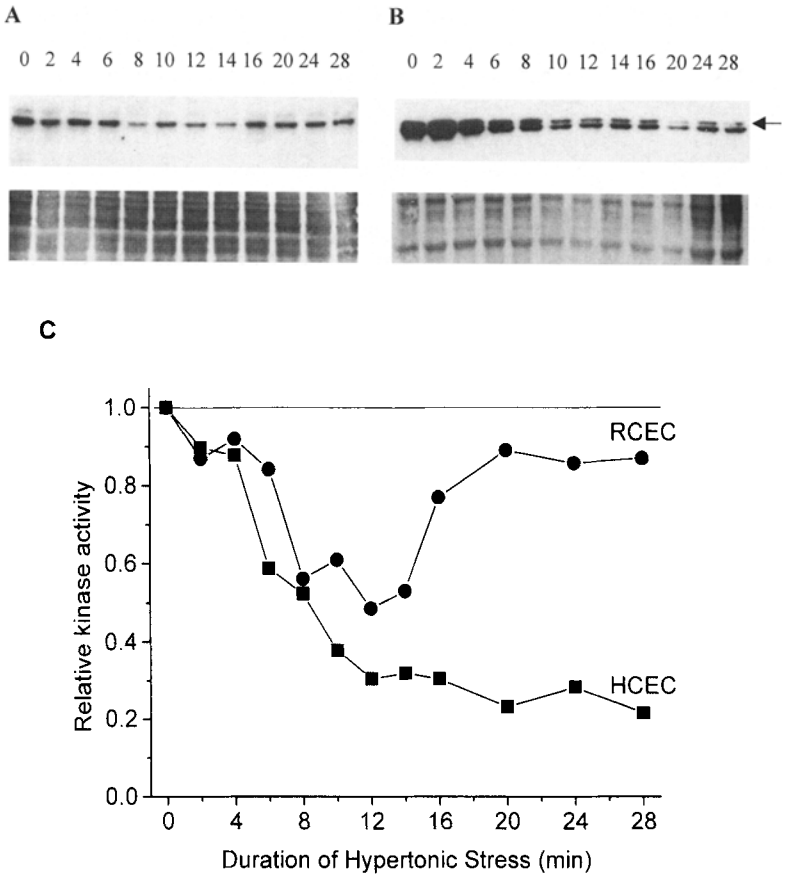

Fig. 8. Hypertonicity-induced changes in RCEC and HCEC JNK activity. The top rows of panels *A* and *B* represent quantitative immunoblotting with phospo-Elk-1 antibody in RCEC and HCEC, respectively. Cell extracts were incubated overnight with 15 μl of immobilized Erk1/2 (Thr202/Tyr204) monoclonal antibody. After extensive washing, the kinase reaction was performed in the presence of 200 μM of cold ATP and 2 μg of Elk-1 fusion protein. Phosphorylation of Elk-1 at Ser383 was measured by Western blot/ECL using phospho-Elk-1 (Ser383) antibody as indicated by the arrow. The bottom rows of these panels demonstrate uniform staining of exposed membranes with colloidal gold. (*C*) The results shown in panels *A* and *B* were quantified with densitometry and normalized to their corresponding value at zero time (control).

Table 2. Role of MAPK superfamily kinases in mediating RVI during 20 min exposure to 600 mOsm challenge

Inhibitor	Concentration μM	RCEC		HCEC	
		RVI (%)	τ (sec)	RVI (%)	τ (sec)
No inhibitor		84.1 ± 0.8	71.5 ± 4.8	65.5 ± 2.4	170.3 ± 4.7
U0126	1	80.6 ± 6.9	62.0 ± 8.1	69.0 ± 8.7	167.6 ± 13.8
SP600125	1	81.7 ± 7.3	65.4 ± 8.0	68.5 ± 7.5	148.4 ± 15.2
SB203580	0.1	69.2 ± 9.2	67.6 ± 6.9	49.1 ± 7.5	205.2 ± 16.5
	0.33	46.5 ± 5.3	59.8 ± 5.4	29.6 ± 6.9	289.2 ± 12.1
	1.0	36.1 ± 7.7	45.6 ± 5.8	none	none

Values represent the mean of 3 to 4 independent experiments (±SEM).

