Osmotic Shrinkage of Human Cervical Cancer Cells Induces an Extracellular Cl⁻-dependent Nonselective Cation Channel, Which Requires p38 MAPK*

Received for publication, August 6, 2002, and in revised form, September 10, 2002 Published, JBC Papers in Press, September 10, 2002 DOI 10.1074/jbc.M207993200

Meng-Ru Shen¶§¶¶, Cheng-Yang Chou§, Keng-Fu Hsu§, and J. Clive Ellory¶
From the ¶Department of Pharmacology, the ¶Department of Obstetrics & Gynecology, College of Medicine, National Cheng Kung University, Tainan 704, Taiwan and the ¶¶University Laboratory of Physiology, Parks Road, University of Oxford, OX1 3PT, United Kingdom

This study is to integrate a functional role of nonselective cation (NSC) channels into a model of volume regulation on osmotic shrinkage for human cervical cancer cells. Application of a hypertonic solution (400 mosm kg⁻¹) induced cell shrinkage, which was accompanied by a 7-fold increase of inward currents at −80 mV from −4.1 ± 0.4 pA pF⁻¹ to −29 ± 1.1 pA pF⁻¹ ($n = 36, p < 0.001$). There is a good correlation of channel activity and cell volume changes. Replacement of bath Na⁺ by K⁺, Cs⁺, Li⁺, or Rb⁺ did not affect the stimulated inward current significantly, but replacement by Ca²⁺, Ba²⁺, or the impermeable cation N-methyl-D-glucamine abolished the inward current; this demonstrates that the shrinkage-induced currents discriminate poorly between monovalent cations but are not carried by divalent cations. Replacement of extracellular Cl⁻ by gluconate abolished the shrinkage-induced currents in a concentration-dependent manner without changing the reversal potential. Gadolinium (Gd³⁺) inhibited the stimulated current, whereas bumetanide and amiloride had no inhibitory effect. Cell shrinkage triggered mitogen-activated protein (MAP) kinase cascades leading to the activation of MAP/extracellular signal-regulated kinase 1/2 (ERK1/2) kinase (MEK1/2), and p38 kinase. Interference with p38 MAPK by either the specific inhibitor (SB202190), or a dominant-negative mutant profoundly suppressed the activation of the shrinkage-induced NSC channels. In contrast, the regulatory mechanism of shrinkage-induced NSC channels was independent of the volume-responsive MEK1/2 signaling pathway. More importantly, the cell volume response to hypertonicity was inhibited significantly in p38 dominant-negative mutant or by SB202190. Therefore, p38 MAPK is critically involved in the activation of a shrinkage-induced NSC channel, which plays an important role in the volume regulation of human cervical cancer cells.

Homeostasis of cell volume is a fundamental cellular property. Even at constant extracellular osmolality, cell volume is frequently challenged by transport of osmotically active substances across the cell membrane and formation or disappearance of cellular osmolytes by metabolism (1). Thus maintenance of cell volume requires the constant operation of volume regulatory mechanisms, including ion transport across plasma membranes as well as accumulation or disposal of organic osmolytes and metabolites (2). In response to osmotic swelling, cells extrude ions and certain organic molecules to accomplish regulatory volume decrease (RVD).¹ In most cell types, the predominant pathway for RVD is the activation of separate volume-regulated K⁺ and Cl⁻ channels (2). On the other hand, osmotic shrinkage can activate uptake systems for ions or trigger the expression of transporters for organic osmolytes. Shrunken cells can thereby increase their volume toward their original level by net uptake of Na⁺, Cl⁻, and often K⁺ as well, and concomitant uptake of water (2, 3). This process is known as regulatory volume increase (RVI). The major ion transport systems accomplishing electrolyte accumulation in shrunken cells are the Na⁺-K⁺-2Cl⁻ cotransporter (NKCC) and the Na⁺/H⁺ exchanger (NHE). The latter alkalinizes the cell leading to parallel activation of the Cl⁻/HCO₃⁻ exchanger. The activation of ion channels in response to a hypertonic challenge has received scant attention, although the participation of Na⁺ and nonselective cation (NSC) channels in RVI has been proposed (4, 5). In addition, very little is known about the signal pathways underlying the activation of the shrinkage-induced ion channels.

