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Differential Dependence of Regulatory Volume Decrease Behavior in Rabbit Corneal Epithelial Cells on MAPK Superfamily Activation

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Abstract

We characterized the dependence of hypotonicity-induced regulatory volume decrease (RVD) responses on mitogen-activated protein kinase (MAPK) pathway signaling in SV40-immortalized rabbit corneal epithelial cells (RCEC). Following calcein-AM loading, RVD was monitored using a microplate fluorescence reader. Western blot analysis determined MAPK activation. After 30 min, the RVD response restored the relative cell volume to nearly isotonic values, whereas it was inhibited when cells were bathed either in a Cl⁻-free solution or with the Cl⁻-channel inhibitors: 5-nitro-2-(3-phenylpropylamino) benzoic acid or niflumic acid. Similar declines occurred with either a high-K⁺ (20 mM) supplemented solution or the K⁺ channel inhibitor 4-aminopyridine. Activation of extracellular signal-regulated kinase (ERK), p38, and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) was time and tonicity-dependent. Stimulation of ERK and SAPK/JNK was maximized earlier than that of p38. Activation of ERK and SAPK/JNK was insensitive to Cl⁻ and K⁺ channel inhibitors, whereas inhibition with either PD98059 or SP600125, respectively, blocked RVD. However, inhibition of p38 with SB203580 had no effect on RVD. Suppression of RVD instead blocked p38 activation. Differences in the dependence of RVD activation on Erk1/2 and p38 signaling were validated in dominant negative (d/n) -Erk1 and d/n-p38 cells. Volume-sensitive Cl⁻ and K⁺ channel activation contributes, in concert, to RVD in RCEC. Therefore, swelling-induced ERK and SAPK/JNK stimulation precedes Cl⁻ and K⁺ channel activation, whereas p38 activation occurs as a consequence of RVD.

Keywords

regulatory volume decrease; hypotonic stress; swelling-sensitive ion channel; MAPK; Erk1/2; p38; SAPK/JNK

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1. Introduction

The corneal epithelium provides the first line of defense against environmental insults. Damage to this layer leads to loss of its protective and refractive functions. One type of challenge to corneal epithelial transparency includes swimming or bathing in fresh water and usage of hypotonic eye drops. These conditions can induce corneal epithelial cell (CEC) swelling and microvilli destruction (Schrage et al., 2004). The influx of water dilutes critical enzymes and electrolytes, which may subsequently compromise related signal transduction pathways and CEC's barrier function. Nevertheless, the corneal epithelium largely tolerates such stress by initiating a regulatory volume decrease (RVD) response described in cultured rabbit CEC (RCEC). This response depends on Ca^{2+} release through ryanodine-sensitive channels localized on intracellular calcium store membranes followed by plasma membrane Ca^{2+} influx from the extracellular medium (Wu et al., 1997). Such signaling in turn leads to the activation of ion efflux pathways underlying RVD. In some epithelia, this efflux results from activation of volume-sensitive K^+ , Cl^- channels (Grunnet et al., 2002; Hazama and Okada, 1988; Pavenstadt et al., 1996; Shen et al., 2001). Another contributor to RVD in human CEC (HCEC) and some other tissues is the K-Cl cotransporter (KCC) (Capo-Aponte et al., 2005, 2007a; Ernest et al., 2005). Even though volume sensitive Cl^- channel activity was identified in many tissues, nothing is known about the role of K^+ as a counterion to Cl^- egress in RCEC (Al-Nakkash et al., 2004; Dupre-Aucouturier et al., 2004; Hazama and Okada, 1988; Inoue et al., 2005).

The ability of cells to regulate their cellular volume during exposure to an anisotonic challenge is essential for sustaining their proliferative (Lang et al., 2005; Schreiber, 2005), migratory (Mao et al., 2005; Soroceanu et al., 1999) and secretory activity (Do et al., 2006; Strbak and Greer, 2000). Such responses are dependent on receptor mediated differential mitogen protein kinase (MAPK) activation. This superfamily includes three signaling pathways: extracellular signal-regulated kinase (ERK), p38 and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK). MAPK signaling control of linked responses can be modulated via crosstalk between each of these pathways through stimulation of protein phosphatase (PP) activity and expression (Hoefen and Berk, 2002; Wang et al., 2006). In RCEC, crosstalk between the p38 and ERK pathways modulates hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF) control of migration (Sharma and Bazan, 2003). Such crosstalk modulation of migration also occurs in response to EGF exposure in RCEC. It is attributable to MAPK-induced up-regulation of PPs (i.e. PP-2A and MAP kinase phosphatase-1) expression and activity (Wang et al., 2006). The timing of PP changes is initiated by changes in the phosphorylation status of each MAPK pathway. Such modulation in CEC and some other tissues dictates MAPK pathway control of such as synthesis, secretion, proliferation and migration (Kusuhara et al., 1998; Mackova et al., 2000; Paumelle et al., 2000; Surapisitchat et al., 2001; Wang et al., 2000; Zhang et al., 2001).