recovery may be related to the magnitude of the RVI response. There is no information in the literature that we are aware of documenting this association. On the other hand, in parallel experiments with both cell lines, the magnitude of R_t changes, which are a reflection of tight junctional ion permeation properties, were only in qualitative agreement with the RVI response and dye permeability. It is of interest that in both cell lines there is a correspondence between the magnitude of the osmometric cell shrinkage, the initial increase in dye penetration rate and the decrease in R_t. In each case, the change was by a factor of about two. Following 8 minutes of hypertonic stress in RCEC, cell volume recovered up to 90% of its isotonic value (Fig. 2) and the dye penetration rate progressively decreased after this time (Fig. 1). In parallel experiments, the R_t recovery was much slower than that of cell volume and dye permeability (Fig. 3). Under the same conditions, in HCEC cell volume recovery reached only 77% of its isotonic value. This extent of RVI seems to be inadequate for restoration of barrier function as neither the increases in dye penetration rate nor the decreases in R_t were reversible (Figs. 1–3). Previously, we showed that the isotonic, steady-state levels of NKCC1 mRNA and protein expression were twofold higher in RCEC than in HCEC (Bildin et al., 2000). This finding could explain why RCEC mediated a more complete RVI response and only in these cells did the dye penetration rate recover to its isotonic value.

It would appear that the magnitude of cell layer barrier recovery is related to the extent of RVI during the challenge. With sulforhodamine B (M_r = 559), a relatively low-molecular weight dye, for evaluating barrier function, the cell volume during the RVI response had to increase to about 90% of its isotonic value for full restoration of the barrier properties. On the other hand, only in RCEC did R_t partially recover after 20 minutes. This difference between the limited level of recovery of R_t and the almost complete recovery of dye permeability rate and cell volume is possibly a reflection of R_t being a much more sensitive measure of tight junctional integrity. Even though cell-to-cell apposition is restored, as reflected by extent of RVI and dye permeability recovery, tight junctional ion permeability may remain disrupted and account for the failure of R_t to recover.

Roles of NKCC and Na/K-ATPase in Translayer Dye Penetration and Cell Volume Regulation

Both NKCC and Na/K-ATPase activity are essential requisites for numerous tissues to mount RVI responses during exposure to a hypertonic challenge (Graf & Haussinger, 1996; Russell, 2000). Our unpublished data show that in HCEC and RCEC the combined activity of NKCC and Na/K-ATPase mediate most of the total ^{86}Rb uptake. In order to evaluate their contributions to the maintenance of translayer dye permeability and RVI capacity during exposure to a short-term hypertonic challenge, we measured the individual effects of bumetanide and ouabain on these parameters.

In RCEC, both inhibitors caused a 14% decrease in the extent of RVI (Table 1) without affecting translayer dye permeability rate (Fig. 4*A*,*B*). However, inhibition of Na/K-ATPase with ouabain extended the τ of cell volume recovery by 1.6-fold. This delay may account for an increase in dye penetration rate observed during the first 3 minutes of the challenge. On the other hand, in HCEC, its RVI and cell layer dye permeability behavior were much more dependent on NKCC and especially Na/K-ATPase activity than in RCEC (Fig. 4*C*,*D*, Table 1). This is indicated because in these cells bumetanide (50 μM) inhibited RVI recovery much more than in RCEC. In addition, τ was prolonged and accompanied by a proportional increase in dye penetration rate. These differences in sensitivity to bumetanide agree with our previous data that bumetanide at concentrations of up to 100 μM decreased ^{86}Rb uptake in RCEC less than in HCEC (Bildin et al., 2000). One possible explanation for these differences is that the NKCC isoform(s) expressed in RCEC have a lower affinity for bumetanide than in HCEC.

In HCEC, inhibition of Na/K-ATPase affected RVI and dye permeability more than in RCEC. This is evident because in HCEC, RVI recovery was much more limited and transient than in RCEC (Fig. 5*D*). Cell volume reversal suggests that inhibition of the Na/K-ATPase prevented expression of even a sustained partial recovery. Similarly, in HCEC the dye penetration rate initially increased, stabilized for 10 minutes and then rose again, whereas in RCEC it transiently became larger followed by a complete recovery to its isotonic value (Fig. 4*B*,*D*). These larger effects of bumetanide and ouabain on HCEC RVI behavior and the rates of translayer dye penetration suggest that in HCEC NKCC and Na/K-ATPase activity are larger contributors to RVI maintenance than in RCEC. However, the identity of other ion (osmolyte) transporters in RCEC that could contribute to this function is unclear. Taken together, the ability to maintain barrier function during acute hypertonic stress is related to cell volume regulatory capacity.