Mitogen-activated protein (MAP) kinases are ubiquitous serine/threonine protein kinases that have been implicated in many cellular processes such as proliferation, differentiation, and apoptosis (6, 7). MAP kinases can be activated by a variety of external signals, including ultraviolet radiation, various growth factors, and cytokines. Osmotic stress also appears to be an important stimulus for the modulation of MAP kinase family. For example, p38 MAPK represents a human homologue of the Saccharomyces cerevisiae HOG-1 gene product, a yeast MAP kinase required for cellular osmoregulation (8, 9, 10). Pharmacological blockade of p38 MAPK activity prevents osmotic induction of multiple mRNAs (11, 12). In addition to the effects on p38, change in cell volume is an important stimulus for modulation of other members of the MAP kinase family, including extracellular signal-regulated kinase 1 and 2 (ERK1/ERK2) and c-Jun N-terminal kinase (JNK) (11). An

¹ The abbreviations used are: RVD, regulatory volume decrease; NKCC, Na⁺-K⁺-2Cl⁻ cotransporter; MAPK, mitogen-activated protein kinase; NMDG, N-methyl-D-glucamine; GFP, green fluorescent protein; NHE, Na⁺/H⁺ exchanger; MEK, MAP kinase; RVI, regulatory volume increase; NSC, nonselective cation; EIPA, ethylisopropylamiloride; ERK, extracellular-regulated kinase.

45776 This paper is available on line at http://www.jbc.org
imported question is whether the cascades of MAP kinases can regulate membrane ion transport because the main physiological function of these signal pathways is to translate extracellular signals to the nucleus.

The physiological role of the shrinkage-induced NSC channels remains elusive. There are two specific aims in the present study: (1) to investigate the effect of cell shrinkage on the whole-cell membrane currents of human cervical cancer cells and characterize the properties of the shrinkage-induced currents, and (2) to determine whether signal cascades of MAP kinases are provoked in shrunken cervical cancer cells and, if so, whether these signaling pathways are linked with cell volume regulation. The results demonstrate that shrinkage of human cervical cancer cells induces an extracellular Cl⁻-dependent NSC channel, which requires p38 MAPK and plays an important role in volume regulation.

MATERIALS AND METHODS

Cell Culture—The human cervical cancer cell line, SiHa cell, was obtained from the American Type Culture Collection (Manassas, VA). These cells, from passage 15 to 35, were maintained at 37°C in a CO₂/air (5:95%) atmosphere and cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), 80 IU/ml penicillin and 80 µg/ml streptomycin (Sigma). Cells were grown in 100-cm² culture flasks at 90% confluence, were starved in serum-free Dulbecco’s modified Eagles medium at 37°C for 40 min and then at 37°C for 1 h. Following washing with TBS-T for three times, the membrane was incubated with primary antibodies at 1:1000 dilution for 24 h at room temperature. At indicated time periods, cells were immediately harvested with ice-cold protein lysis solution containing a protease inhibitor mixture (Roche Diagnostics), 100 mM KCl, 80 mM NaF, 10 mM EDTA, 50 mM β-glycerophosphate, 10 mM p-nitrophenyl phosphate, 1 mM vanadate, 0.5% sodium deoxycholate, 1% Nonidet P-40. The lysates were centrifuged at 10,000 × g for 20 min at 4°C, and the supernatants were collected. Protein concentrations were determined with a Bio-Rad protein assay. Equal amounts of proteins were separated by 10% SDS-polyacrylamide gel electrophoresis, then transferred to polyvinylidene difluoride (PVDF; Stratagene, La Jolla, CA) membranes. Nonspecific binding was blocked with 5% (w/v) nonfat dried milk in TBS-T (20 mM Tris, pH 7.5, 137 mM NaCl, 0.2% Tween-20) for 1 hour at room temperature. The blots were incubated with primary antibodies at 1:1000 dilution of TBS-T overnight at 4°C, washed with TBS-T four times (10 min/wash), and then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase at 1:5000 dilution for 1 h at room temperature. Following washing with TBS-T for three times, the membrane was developed with enhanced chemiluminescence according to the manufacturer’s instructions (Amersham Biosciences). Phosphorylated levels of MAP kinases were analyzed by scanning densitometry, and the results were expressed as arbitrary units.