As with cell proliferation and migration, the extent of interaction between MAPK components may modulate regulatory volume responses needed for restoration of isotonic cell volume during exposure to anisotonic challenges. In many tissues, the ion transport activation that is required for inducing a regulatory volume response is dependent on MAPK stimulation, which may induce changes in cytoskeletal and scaffolding protein organization. In RCEC, regulatory volume increase (RVI) activation in response to a hypertonic challenge is solely dependent on stimulation of the p38 pathway. Its inhibition suppressed such response. RVI is mediated by p38 protein-protein interaction with the Na:K:2Cl cotransporter 1 (NKCC1), which induces increases in osmolyte uptake followed by rises in NKCC1 gene and protein expression (Bildin et al., 2003; Capo-Aponte et al., 2007b). This dependence of RVI induction on p38 activation also exists in the renal medullary thick ascending limb of Henle's loop (Bustamante et al., 2003; Roger et al., 1999).

Unlike with RVI, the dependence of RVD on MAPK activation is tissue specific. In cervical cancer cells (Shen et al., 2001), RVD is dependent on ERK activation whereas in renal epithelial cells p38 activation instead stimulates this response (Chiri et al., 2004; Shen et al., 2001). There is also tissue specificity regarding the identity of the upstream mediators of ERK activation. In human cervical cancer cells (Shen et al., 2001) and astrocytes (Schliess et al., 1996), hypotonicity induced Ca^{2+} transients result in ERK activation. However, in renal epithelial cells (Chiri et al., 2004) and hepatoma cells (Schliess et al., 1995), ERK stimulation is instead solely dependent on tyrosine kinase and G-protein activation. In RCEC, the dependence of RVD on MAPK signaling cascade activation is unclear.

In the present study, we characterized in RCEC the dependence of RVD on MAPK activation. RVD is dependent on increases in K^{+} and Cl^{-} efflux. Furthermore, RVD is solely dependent on ERK and SAPK/JNK activation. On the other hand, p38 activation occurred in response to RVD. Therefore, stimulation by ERK and SAPK/JNK is requisite for inducing the RVD response, whereas p38 activation occurs as a result of the RVD response.

2. Materials and methods

2.1 Cell culture

SV40-adenovirus-immortalized RCEC (a generous gift from Dr. Araki-Sasaki, Kagoshima Miyata Eye Clinic, Kagoshima, Japan) is a relevant model for RCEC-based studies (Kang et al., 2001). Cells were cultured in supplemented Dulbecco's Modified Eagle's Medium (DMED/F12), as previously described (Araki-Sasaki et al., 1995). Briefly, upon reaching 80–90% confluence, cells were detached with 0.5% trypsin-EDTA, and subcultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS), 5 ng/ml EGF, 5 $\mu\text{g}/\text{ml}$ insulin, and 40 $\mu\text{g}/\text{ml}$ gentamicin in a humidified incubator with 5% CO_2 /95% atmosphere air at 37°C. Stable tetracycline-inducible RCEC lines expressing dominant negative (d/n)-Erk1 and d/n-p38 were developed, as previously described (Yao et al., 1998), and maintained in the culture medium described above but without gentamicin. Expression of d/n-Erk1 and d/n-p38 was elicited using 1 $\mu\text{g}/\text{ml}$ tetracycline (Sigma, St. Louis, MO, USA) for 24 h.

2.2 Experimental solutions

Isotonic (300 ± 5 mOsm) Ringer's solution (control) contained 147.8 mM NaCl, 4.7 mM KCl, 0.4 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5.5 mM glucose, 1.8 mM CaCl_2 , and 5.3 mM HEPES Na^{+} (pH 7.4). High- K^{+} (20 mM) solution was isosmolar to the control solution and was prepared by decreasing the NaCl concentration to 132.8 mM and increasing the KCl concentration to 20 mM. The Cl^{-} -free solution (300 ± 5 mOsm) was comprised of 141.6 mM sodium D-gluconic acid, 2.5 mM K_2SO_4 , 2.0 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0 mM glucose, 5.4 mM $\text{CaSO}_4 \cdot \text{H}_2\text{O}$, 5.3 mM HEPES Na^{+} (pH 7.4). All drugs were dissolved in the appropriate control solution. The osmolality of solutions was measured using a $\mu\text{Osmette}$ Osmometer (Precision System, Natick, MA, USA) based on measurement of freezing-point depression.

2.3 Cell volume measurement and RVD analysis

Measurement of time-dependent changes in relative cell volume was made using a fluorescence microplate analyzer (Fusion™ Universal Microplate Analyzer (Perkin-Elmer, Boston, MA, USA)(Capo-Aponte et al., 2005). Briefly, RCEC were plated onto 24-well culture plates (10^4 cells/well) (Fisher Scientific, Pittsburgh, PA, USA) and grown to 80–90% confluence for 24–48 h prior to experimental use. RCEC were washed twice with pre-warmed phosphate-buffered saline (PBS) and loaded with 10 μM calcein-AM for 1 h. Cells were then washed with PBS and treated with the desired reagents for 30 min at 37°C. Calcein fluorescence was excited at 485 nm and emission was measured at 530 nm. A 5-min isotonic baseline was recorded prior to hypotonic challenges. Hypotonic challenges were created by simultaneously adding pre-

warmed (37°C) distilled water to isotonic Ringer's solution for each condition. Fluorescence was recorded at 30 s intervals up to 30 min. Preliminary experiments showed that both dilution and solution replacement methods produced similar osmotic stress and volume responses.