Role of MAPK Superfamily Kinases Activity in Cell Responses to Hypertonicity

The osmosensors that detect a hypertonic stress, and which in turn activate signaling pathways that mediate a RVI response, are still unknown. In some tissues, the RVI response significantly depends on increases in NKCC activity in response to a hypertonic challenge. Such increases can be mediated through stimulation of signaling pathways that include protein kinase A or protein kinase C as well as possibly inhibition of protein phosphatase(s) (Pewitt et al., 1990; Palfrey & Pewitt, 1993). During a hypertonic challenge, NKCC phosphorylation initially plays an important role in the regulation of its activity (Haas, McBrayer & Lytle, 1995; Sun & O'Donnell, 1996). The cell signaling pathways that could be involved in activating cell volume regulation include increases in the activity of different members of the MAPK superfamily. Modulation of their activity in response to hypertonic stress is very diverse and tissue-specific. In some tissues, activation of membrane ion transporters and RVI in response to exposure to a hypertonic stress is not even directly linked to MAPK activation. For example, in U937 cells, analysis of the time course of hypertonic activation of NHE and of the three subfamilies of MAPK and sensitivity of these processes to specific inhibitors revealed that the stimulation of NHE and the activation of Erk1/2, p38MAPK and JNK are parallel but independent events (Gillis et al., 2001). Therefore, it is not possible to generalize based on studies in one tissue, which limb of the MAPK superfamily cascade is involved in eliciting a regulatory volume response to a hypertonic challenge.

In the current study, the hypertonic challenge in both cell lines induced fast and large increases in p38 activity (Fig. 6*C*). However, the kinetics of this acti-

vation were completely different: 1) in RCEC, p38 was maximally phosphorylated within the first 2 minutes, whereas in HCEC it occurred only after 8 minutes; 2) RCEC peak activation was 5-fold greater than in HCEC. In both cell lines, p38 activation preceded RVI and recovery of cell layer barrier properties, and the profile of this activation correlated with the kinetics for both of these processes (Figs. 1 and 2). Our results comparing the ability of MAPK inhibitors to suppress RVI indicate that the p38 limb activation is required to elicit this response. The results shown in Table 2 make it apparent that only inhibition of the p38 limb with SB203580 mediated dose-dependent declines in RVI in both cell lines. In contrast, exposure to ERK- and JNK-pathway inhibitors over the same concentration range had no effect on RVI. These data are in good agreement with the report that in the rat medullary thick ascending limb of Henle, SB203580, a specific inhibitor of p38MAPK, almost completely inhibited RVI induced by hypertonicity, whereas inhibition of Erk1/2 activity did not alter this response (Roger et al., 1999).

A noteworthy finding in the current study is that p38 activation exhibited a two-wave pattern (i.e., oscillation) (Fig. 6*C*). Equivalent band intensity in different lanes shown in the bottom of Fig. 6*A*,*B*, documents that all samples contained a similar amount of total p38. Additional evidence that the wave pattern is not artifactual is that it was consistently observed in four independent experiments for each cell line. One possible reason that such oscillations are rarely described is that we measured MAPK activity at 2 minutes intervals, whereas others generally measure it only at 5 to 20 minute intervals. Furthermore, the absence of oscillations in most other studies could be explained by the fact that they provide average data rather than individual representative experiments.

Activation of the p38MAPK cascade in hypertonicity-stressed cells is involved in eliciting a number of other functions needed for adaptation to such stress. For example, in human peripheral blood monocytes and human peripheral blood-derived macrophages, p38MAPK is involved in hyperosmolality-induced upregulation of betaine and myo-inositol transporters (Denkert et al., 1998). In renal cells, betaine transporter expression was also dependent on p38 activation (Sheikh-Hamad et al., 1998). Taken together, these data suggest that p38 kinase is responsible for cell adaptation to acute and prolonged hypertonic stress and activation of inorganic and organic ion transporters, which lead to recovery of isotonic cell volume.