Electrophysiological Measurements—The whole-cell mode of the patch-clamp technique was used to measure membrane currents at room temperature (22–25°C) as previously described [14, 15]. When the pipettes were connected to the input stage of an Axopatch-200A amplifier (Axon Instruments, Union City, CA), their DC resistance varied between 2 and 5 MΩ. The current-voltage relationship and time course of the whole-cell membrane current were obtained from either a ramp or a step protocol. The ramp protocol consisted of linear voltage ramps changing from −100 mV to +100 mV in 400 ms, applied every 15 s. The step protocol consisted of a series of 400-ms-long voltage steps applied from a holding potential of −40 mV to voltages between −100 and +100 mV with an increment of 20 mV. Currents were sampled at 2 ms intervals. Current densities were determined by normalizing the whole-cell current to the membrane capacitance, which was 20 ± 1.7 pF (n = 95) in this study. The normalized shrinkage-induced current is defined as the difference in current density between isotonic and hypertonic solutions and is expressed per unit membrane capacitance.

To calculate ion permeability, liquid junction potentials were corrected as described previously [16]. Briefly, the reference electrode was an Ag/AgCl pellet bathed in the same solution as that used in the pipette, and connected to the bath via an agar/pipette-solution bridge in the outflow path of the chamber. Liquid junction potentials occurring at the bridge region were measured using a 3 M KCl flowing boundary electrode and were 4−2, 3, 0.4, −3, 4, or 5 mV when bath Na⁺ was replaced by NMDG⁺, K⁺, Li⁺, Ca²⁺, Ba²⁺, and Ba²⁺, respectively. For data analysis the measured membrane voltage values were corrected accordingly and in the whole-cell current recordings the pipette holding potential was corrected for liquid junction potentials as appropriate. The membrane permeability of various monovalent cations (I⁺) was calculated to that of Na⁺ (PNa/P蛙) was determined from the shift of the reversal potential (AVrev) in cation substitution experiments and calculated from the modified Goldman-Hodgkin-Katz Equation 1.

\[
P_{Na}/P_{蛙} = \exp(\Delta V_{rev} \times (F/RT))
\]

(Eq. 1) where F is the Faraday constant, R the gas constant, and T absolute temperature. Data from electrophysiological experiments were digitized and analyzed using pCLAMP software (Version 6.0.3, Axon Instruments, Union City, CA).

Transient Transfection with a Dominant Negative Mutant of raf-1 or p38 MAPK—To provide genetic evidence that the signal pathways of MEK1/2 and p38 MAPK were involved in the activation of shrinkage-induced currents, transient transfection was used. Using the calcium phosphate coprecipitation method (17), SiHa cells cultured in 6-well plates were cotransfected with green fluorescence protein (GFP, 100 ng/well, Clontech) together with pRSVC4Braf/1 (500 ng/well), or pCMV-FLAG-p38AGF (500 ng/well), or with an empty vector (500 ng/well). The pRSVC4Braf/1 encoding dominant-negative mutant of raf-1 (18) was a gift from Dr. H. S. Liu, Department of Immunology and Microbiology, National Cheng Kung University, Taiwan. The pCMV-FLAG-p38AGF (a gift from Dr. P. Stambrook, University of Cincinnati) is a kinase-deficient p38 mutant made by substituting threonine with alanine and tyrosine with phenylalanine in the C-terminal sequence of the p38 kinase. Using the ratio (GFP/cDNA of interest) > 1.5, more than 90% of the GFP-positive cells also expressed the vector of interest, as determined in separate experiments by immunostaining the epitope tag in dominant-negative raf-1 or p38 mutants. Electrophysiological recordings and cell volume measurements were done 48 h after transfection.