To convert fluorescence values to relative cell volume, the following equation was applied (Capo-Aponte et al., 2005; Crowe et al., 1995; Hamann et al., 2002)

$$\frac{V_t}{V_0} = \frac{\frac{F_t}{F_0} - f_b}{1 - f_b}$$

where V_t is the cell volume at time t , V_0 is initial volume at time = 0, F_0 is the fluorescence of cells in isotonic solution at time = 0 (π_0), F_t is the fluorescence measured subsequent to a hypotonic challenge at time t (π_t), and f_b is the background fluorescence, which represents the fraction of intracellular calcein insensitive to external osmolarity (i.e. $f_b < 1$). The conversion was based on the linear relationship between normalized drift-corrected fluorescence (F_t/F_0) during steady-state volume and the reciprocal of the relative osmotic pressure of the external solution (π_t/π_0). The relative volumes (V_t/V_0) were plotted as a function of time.

The percentage of volume recovery was calculated as

$$\text{Volume (\%)} = \frac{V_{\max} - V_f}{V_{\max} - V_0}$$

where $V_{\max}$ is maximum volume reached during the challenge and V_f is the final volume at 30 min after the challenge (Capo-Aponte et al., 2005). Data were analyzed using independent Student's two-tailed t -test ($p < 0.05$). Results are reported as mean RVD value $\pm$ standard error mean (SEM) for at least four independent experiments unless otherwise indicated.

2.4 Western blot analysis

Western blotting was carried out as described previously, with some modifications (Bildin et al., 2000). Briefly, RCEC were subcultured and deprived of FBS and EGF for 24 h prior to experimentation. Cells were then treated for 30 min with the desired reagents dissolved in control solution, and were subsequently challenged by exposing them to either 25 or 50% hypotonic stress. Cell lysates were prepared using lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerolphosphate and 1 mM Na_3VO_4 , pH 7.5) with a protease inhibitor mixture (1 mM PMSF, 1 mM benzamidine, 10 $\mu\text{g/ml}$ leupeptin, and 10 $\mu\text{g/ml}$ aprotinin) for at least 10 min. Cells were scraped with a rubber policeman followed by four sonication pulses for 4 s each at 50 mV (Fisher, Swannee, GA, USA) and centrifugation at 13 000 rpm for 15 min at 5°C. Supernatants were harvested and stored at -80°C until analysis. The protein concentration of each lysate was determined by BCA assay. After boiling the sample for 5 min, equal amounts of proteins were fractionated onto 10% sodium dodecylsulphate (SDS)-polyacrylamide gels, followed by electrophoresis and blotting onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked with blocking buffer for 1 h at room temperature and then probed overnight at 5°C with the antibody of interest. After three washes with blocking buffer, membranes were incubated with goat anti-rabbit or anti-mouse IgG-HRP secondary antibody for 1 h at room temperature, followed by consecutive washes with TBS-T, TBS, and distilled water for 10 min. Immunobound materials were visualized using the ECL-plus assay. Images were analyzed by densitometry using Sigma Scan Pro 5 (Aspire

Software, Leesburg, VA, USA). Experiments were performed in triplicate and the results were normalized to controls. They are represented as mean $\pm$ SEM, unless otherwise indicated.

MAPK activation was measured by probing with anti-phospho-Erk1/2, anti-phospho-p38, and anti-phospho-SAPK/JNK. To validate that changes in MAPK phosphorylation status were not due to differences in total MAPK component levels, membranes were reprobed with anti-Erk1, anti-p38, or anti-SAPK/JNK antibody after being stripped twice for 15 min with stripping buffer (0.2 M glycine, 0.1% SDS, 0.1% Tween-20, pH 2.2).

2.5 Reagents

Calcein-AM was purchased from Molecular Probes (Eugene, OR, USA). Five-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), niflumic acid (NA), 4-aminopyridine (4-AP), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). PD98059, U0126, SB203580, and SP600125 were purchased from Biomol (Plymouth Meeting, PA, USA). Phospho-Erk1/2 antibody, Erk1 and p38 MAPK antibody, as well as goat anti-rabbit and goat anti-mouse IgG-horseradish peroxidase (HRP) secondary antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Phospho-p38 antibody, phospho-SAPK/JNK antibody, and SAPK/JNK antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). Blocking buffer consisted of 5% nonfat dry milk (Carnation) in 0.1% Tris-buffered solution-Tween-20 (TBS-T). Antibodies were prepared to the desired concentration in blocking buffer. A bichinchoninic acid assay (BCA) kit was purchased from Pierce Biotechnology (Rockford, IL, USA). The enhanced chemiluminescence (ECL)-plus Western blotting detection assay kit was purchased from Amersham Biosciences (Piscataway, NJ, USA).