The variations of JNK activity in RCEC and HCEC were negligible in comparison to those of p38 (Fig. 7*C*). Only in HCEC a relatively moderate increase, an about twofold elevation in its activity, was observed. In RCEC, there were no changes in SAPK/JNK activity, a result which strongly supports the conclusion that this module of the MAPK cascade is not involved in eliciting the RVI response or in maintaining cell layer integrity. Two waves of moderate increases in SAPK/JNK activity in HCEC could reflect comparatively lower, perhaps inadequate, activation of p38 in these cells, as we suggested above.

Our results are in agreement with those of others dealing with the profile of activation of the SAPK/JNK limb in response to osmotic stress. In rat PC12 and human small-cell lung cancer cells, there were cell type-dependent increases in activity of selective JNK isoforms (Butterfield et al., 1999). In IMCD-3 cells and in bovine aortic endothelial cells, hypertonicity-induced JNK activation appears to be linked to promoting cell survival and suppressing apoptosis rather than stimulation of membrane ion transport (Malek et al., 1998; Wojtaszek et al., 1998). One observation suggesting the possibility of SAPK/JNK involvement in the RVI response in some cells is that hypertonic activation of Na^+/H^+ exchange in *Xenopus* oocytes is mediated by activation of JNK and requires Cl^- (Goss et al., 2001). Taken together, our results are in agreement with many other studies suggesting the importance of the SAPK/JNK limb of MAPK to cell adaptation to chronic rather than acute hypertonic exposure.

Erk1/2 activation by osmotic stress is a consistent finding in many different types of cells (Cohen, 1999). However, there is still limited information on the adaptive significance of such activation to restoration of cellular function. Some examples of hypertonicity-induced effects include: 1) Erk1/2 activation in mouse lung epithelial cells (MLE-15), resulting in increases in AQP5 expression (Hoffert et al., 2000); 2) inhibition of Erk1/2 activation by PD98059 in ANA1 murine macrophage cell line, abrogating upregulation of taurine uptake (Romio et al., 2001). A recent study in a secretory epithelium, human tracheal epithelial cells, suggests that hyperosmotic-induced activation of NKCC1 is regulated by stimulation of PKC-delta and ERK (Liedtke & Cole, 2002).

Unlike in those studies, we found a negative response by Erk1/2 to such a challenge (Fig. 8*C*). We are aware of only one study where a similar effect was described. In C6 glioma cells, Erk1/2 activity increased in hypoosmotic (205 mOsm) medium but decreased under a hyperosmotic (405 mOsm) condition, relative to its isotonic control (Sinning et al., 1997). In our study, Erk1/2 activity does not appear to be associated with activation of the RVI responses, but instead seems to be dependent on cell volume and integrity recovery. This is indicated because we observed: 1) a significant decline in Erk1/2 activity during the first 10 minutes of the hypertonic stress in both cell lines; 2) its activity almost completely

recovered in RCEC, which was the only cell type able to recover its volume and layer integrity; 3) the kinetics of RVI precede the temporal pattern of Erk1/2 recovery. Our data pertaining to RVI behavior and Erk1/2 activation agrees with a report that in the rat medullary thick ascending limb of Henle SB203580, inhibition of Erk1/2 activity did not alter RVI induced by hypertonicity (Roger et al., 1999).

In summary, there is a temporal relationship between hypertonic stress-induced p38 activation, RVI kinetics and recovery of cell layer barrier function. Activation (phosphorylation) of p38MAPK was faster and larger in RCEC than in HCEC and this correlates with the cells' respective regulatory volume capacities. Furthermore, inhibitor analysis shows that p38 activation appears to be essential for mediating RVI. In both cell lines 1) the variations in SAPK/JNK activity were negligible in comparison to those of p38; 2) the changes in Erk activity reflected profiles of RVI and cell layer recovery. Taken together, during an acute hypertonic challenge lasting 30 minutes maintenance of cell volume and barrier function are dependent on sufficient activation of the p38 limb of the MAPK cascade.