Western Immunoblotting—SiHa cells, grown in 100-mm dishes to 90% confluence, were starved in serum-free Dulbecco’s modified Eagle’s medium overnight before experiments. After serum starvation, the culture medium was replaced by isotonic or hypertonic solution at room temperature. At indicated time periods, cells were immediately harvested with ice-cold protein lysis solution containing a protease inhibitor mixture (Roche Diagnostics), 100 mM KCl, 80 mM NaF, 10 mM EDTA, 50 mM β-glycerophosphate, 10 mM p-nitrophenyl phosphate, 1 mM vanadate, 0.5% sodium deoxycholate, 1% Nonidet P-40. The lysates were centrifuged at 10,000 × g for 20 min at 4°C, and the supernatants were collected. Protein concentrations were determined with a Bio-Rad protein assay. Equal amounts of proteins were separated by 10% SDS-polyacrylamide gel electrophoresis, then transferred to polyvinylidene difluoride (PVDF; Stratagene, La Jolla, CA) membranes. Nonspecific binding was blocked with 5% (w/v) nonfat dried milk in TBS-T (20 mM Tris, pH 7.5, 137 mM NaCl, 0.2% Tween-20) for 1 h at room temperature. The blots were incubated with primary antibodies at 1:1000 dilution of TBS-T overnight at 4°C, washed with TBS-T four times (10 min/wash), and then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase at 1:5000 dilution for 1 h at room temperature. Following washing with TBS-T for three times, the membrane was developed with enhanced chemiluminescence according to the manufacturer’s instructions (Amersham Biosciences). Phosphorylated levels of MAP kinases were analyzed by scanning densitometry, and the results were expressed as arbitrary units.

Fluorescence Measurements of Intracellular Free Ca²⁺ Concentration ([Ca²⁺]i)—[Ca²⁺]i was measured with the fura-2 fluorescence ratio method on an a fluorimeter (F-2000, spectrophotometer, Hitachi, Tokyo, Japan) as previously described [15, 19]. In brief, cells attached on coverslips were loaded with 2 µM fura-2/acetoxymethyl ester (fura-2/AM) in Dulbecco’s modified Eagle’s medium culture medium at room temperature for 40 min and then at 37°C for 20 min. After loading, cells were washed three times with phosphate-buffered saline. After washing, the coverslip was mounted in a custom-made holder and placed in
a 5-ml quartz cuvette. Fluorescence emission was collected from a group of 102 cells located in the excitation path. Excitation wavelength was alternated between 340 nm (λ340) and 380 nm (λ380), and fluorescence intensity was monitored at 510 nm. [Ca2+] was calculated from the I340/I380 ratio using Equation 2 proposed by Grynkiewicz, Poenie, and Tsien (20).

\[
[Ca^{2+}] = K_d \times \frac{(F_{\text{max}}/F_{\text{rest}}) \times ([R-R_{\text{min}}])}{(R_{\text{max}} - R_{\text{min}})}
\]

(Eq. 2)

where \(K_d\) is the dissociation constant for fura-2 in the cytosol (250 nm), \(F_{\text{max}}\) and \(R_{\text{max}}\) are the 380 nm fluorescence intensity and I340/I380 ratio at low [Ca2+] respectively, \(F_{\text{rest}}\) and \(R_{\text{rest}}\) are the 380-nm fluorescence intensity and I340/I380 ratio at high [Ca2+], \(R\) is the I340/I380 ratio recorded during experiments. Calibration measurements of \(F_{\text{rest}}\) and \(R_{\text{rest}}\) were performed after incubating cells for 10 min in nominally Ca2+-free isotonic solution containing 3 mM EGTA. Cells were then superfused with isotonic solution containing 1 µM thapsigargin, 5 µM ionomycin, and 10 mM Ca2+ to evaluate \(F_{\text{max}}\) and \(R_{\text{max}}\).

**Measurements of Cell Volume**—Cell volume was measured as described previously (15, 19). Briefly, cells were harvested, transferred, and allowed to achieve cell attachment in Petri dish for ~30 min. A 2-ml bath, which was continuously superfused with isotonic solution or hypertonic solution, was used to monitor the change of cell size, the microscope was coupled to a video camera system and the images were recorded in real-time and stored on a video cassette recorder (Panasonic Inc., Tokyo, Japan). Images were then analyzed by the public domain NIH Image program. The majority of cells observed were spherical and the relative volume change (VV/V0) was calculated from the cross-sectional area at the beginning (S1) of experiment and during (S) the experiments from the relation: \(V/V_0 = (S/S_0)^{3/2}\) (15, 19). Data were presented as the percentage of starting volume (VV/V0) as a function of time. The validity of this approach to measure cell volume has been demonstrated in mouse thymocytes (21), renal A6 cells (22), lymphocytes (23), and human cervical cancer cells (19).