3. Results

3.1 Dependence of RVD activation on Cl^- and K^+ channel stimulation

The ion channel activity underlying RVD behavior was determined by evaluating the effects of inhibiting Cl^- and K^+ channel activity on this response. This was accomplished by assessing whether or not exposure to a 50% hypotonic challenge in the presence or absence of either a Cl^- or K^+ channel inhibitor affects time-dependent recovery of calcein-originated fluorescence. As shown in Figs. 1A and 2A, the relative cell volume remained relatively unchanged (~ 1.0) during the first 5-min exposure to the control NaCl Ringer's solution, in the presence or absence of the respective inhibitor prior to exposure to the indicated solution substitution. This hypotonic challenge increased the relative cell volume by 40% within 30 s. This level was close to the ideal calculated osmotic swelling. The RVD response ensued at an initial rate of 6%/min, with a partial recovery of $88 \pm 2\%$ at 30 min. Interestingly, with the Cl^- channel inhibitor NA, NPPB, or a Cl^- -free solution, additional initial swellings occurred of 2, 9 and 13%, respectively, the amounts greater than those measured under control conditions (Fig. 1A).

Subsequently, RVD recovery was partially blocked with NPPB (100 μM) or NA (100 μM) by 35 and 27% ($p < 0.05$), respectively, compared to control. Cl^- -free solution suppressed hypotonicity-induced RVD by 53% ($p < 0.001$) compared to the response obtained in control NaCl Ringer's solution (Figs. 1A and B). These results show that Cl^- channel activity contributes to the RVD response.

To evaluate the role of swelling-sensitive K^+ channel activity in mediating RVD, either the K^+ channel inhibitor 4-AP (1 mM) or an isotonic high- K^+ solution (20 mM) was used. Figure 2A illustrates the average effects of such exposure. An isotonic NaCl solution containing 4-AP- or a high- K^+ solution also caused additional swellings of 6 and 2%, respectively. With 20

mM K^+ -containing solution, RVD was maximally inhibited. 4-AP suppressed this response to a lesser extent. Figure 2B summarizes their inhibitory effects on RVD.

Hence volume-sensitive Cl^- and K^+ channel activation equally contributes in mediating RVD, i.e., NA and 4-AP individually inhibited this response by 27%, a result similar to those for HCEC (Capo-Aponte et al., 2005).

3.2 Time and hypotonicity dependence of MAPK activation

Figures 3A–C depict the time-dependent effects of exposure to 50% hypotonicity on Erk1/2, p38, and SAPK/JNK activation. As shown in Fig. 3A, Erk1/2 activation was maximized at 2.5 min and then decayed to the control level by 60 min. The slight activation of Erk1/2 prior to this challenge was possibly associated with solution dilution-induced cellular stress (Schliess et al., 1996).

Figure 3A shows that Erk2 expression was greater than that of Erk1. However, the relative hypotonicity-induced increase in activated Erk1 was more pronounced than that of Erk2 throughout the time course. This difference, which peaked at 2.5 min, was threefold greater for Erk1 than for Erk2, and waned before completely disappearing at 60 min. On the other hand, p38 activation was undetectable after 24-h serum starvation (Fig. 3B, lane 1), increasing slightly within 2.5 min of exposure to the hypotonic challenge. Activation reached a maximal level at 30 min and returned to control level during the next 30 min (Fig. 3B). The time-dependence of SAPK/JNK activation resembles that of Erk1/2, with a maximum at 2.5 min and partial recovery to baseline level at 60 min (Fig. 3C). Therefore, Erk1/2 and SAPK/JNK activation kinetics were much more rapid than that of p38.

Figure 4(A–C) shows that Erk1/2, p38, and SAPK/JNK phosphorylation levels increased as the osmolarity declined. During a 25% hypotonic challenge (225 mOsm), activation of Erk1/2, p38, and SAPK/JNK increased by 22, 112, and 45%, respectively, whereas during a 50% hypotonic challenge (150 mOsm), increases were 250, 310, and 215%, respectively.

Taken together, MAPK pathway activation is hypotonicity dependent with ERK and SAPK/JNK being more rapid than that of p38.

3.3 Dependence of RVD on Erk1/2 activation

To determine if Erk1/2 activation is dependent on induction of the RVD response, the aforementioned Cl^- and K^+ channel inhibitors were applied 30 min prior to the 50% hypotonic challenge. In the presence of either the isotonic Cl^- -free solution or Cl^- inhibitors NPPB (100 μ M) and NA (100 μ M), hypotonicity-activated Erk1/2 remained relatively unaffected (Fig. 5A). Similarly, inhibition of K^+ channel activity with 4-AP (1 mM) and high- K^+ solution (20 mM) had no effect on Erk1/2 activation (Fig. 5B). These results indicate that swelling-induced Erk1/2 activation is either upstream of channel activation or independent of the volume-sensitive Cl^-/K^+ efflux induced by RVD.