This work was supported by USPHS Grants EY04795 (to PSR), RPB Inc. (to PI), and the Schnurmacher Foundation (to VNB).

References

Araie, M., Maurice, D. 1987. The rate of diffusion of fluorophores through the corneal epithelium and stroma. *Exp. Eye. Res.* **44:**73–87

Araki, K., Ohashi, Y., Sasabe, T., Kinoshita, S., Hayashi, K., Yang, X.Z., Hosaka, Y., Aizawa, S., Handa, H. 1993. Immortalization of rabbit corneal epithelial cells by a recombinant SV40-adenovirus vector. *Invest. Ophthalmol. Vis. Sci.* **34:**2665–2671

Bildin, V.N., Yang, H., Crook, R.B., Fischbarg, J., Reinach, P.S. 2000. 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. *J. Membrane Biol.* **177:**41–50

Bildin, V.N., Yang, H., Fischbarg, J., Reinach, P.S. 1998. Effects of chronic hypertonic stress on regulatory volume increase and Na-K-2Cl cotransporter expression in cultured corneal epithelial cells. *Adv. Exp. Med. Biol.* **438:**637–642

Butterfield, L., Zentrich, E., Beekman, A., Heasley, L.E. 1999. Stress- and cell type-dependent regulation of transfected c-Jun N-terminal kinase and mitogen-activated protein kinase kinase isoforms. *Biochem. J.* **338:**681–686

Candia, O.A., Patarca, R., Alvarez, L.J. 1998. Reduction of water permeability by anisotonic solutions in frog corneal epithelium. *Invest. Ophthalmol. Vis. Sci.* **39:**378–384

Cohen, D.M. 1999. Signalling and gene regulation by urea and NaCl in the renal medulla. *Clin. Exp. Pharmacol. Physiol.* **26:**69–73

Denkert, C., Warskulat, U., Hensel, F., Haussinger, D. 1998. Osmolyte strategy in human monocytes and macrophages: involvement of p38MAPK in hyperosmotic induction of betaine and myoinositol transporters. *Arch. Biochem. Biophys.* **354:**172–180

Duzgun, S.A., Rasque, H., Kito, H., Azuma, N., Li, W., Basson, M.D., Gahtan, V., Dudrick, S.J., Sumpio, B.E. 2000. Mitogen-activated protein phosphorylation in endothelial cells exposed to hyperosmolar conditions. *J. Cell Biochem.* **76:**567–571

Fischbarg, J., Li, J., Kuang, K., Echevarria, M., Iserovich, P. 1993. Determination of volume and water permeability of plated cells from measurements of light scattering. *Am. J. Physiol.* **265:**C1412–1423

Gillis, D., Shrode, L.D., Krump, E., Howard, C.M., Rubie, E.A., Tibbles, L.A., Woodgett, J., Grinstein, S. 2001. Osmotic stimulation of the Na^+/H^+ exchanger NHE1: relationship to the activation of three MAPK pathways. *J. Membrane Biol.* **181:**205–214

Goss, G.G., Jiang, L., Vandorpe, D.H., Kieller, D., Chernova, M.N., Robertson, M., Alper, S.L. 2001. Role of JNK in hypertonic activation of Cl^--dependent Na^+/H^+ exchange in *Xenopus* oocytes. *Am. J. Physiol.* **281:**1978–1990

Graf, J., Haussinger, D. 1996. Ion transport in hepatocytes: mechanisms and correlations to cell volume, hormone actions and metabolism. *J. Hepatol.* **24:**53–77

Haas, M., McBrayer, D., Lytle, C. 1995. $[Cl^-]_i$-dependent phosphorylation of the Na-K-Cl cotransport protein of dog tracheal epithelial cells. *J. Biol. Chem.* **270:**28955–28961

Hallows, K.R., Knauf, P.A. 1994. Regulatory volume decrease in HL-60 cells: importance of rapid changes in permeability of Cl^- and organic solutes. *Am. J. Physiol.* **267:**C1045–1056

Hoffert, J.D., Leitch, V., Agre, P., King, L.S. 2000. Hypertonic induction of aquaporin-5 expression through an ERK-dependent pathway. *J. Biol. Chem.* **275:**9070–9077