**Statistics**—All values in the present study were reported as mean ± S.E. Student’s paired or unpaired t test was used for statistical analyses. Differences between values were considered significantly when \(p < 0.05\).

**RESULTS**

**Effect of Hypertonicity on the Whole-cell Membrane Currents**—Whole-cell voltage-clamp recordings were made from cervical cancer SiHa cells with simultaneous measurement of membrane current and cell diameter. Membrane currents recorded during the ramp protocol applied to SiHa cells in isotonic solution were small (Fig. 1A). Application of a hypertonic solution (400 mos M NaCl) increased the inward currents, which was accompanied by an activation of mild outwardly rectifying currents (Fig. 1A). The time courses of channel activity and cell diameter indicate that there is a good correlation of membrane current and cell volume changes.

Membrane currents were also recorded during the step protocol applied to SiHa cells (Fig. 2A). The baseline membrane currents were small (−4.1 ± 0.4 pA pF−1 at −80 mV, \(n = 36\)) and time-independent (Fig. 2A). Replacement of Na+ in the bath solution by the impermeable cation NMDG only slightly decreased the inward current. Application of a hypertonic solution (400 mosm kg−1) induced a 7-fold increase of inward current from −4.1 ± 0.4 pA pF−1 to −29 ± 1.1 pA pF−1 measured at −80 mV (\(n = 36, p < 0.001\)). The hypertonicity-induced currents did not exhibit any major voltage-dependent activation or inactivation when voltage pulses of 400 ms duration were applied between −100 and +100 mV (Fig. 2A). The current-voltage relationship in hypertonic solution, obtained from the step protocols, reversed close to the theoretical equilibrium potential for cations (\(E_{\text{cation}} = 0\) mV) with the standard bath and pipette solutions. This shrinkage-induced current is reversible after changing back to the isotonic bath solution.

The cation selectivity of the shrinkage-induced current was investigated further. Following the plateau phase of current stimulus in the hypertonic solution, extracellular Na+ was replaced by the monovalent cation Li+, K+, Cs+, Rb+, or NMDG. The replacement of extracellular Na+ with the impermeable cation NMDG shifted the reversal potential from −0.5 ± 1.6 mV to −50 ± 3.1 mV (\(n = 6\)) and abolished the shrinkage-induced inward current (Fig. 2, A and B), whereas replacement by Li+, K+, Cs+, or Rb+ only slightly changed the inward currents. The sequence of cation permeability, calculated from the shifts in reversal potential, was Na+ : Li+ : K+ : Cs+ : Rb+ : NMDG = 1 : 0.91 ± 0.08 (\(n = 3\)) : 1.07 ± 0.07 (\(n = 5\)) : 0.88 ± 0.08 (\(n = 3\)) : 1.08 ± 0.09 (\(n = 3\)) : 0.13 ± 0.05 (\(n = 6\)) (Fig. 2C). This indicates that the shrinkage-induced currents discriminate poorly among these monovalent cations.

To investigate whether the shrinkage-induced currents will carry divalent cations, we performed experiments in which extracellular 140 mos M NaCl was replaced with 70 mos M CaCl2 at the plateau phase of current stimulation in the hypertonic condition. Preliminary experiments (\(n = 5\)) indicated that with a standard pipette solution containing 1 mM EGTA a change of the bath solution to 70 mos M CaCl2 could raise the cytosol Ca2+ sufficiently to activate Ca2+-activated Cl− channels, which have been demonstrated in cervical cancer SiHa cells (13). To prevent the interference from these Ca2+-activated Cl− channels, we used pipette solutions containing 10 mM BAPTA. A typical experiment with 10 mM BAPTA in the pipette solution is shown in Fig. 3, A and B. During hypertonic stimulation, replacement of extracellular Na+ with Ca2+ decreased significantly the inward currents and was accompanied with a change of the reversal potential from −0.3 ± 0.6 mV to −60.8 ± 2.5 mV (\(n = 6\)). Similar effects were observed in the replacement of Na+ with Ba2+ (\(n = 3\)). These results suggest that shrinkage-induced NSC channels are poorly permeable to divalent cations.