To determine if RVD activation is a consequence of ERK stimulation, RVD responses were evaluated in the presence of the MEK1/2 inhibitors PD98059 (20 and 40 μ M) or U0126 (10 μ M). Such MAPK inhibitor concentrations were sufficient to inhibit Erk1/2 activation (Fig. 6A, inset). Figure 6A shows that PD98059 and U0126 partially inhibited the RVD response to the 50% hypotonic challenge. With 40 μ M PD98059, RVD inhibition was 62% whereas with either 20 μ M PD98059 or 10 μ M U0126, inhibitions were 35 and 37%, respectively. To validate the inhibitory selectivity of PD98059 and U0126 on RVD, RVD responses were measured in d/n-Erk1 cells in the presence or absence of tetracycline (1 μ g/ml) for 24 h. The selectivity of tetracycline to induce d/n-Erk1 expression was also evaluated by first determining whether this antibiotic in nontransfected cells had an inhibitory effect on RVD. As shown in

Fig. 6B, RVD recovery was similar in d/n-Erk1 cells and their nontransfected counterpart in the absence of tetracycline. With tetracycline, however, RVD was inhibited by 12% ($p < 0.05$) in d/n-Erk1 cells. Furthermore, the initial cell swelling increased by an additional 10% in both the untreated d/n-Erk1 cells and in nontransfected counterpart exposed to either PD98059 or U0126 (Fig. 6A). The difference of the inhibitory efficacy between the drugs and d/n-Erk1 cells is possibly attributable to Erk2 activity. Therefore, stimulation of volume-sensitive Cl^- and K^+ channel activity and RVD is dependent on Erk1/2 MAPK pathway activation.

3.4 Dependence of p38 Activation on RVD

We probed for the dependence of RVD on p38 activation by determining whether (i) p38 activation occurs as a consequence of RVD or (ii) RVD is inhibited by suppression of p38 activation. Figure 7A reveals that blockage of RVD by the Cl^- channel inhibitors NPPB (100 μM) or NA (100 μM) inhibited p38 activation by 96 and 89%, respectively ($p < 0.001$). In the presence of Cl^- -free solution, hypotonicity-induced p38 activation was abolished by 99% ($p < 0.001$). It should be noted that there is an association between the magnitude of RVD inhibition and the decline in p38 activation. In the Cl^- -free solution, RVD inhibition was maximal (Fig. 1); and under this condition as well, p38 activation was maximally suppressed (Fig. 7A). In contrast, NA induced minimal reduction of RVD and p38 activation. Blockage of RVD resulting from exposure to either the K^+ channel blocker 4-AP (1mM) or the high- K^+ solution (20 mM) inhibited p38 activation by 83 and 92%, respectively (Fig. 7B). Similarly, there is an association between the magnitude of RVD inhibition and declines in p38 activation since 20 mM high- K^+ solution suppressed RVD more than did 4-AP (Fig. 2); p38 activation was inhibited more with this high- K^+ solution than with 4-AP (Fig. 7B). A recent study (vom Dahl et al., 2001) on rat liver indicated that SB203580, a specific p38 MAPK inhibitor, inhibited RVD. SB203580 was applied to RCEC 30 min prior to the hypotonic challenge to determine if the aforementioned result could be replicated in RCEC. At 10 and 20 μM SB203580, p38 activation was sufficiently suppressed, but the RVD response was unchanged (Fig. 8A). More specifically, the extent of volume recovery was similar in the presence or absence of SB203580, indicating that swelling-induced RVD is not dependent on p38 activation. To validate the selectivity of the negative effect of SB203580 on the RVD response, RVD was evaluated in d/n-p38 cells in the presence or absence of tetracycline. Figure 8B shows that in comparison to untreated d/n-p38 cells, tetracycline-induced expression of mutant p38 did not alter the RVD response. That this RVD response was unaltered in d/n-p38 cells and was the same as that in nontransfected RCEC exposed to SB203580 validates the notion that inhibition of p38 does not suppress the RVD response. Taken together, hypotonic activation of p38 occurs as a consequence of swelling-induced increases in Cl^-/K^+ channel activity rather than inducing activation of this response.

3.5 Dependence of RVD on SAPK/JNK activation

To determine the involvement of SAPK/JNK in eliciting RVD, SAPK/JNK activation was first inhibited with 30 μM SP600125. This inhibitor suppressed RVD recovery by 46%, but also increased the hypotonicity-induced relative cell swelling by 16% more than that without the inhibitor (Fig. 9).