Liedtke, C.M., Cole, T.S. 2002. Activation of NKCC1 by hyperosmotic stress in human tracheal epithelial cells involves PKC-delta and ERK. *Biochim. Biophys. Acta.* **1589:**77–88

Lu, L., Reinach, P.S., Kao, W.W. 2001. Corneal epithelial wound healing. *Exp. Biol. Med. (Maywood)* **226:**653–664

Malek, A.M., Goss, G.G., Jiang, L., Izumo, S., Alper, S.L. 1998. Mannitol at clinical concentrations activates multiple signaling pathways and induces apoptosis in endothelial cells. *Stroke* **29:**2631–2640

O'Neill, W.C. 1999. Physiological significance of volume-regulatory transporters. *Am. J. Physiol.* **276:**C995–C1011

Palfrey, H.C., Pewitt, E.B. 1993. The ATP and Mg^{2+} dependence of Na^+-K^+-$2Cl^-$-cotransport reflects a requirement for protein phosphorylation: studies using calyculin A. *Pfluegers Arch.* **425:**321–328

Pewitt, E.B., Hegde, R.S., Haas, M., Palfrey, H.C. 1990. The regulation of Na/K/2Cl cotransport and bumetanide binding in avian erythrocytes by protein phosphorylation and dephosphorylation. Effects of kinase inhibitors and okadaic acid. *J. Biol. Chem.* **265:**20747–20756

Reinach, P., Ganapathy, V., Torres-Zamorano, V. 1994. A Na:H exchanger subtype mediates volume regulation in bovine corneal epithelial cells. *Adv. Exp. Med. Biol.* **350:**105–110

Roger, F., Martin, P.Y., Rousselot, M., Favre, H., Feraille, E. 1999. Cell shrinkage triggers the activation of mitogen-activated protein kinases by hypertonicity in the rat kidney medullary thick ascending limb of the Henle's loop. Requirement of p38 kinase for the regulatory volume increase response. *J. Biol. Chem.* **274:**34103–34110

Romio, L., Zegarra, M., Varesio, L., Galietta, L.J.V. 2001. Regulation of taurine transport in murine macrophages. *Amino Acids.* **21:**151–160

Russell, J.M. 2000. Sodium-potassium-chloride cotransport. *Physiol. Rev.* **80:**211–276

Sheikh-Hamad, D., Di Mari, J., Suki, W.N., Safirstein, R., Watts, B.A., 3rd, Rouse, D. 1998. p38 kinase activity is essential for osmotic induction of mRNAs for HSP70 and transporter for organic solute betaine in Madin-Darby canine kidney cells. *J. Biol. Chem.* **273:**1832–1837

Sinning, R., Schliess, F., Kubitz, R., Haussinger, D. 1997. Osmosignalling in C6 glioma cells. *FEBS Lett.* **400:**163–167

Sun, D., O'Donnell, M.E. 1996. Astroglial-mediated phosphorylation of the Na-K-Cl cotransporter in brain microvessel endothelial cells. *Am. J. Physiol.* **271:**C620–627

Terada, Y., Tomita, K., Homma, M.K., Nonoguchi, H., Yang, T., Yamada, T., Yuasa, Y., Krebs, E.G., Sasaki, S., Marumo, F. 1994. Sequential activation of Raf-1 kinase, mitogen-activated protein (MAP) kinase kinase, MAP kinase, and S6 kinase by hyperosmolality in renal cells. *J. Biol. Chem.* **269:**31296–31301

Watts, B.A., 3rd, Di Mari, J.F., Davis, R.J., Good, D.W. 1998. Hypertonicity activates MAP kinases and inhibits HCO-3 absorption via distinct pathways in thick ascending limb. *Am. J. Physiol.* **275:**F478–486

Wojtaszek, P.A., Heasley, L.E., Siriwardana, G., Berl, T. 1998. Dominant-negative c-Jun NH2-terminal kinase 2 sensitizes renal inner medullary collecting duct cells to hypertonicity-induced lethality independent of organic osmolyte transport. *J. Biol. Chem.* **273:**800–804

Zhuang, S., Hirai, S.I., Ohno, S. 2000. Hyperosmolality induces activation of cPKC and nPKC, a requirement for ERK1/2 activation in NIH/3T3 cells. *Am. J. Physiol.* **278:**C102–109.