[Ca2+] was subsequently measured to ascertain no Ca2+ entry during cell shrinkage. Superfusion of SiHa cells with the hypertonic solution elicited a rise of [Ca2+] from the basal level of 105 ± 6 na to a peak of 145 ± 5 nm (\(p < 0.05\), Student’s paired t test, \(n = 12\) experiments). In the absence of extracellular Ca2+, the basal [Ca2+] was 50 ± 5 nm and hypertonicity induced an increase of [Ca2+] to 88 ± 5 nm (Fig. 3C, \(p < 0.05\), Student’s paired t test, \(n = 12\) experiments). The pattern of

**FIG. 1.** Hypertonicity activates membrane currents, which correlate well with the cell size change. Whole-cell voltage-clamp recordings were made from cervical cancer SiHa cells with simultaneous measurement of membrane current and cell diameter. A, representative whole-cell current traces obtained from ramp protocols recorded in isotonic (300 mos M NaCl, trace a) solution and hypertonic (400 mos M NaCl, trace b). B, the time course of membrane current at −80 mV and the corresponding cell diameter. Each point represents mean ± S.E. (\(n = 12\)).
moval of extracellular Cl\textsuperscript{−} by replacement with the impermeable anion gluconate decreased significantly the amplitude of shrinkage-induced currents without changing the reversal potential (Fig. 5). For example, when [Cl\textsuperscript{−}], was decreased from 145 to 70 or 35 mM, the shrinkage-induced current measured at −80 mV was reduced by 35 ± 3% (n = 6, p < 0.05) and 70 ± 3% (n = 6, p < 0.01), respectively (Fig. 5B). In contrast, replacement of extracellular Cl\textsuperscript{−} with equimolar amounts of Br\textsuperscript{−} or I\textsuperscript{−} did not produce either a shift of reversal potential or a decrease in the amplitude of the shrinkage-induced currents (n = 4).

Furthermore, lowering the intracellular Cl\textsuperscript{−} concentration from 145 to 70 mM by changing the pipette solutions (i.e., 140 mM KCl and 5 mM NaCl were changed to 65 mM KCl, 75 mM K-aspartate and 5 mM NaCl) failed to affect the reversal potential or the amplitude of shrinkage-induced currents (n = 4). These results indicate the possible presence of an external anion binding site, which may act as a modulator for the shrinkage-induced cation conductance.

The Effects of Inhibitors on Shrinkage-induced Currents—Gd\textsuperscript{3+} is a known inhibitor of NSC channel (4, 5). As shown in Fig. 6, Gd\textsuperscript{3+} inhibited the shrinkage-induced NSC channel in a dose-dependent manner. However, by contrast, activation of the shrinkage-induced current was not sensitive to 10 and 50 μM bumetanide (n = 5), an inhibitor of the basolateral membrane Na\textsuperscript{+}−K\textsuperscript{+}−2Cl\textsuperscript{−} cotransporter (Fig. 6C).

The amiloride-sensitive epithelial Na\textsuperscript{+} channel has been described as sensitive to osmotic change and is responsible for the shrinkage-induced NSC channels in some cell types (24, 25). However, amiloride at concentrations from 1 to 100 μM showed no effect on the activation of shrinkage-induced currents SiHa cells (Fig. 6C).

Osmotic Shrinkage Induces the Activation of MAPK—We investigated the roles of two major MAP kinases in cell volume regulation: 1) MEK1/2 and 2) the p38 MAPK (6, 7). As shown in Fig. 8A, the phosphorylation status of p38 MAPK in SiHa cells was constant in the isotonic solution. Osmotic shrinkage of SiHa cells led to a significant increase in the phosphorylation of p38 MAPK. Compared with the level in isotonic solution, the phosphorylated form of p38 MAPK was increased by 60 ± 17% (n = 3, p < 0.05) and 120 ± 12% (n = 3, p < 0.01) on exposure of hypertonicity for 3 and 10 min, respectively. SB202190 (1 μM), a specific and membrane-permeable inhibitor of p38 MAPK, could abolish the hypertonicity-induced phosphorylation of p38 MAPK. Furthermore, SB202190 inhibited the activation of shrinkage-induced nonselective cation channels with an IC\textsubscript{50} of 0.5 μM (Fig. 7, B–D). The inhibitory effect of high concentrations of SB202190 was only poorly reversible (Fig. 7C).
(pRSV or pCMV) did not change the activation of shrinkage-induced currents ($n=5$). Therefore, interference with p38 MAP kinase activity by either a specific inhibitor or a dominant-negative mutant profoundly reduced the activity of the shrinkage-induced current, indicating that p38 MAP kinase is critically involved in the activation of shrinkage-induced NSC channels. On the other hand, the volume-sensitive MEK1/2 does not serve as a regulator in the activation of shrinkage-induced currents.