To determine the dependence of RVD on SAPK/JNK activation, the effect of a hypotonic challenge on SAPK/JNK activation was compared in the presence and absence of either a Cl^- or K^+ channel inhibitor. Their selectivity was validated using either a Cl^- -free or high- K^+ containing solution. Under all of these conditions, hypotonicity-induced SAPK/JNK activation was unaffected even though the RVD responses were suppressed (Figs. 10A–B). Therefore, as with ERK activation, SAPK/JNK stimulation is not dependent on RVD but rather it contributes towards inducing RVD.

4. Discussion

As in a number of different vertebrate cells, we found in RCEC that K^+ and Cl^- channel activation contributes to the RVD response (Christensen and Hoffmann, 1992; Furuya et al., 1989; Hazama and Okada, 1988; Kubo and Okada, 1992). Unlike the relationship between activation of MAPK signaling and RVD in any other tissue described in the literature, RVD is dependent on activation of the ERK and SAPK/JNK branches of the MAPK cascade. Conversely, p38 phosphorylation is instead dependent on RVD stimulation.

It is relevant to evaluate whether the results of the present study employing cultured cells grown in multiwell plates are related to those in which the corneal epithelial layer was in a polarized configuration. Such an assessment is needed since anisotonicity-induced changes of epithelial membrane osmolyte and fluid permeability are affected by whether or not the tissue is polarized. This dependence is based on the finding in the intact cornea that hypotonic-induced changes in basolateral membrane permeability mediate changes in the opposing apical membrane permeability (Candia et al., 1998). To evaluate whether there is any cell membrane polarization in cultured cells on a solid surface, we compared our reported electrophysiological results and the present RVD findings with those in which there was epithelial layer polarization (Al-Nakkash et al., 2004; Klyce and Wong, 1977). Cl^- dependent membrane depolarization occurred both in cultured RCEC and in the intact tissue in response to either Cl^- removal from the tear-side bathing solution or exposure to a hypotonic challenge. Since in the intact tissue this depolarization effect was solely observed if Cl^- was removed from the tear side bathing solution, the apical membrane has an appreciable Cl^- permeability. The fact that either Cl^- removal or exposure to hypotonicity induced a similar depolarizing response in cultured cells suggests that at least some of the cultured cells have their apical membrane exposed to the bathing solution rather than to the supporting surface on which they are grown. This polarized orientation accounts for why cultured cells can undergo RVD. Therefore, studies employing cultured cells have a physiological relevance to those obtained using intact polarized tissue.

We determined the dependence of the RVD response on increases in Cl^- and K^+ conductance and the relationship of their stimulation to MAPK superfamily signaling. Since Cl^- and K^+ channels are the major contributors with respect to RVD in other tissues, their involvement in this response was probed by inhibiting Cl^- and K^+ channels prior to a hypotonic challenge. In RCEC, we found that inhibition of each of these channels did not fully suppress RVD since NA and 4-AP by themselves both suppressed this response by 27%. This equivalence between their inhibitory effects indicates that Cl^- and K^+ effluxes are electrically coupled. On the other hand, the fact that the suppressions of RVD were greater in Cl^- -free and high- K^+ solutions (53 and 44%, respectively) than those obtained with individual Cl^- and K^+ channel inhibitors (27% and 27%, respectively), suggests the existence of a heterogeneous family of volume-sensitive ion transients mechanisms, only some of which are sensitive to NA and 4-AP (Nilius et al., 1996). KCl cotransporter (KCC) is another type of contributor to RVD, which has been described in multiple cell lines, including HCEC and RCEC (Capo-Aponte et al., 2005, 2007a; Ernest et al., 2005; Lauf and Adragna, 2000; Shen et al., 2000; Wehner et al., 2003). The contribution of this alternative mechanism to RVD is variable in different species, but less than that mediated by Cl^- and K^+ channel activity. These individual contributions by different ion transport mechanisms to RVD are similar to what has been reported in human glioma cells (Ernest et al., 2005).

It is well documented that MAPK superfamily signaling is activated in response to osmotic challenges. The dependence of RVD on MAPK activation appears to be tissue-specific. We found that ERK and SAPK/JNK activations peak early (i.e., after 2.5 min) in response to a 50% hypotonic challenge, while p38 activation peaks later (i.e., after 30 min). These different peak times suggest that ERK, SAPK/JNK, and p38 have different roles in mediating responses

to hypotonic stress. This separation of function is supported by our finding that activation of ERK and SAPK/JNK is requisite for eliciting the RVD response whereas p38 activation is dependent on RVD. The ERK inhibitors PD98059 and U0126, at concentrations that block Erk1/2 activation, inhibited RVD in a concentration-dependent manner (Fig. 6A). This inhibition appears not to be dependent on selective suppression of Erk1 activation since in a tetracycline-inducible d/n-Erk1 cell line the RVD response was unchanged from its nontransfected counterpart (Fig. 6B). The SAPK/JNK inhibitor SP600125 also had an inhibitory effect on the RVD response (Fig. 9) whereas SB203580 had no effect (Fig. 8A). This negative effect of SB203580 was replicated in a tetracycline-inducible d/n-p38 cell line (Fig. 8B). Conversely, pre-treatment with K^+ and Cl^- inhibitors suppressed RVD without inhibiting Erk1/2 and SAPK/JNK activation. Consistent with the dependence of p38 activation on RVD, K^+ and Cl^- inhibitors blocked p38 phosphorylation. These results suggest that activation of both ERK and SAPK/JNK pathways are requisite for RVD induction, whereas the converse relationship exists between RVD and p38 activation.