**RVI in SiHa Cells**—Finally we studied the volume changes of SiHa cells in response to hypertonicity. As shown in Fig. 10A, increasing osmolality from 300 to 400 mosM kg$^{-1}$ by the addition of mannitol decreased cell volumes to 85% within 3 min ($n=30$; $p<0.01$). Thereafter, cell volumes gradually increased to 96% of the control value within 15 min, equivalent to a RVI of 73% (Fig. 10C). In contrast to the typical response process, volume recovery was significantly inhibited in p38 kinase-deficient cells, in which the RVI was significantly reduced to 36% ($n=30$, $p<0.05$, Fig. 10, A and C). More importantly, the volume responsive curve of p38 kinase-deficient cells was similar to that of SiHa cells treated with 1 μM SB202190 to abolish the activity of p38 MAP kinase (Fig. 10B). The inhibitory effect on RVI was also no significant difference between cells transfected with p38 kinase-deficient...
tissues, the RVI was significantly reduced to 15 ± 3% (n = 30, p < 0.01, Fig. 10C) indicative of a sizeable contribution of the NKCC to this process. Most importantly, in the presence of 50 μM Gd³⁺, 100 μM EIPA, and 20 μM bumetanide, i.e. with cation conductance, NHE and NKCC blocked, cell shrinkage was most pronounced yielding a negative RVI value of −10 ± 3% within 15 min (n = 30, Fig. 10C). A negative RVI value means that the initial period of shrinkage is followed by a slow further decrease in cell volume. These results indicate that the activities of cation conductance, NHE and NKCC are involved in the regulatory mechanisms of RVI of human cervical cancer cells.

**DISCUSSION**

We have characterized in human cervical cancer cells a NSC channel that is activated during osmotic shrinkage, has equal permeability for monovalent cations, depends on [Cl⁻]o, and is inhibited by Gd³⁺ and a specific p38 MAPK inhibitor. Substitution of extracellular Na⁺ by NMDG abolishes the shrinkage-induced inward currents and causes a negative shift in reversal potential, as anticipated from the conductance of NSC channel. NSC channel, which has been identified in a number of epithelial tissues, can be activated by different stimuli, including anisotropic stress (26), flow-induced shear stress (27), pressure-induced membrane tension (28), and magnetic stimulation (29). However, the pharmacological and electrophysiological proper-
FIG. 7. Hypertonicity activates the p38 MAPK, which can modulate the activation of shrinkage-induced currents. A, hypertonicity increases the phosphorylation of p38 MAPK, which can be abolished by 1 μM SB202190, a specific and membrane permeable inhibitor for p38 MAPK. SiHa cells were exposed to either isotonic or hypertonic solution (ISO, 300 mosM kg⁻¹; HYPER, 400 mosM kg⁻¹) for 3 or 10 min or hypertonic solution containing 1 μM SB202190 for 3 min. Whole-cell extracts were separated by SDS-PAGE (50 μg/lane), transferred to polyvinylidene difluoride, and immunoblotted with anti-phospho-p38 MAPK (Thr-180/Tyr-182) or anti-p38 MAPK. Phosphorylated and total p38 levels were analyzed by scanning densitometry, and the results were expressed as arbitrary units. Each column represents mean ± S.E. (n = 3 independent experiments). p-p38, phosphorylated p38 MAPK; *, p < 0.05; **, p < 0.01 compared with phosphorylated p38 levels in isotonic solution for 3 min.

B, representative recordings of shrinkage-induced currents from ramp protocol. Trace a, isotonic membrane current; trace b, hypertonic membrane current; trace c, currents recorded after perfusion with hypertonic solution containing 1 μM SB202190.

C, time course of membrane currents measured at membrane potential of −80 mV. Data points were obtained from the voltage ramp protocol that was applied every 15 s. The filled points correspond to the current traces recorded in B. Thick horizontal bar, application of hypertonic solution or 1 μM SB202190.