Activation of ERK plays a crucial role in the activation of volume-sensitive Cl^- channels. Several studies have shown that PD98059—but not SB203580—reversibly blocks hypotonic solution activation of the swelling-sensitive Cl^- current in astrocytes (Crepel et al., 1998), cardiac muscle (Du and Sorota, 2000) and cervical cancer cells (Shen et al., 2001). PD98059 also blocks swelling-sensitive K^+ efflux without affecting the KCC co-transporter (Shen et al., 2001). Therefore, swelling-activated ERK signaling mediates increases in volume-sensitive ion channel activity. With regard to the effects of the various MAPK inhibitors on RVD, our results are similar with those in which these inhibitors were delivered to directly inhibit activation of volume-sensitive K^+ and Cl^- currents. This agreement shows that RVD dependence on ERK activation is characteristic of a variety of different tissue types (Bolshakov et al., 2000; Wilk-Blaszczak et al., 1998). This notion is supported by the finding that K^+ and Cl^- channel inhibitors did not affect swelling-activated ERK.

The relationship between SAPK/JNK activation and RVD is variable in different cell types. In some cell types, SAPK/JNK activation precedes RVD; in other cases, however, RVD occurs prior to SAPK/JNK activation. In renal epithelial cells (Niisato et al., 1999), rat hepatocytes (Kim et al., 2000) and RCEC (as in the present study), SAPK/JNK activation peaks within 2.5–5 min of exposure to hypotonic challenge. On the other hand, in cardiac myocytes (Sadoshima et al., 1996), human Caco-2 cells (Hubert et al., 2004), and hepatocytes (Kurz et al., 1998), activation of SAPK/JNK—a rather slow-response kinase—occurs 30–60 min after the RVD response. This difference suggests that SAPK/JNK plays a variable role in inducing RVD in different cell types. In RCEC, SAPK/JNK along with ERK, contributes to RVD induction.

Compared to the rapid activation of ERK and SAPK/JNK, p38 MAPK swelling-induced activation is slower, maximizing at 30 min following the osmotic challenge. The time lag between fast RVD response and slow p38 activation suggests that p38 is not involved in ion channel stimulation. The failure of either (i) chemical inhibition of p38 or (ii) tetracycline-induced d/n-p38 expression to suppress hypotonicity-induced RVD excludes p38 as a mediator of volume-sensitive ion channel stimulation. A similar result was found in cervical cancer cells and intestinal epithelial cells (Shen et al., 2001; Tilly et al., 1996). The dependence of p38 on RVD is further supported by the finding that blockage of RVD—by exposure to either Cl^- -free solution, NPPB or NA, as well as to high- K^+ or 4-AP—inhibited p38 stimulation. Furthermore, there is a correspondence between efficacy of inhibition of RVD and p38 activation by either Cl^- -free solution, NPPB, or NA, as well as high- K^+ or 4-AP. Their rank order of inhibition was: Cl^- -free > NPPB > NA and high- K^+ > 4-AP. This relationship further supports the dependence of p38 activation on RVD.

In conclusion, in RCEC, volume-sensitive Cl^- and K^+ channels, which include a rather wide spectrum of different types of K^+ and Cl^- channels, contribute in concert to cellular volume regulation during exposure to a hypotonic. Each of the three pathways of the MAPK superfamily has a different association with RVD. ERK and SAPK/JNK mediate increases in Cl^- and K^+ efflux and, consequently, induce RVD. On the other hand, p38 stimulation occurs as a consequence of RVD activation.

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Fig. 1. Inhibition of RVD by Cl⁻ channel inhibitors. (A) RCEC were exposed to a 150 mOsm hypotonic challenge (underline) in the absence (control) and presence of Cl⁻-free solution, NPPB (100 μ M), or NA (100 μ M). Cell volume was monitored for 30 min at 37°C. Error bars are not shown when smaller than symbols. (B) Summary of volume recovery (%). Data are presented as mean $\pm$ SEM ($n=4$). * $p < 0.05$, ** $p < 0.001$ versus untreated control.

Fig. 2. Inhibition of RVD by K^+ channel inhibitors. (A) RVD was evaluated in the absence (control) and presence of 4-AP (1 mM) or high- K^+ solution (20 mM) in response to 150 mOsm hypotonic challenge (underline). The cell volume was monitored for 30 min at 37°C. (B) Summary of volume recovery. Data are presented as mean $\pm$ SEM ($n=4$). * $p < 0.05$, ** $p < 0.001$ versus untreated control.

Fig. 3.

Time-dependent activation of Erk1/2, p38 and SAPK/JNK induced by hypotonic challenge. RCEC were exposed to either isotonic (control) solution (300 mOsm) or 150 mOsm hypotonic challenge for the time periods indicated. Activation of Erk1/2 (A), p38 (B), and SAPK/JNK (C) were evaluated by probing the phosphorylated form of the MAPK pathway with phospho-specific Erk1/2 (p-Erk1/2), p38 (p-p38), and SAPK/JNK (p-SAPK/JNK) antibodies, respectively. Membranes were stripped and reprobed with their respective monoclonal anti-Erk1, anti-p38, and anti-SAPK/JNK antibodies as internal controls to test for equal protein loading. As internal controls, results were analyzed by densitometry and expressed as arbitrary units (a.u.). Data are presented as mean $\pm$ SEM ($n=3$).

Fig. 4.

Osmolarity-dependent activation of Erk1/2, p38 and SAPK/JNK by hypotonic challenges. RCEC were exposed to either an isotonic control solution (300 mOsm), 225, or 150 mOsm hypotonic challenge for 2.5 min for Erk1/2 and SAPK/JNK experiments, or 30 min for p38 detection. Activation of Erk1/2 (A), p38 (B), and SAPK/JNK (C) were evaluated by probing the phosphorylated form of MAPK with p-Erk1/2, p-p38, and p-SAPK/JNK antibodies, respectively.

Fig. 5.

Activation of Erk1/2 unaffected by Cl⁻ and K⁺ channel inhibition. Activation of Erk1/2 was probed either in isotonic (control) solution (300 mOsm), or in 150 mOsm hypotonic solution with or without Cl⁻-free solution, NPPB (100 μ M), or NA (100 μ M) in (A) and 4-AP (1 mM) or high-K⁺ (20 mM) solution in (B). Membranes were stripped and reprobed with anti-Erk1 as internal control. Results are summarized in the lower panels and expressed as a.u.. Data are presented as mean $\pm$ SEM ($n=3$).

Fig. 6. Inhibition of RVD by suppression of Erk1/2. (A) RVD was evaluated for 30 min in the absence (control) or presence of 20 μ M, 40 μ M PD98059 (PD), or 10 μ M U0126 (U0) in response to 150 mOsm hypotonic challenge (underline). The inset indicated PD98059 and U0126 inhibited Erk1/2. (B) RVD was evaluated for 30 min in the absence or presence of 24-h pretreatment with 1 μ g/ml tetracycline (tet) in RCEC and d/n-Erk1 cells. Results are summarized in the lower panels. * $p < 0.05$, ** $p < 0.001$ versus untreated control following hypotonic challenge. Data are presented as mean $\pm$ SEM ($n=4$).

Fig. 7.

Inhibition of p38 by Cl⁻ and K⁺ channels inhibition. Activation of p38 was probed either in isotonic (control) solution (300 mOsm), or in 150 mOsm hypotonic solution with or without (A) Cl⁻-free solution, NPPB (100 μ M), or NA (100 μ M) and (B) 4-AP (1 mM) or high-K⁺ (20 mM) solution. * $p < 0.05$, ** $p < 0.001$ versus untreated cells following hypotonic challenge. Membranes were stripped and reprobed with anti-p38 as loading control. Results are summarized in the lower panels and are expressed as a.u.. Data are presented mean $\pm$ SEM ($n=3$).

Fig. 8.

RVD insensitive to p38 inhibition. (A) RVD was evaluated for 30 min in the absence (control) or presence of 10 and 20 μ M SB203580 (SB) in response to 150 mOsm hypotonic challenge (underline). The inset indicated SB203580 that inhibited p38. (B) RVD was evaluated for 30 min in the absence or presence of 24-h pretreatment with 1 μ g/ml tetracycline (tet) in RCEC and d/n-p38 cells. Results are summarized in the lower panel. * $p < 0.05$, ** $p < 0.001$ versus untreated cells following hypotonic challenge. Data are presented as mean $\pm$ SEM ($n=4$).

Fig. 9. Inhibition of RVD by SAPK/JNK inhibition. RVD was evaluated for 30 min in the absence (control) or presence of 30 μ M SP600125 (SP) in response to 150 mOsm hypotonic challenge (underline) (A). The inset indicated SP600125 that inhibited SAPK/JNK. Results are summarized in (B). * $p < 0.05$, ** $p < 0.001$ versus untreated control following hypotonic challenge. Data are presented as mean $\pm$ SEM ($n=4$).

Fig. 10.

Activation of SAPK/JNK unaffected by Cl⁻ and K⁺ channel inhibition. Activation of SAPK/JNK was probed either in isotonic (control) solution (300 mOsm), or in 150 mOsm hypotonic solution with or without (A) Cl⁻-free solution, NPPB (100 μ M), or NA (100 μ M) and (B) 4-AP (1 mM) or high-K⁺ (20 mM) solution. Membranes were stripped and reprobed with anti-SAPK/JNK antibodies as control. Results are summarized in the lower panels and are expressed as a.u.. Data are presented as mean $\pm$ SEM ($n=3$).