D, dose-response curves for the inhibition of shrinkage-induced currents by SB202190, measured at −80 mV. Each point represents mean ± S.E. The numbers of cells examined are indicated in parentheses beside each point.

FIG. 8. Hypertonicity activates the MAPK/extracellular signal-regulated kinase 1/2 (ERK1/2) kinase (MEK1/2), which is not critically involved in the activation of shrinkage-induced currents. A, hypertonicity increases the phosphorylation of MEK1/2, which can be inhibited by PD98059, a specific and membrane permeable inhibitor for MEK1/2. SiHa cells were exposed to either isotonic or hypertonic solution (ISO, 300 mosM kg⁻¹; HYPER, 400 mosM kg⁻¹) or hypertonic solution containing PD98059 for 3 or 10 min. Whole-cell extracts were separated by SDS-PAGE (50 μg/lane), transferred to polyvinylidene difluoride, and immunoblotted with anti-phospho-MEK (Ser-(217/221)) or anti-MEK. Phosphorylated and total MEK1/2 levels were analyzed by scanning densitometry, and the results were expressed as arbitrary units. Each column represents mean ± S.E. (n = 3 independent experiments). p-MEK1/2: phosphorylated MEK1/2. *, p < 0.05; **, p < 0.01; #, p < 0.001 compared with phosphorylated MEK1/2 levels in isotonic solution for 3 min. B, representative time course of membrane currents measured at membrane potential of −80 mV. Data points were obtained from the voltage ramp protocol that was applied every 15 s. Thick horizontal bar, application of hypertonic solution or 50 μM PD98059.

C, no significant inhibitory effects of PD98059 (1, 10, and 50 μM) on shrinkage-induced currents measured at −80 mV. Each column represents mean ± S.E. (n = 8).
ties vary and this variability may reflect different types of NSC channels (30). For example, the epithelial Na$^+$ channel (ENaC) has been suggested as the shrinkage-induced NSC channel in rat hepatocytes, which is inhibited by amiloride and highly selective for Na$^+$ and Li$^+$ over K$^+$ (25). But the shrinkage-induced NSC channel reported here is not inhibited by amiloride at concentrations up to 100 μM, suggesting that ENaC is not involved in the shrinkage-induced NSC channel of human cervical cancer cells. The NSC channels previously described were also characterized by a variable Ca$^{2+}$ dependence. In our experimental conditions, the [Ca$^{2+}$]i was buffered at 100 nM or near 0 nM, and the extracellular bath contained 0 or 1.5 mM Ca$^{2+}$. The amplitude of current stimulation induced by hypertonicity was not significantly different in these experimental conditions, indicating that the NSC channel observed in the present study was not Ca$^{2+}$ dependent. This result agrees well with the generally accepted view that Ca$^{2+}$ signaling is not critically involved in the control of RVI (31). In contrast, opening of NSC channels requires the presence of 1 μM Ca$^{2+}$ on the cytoplasmic face of rat pancreatic duct cells (32). The activation of NSC channels in endothelial cells dispersed from human umbilical vein also depends on the Ca$^{2+}$ entry (33). The molecular identity of the shrinkage-induced NSC channel has not been defined. With respect to the hypertonic stimulation, cation permeability, blockade by Gd$^{3+}$, and insensitivity to amiloride, this cation conductance of human cervical cancer cells is reminiscent of NSC channel found in human nasal epithelial cells (4), M-1 mouse cortical collecting duct cells (5, 16) and Caco-2 cells (34). The physiological role of this NSC channel has remained elusive. Here we demonstrate that abolishing the shrinkage-induced NSC channel activity by Gd$^{3+}$, or p38 kinase inhibitor SB202190 or in p38 kinase-deficient mutant significantly inhibited RVI, suggesting that this channel can play an important role in cell volume regulation. Na$^+$ entry via this NSC channel must account for a significant fraction of the RVI, although the low selectivity and the monovalent cation electrochemical gradients suggest that K$^+$ loss via the NSC channel may attenuate the net effect.

Interestingly, the shrinkage-induced NSC channel in human cervical cancer cells has an unusual dependence on [Cl$^{-}$]: partial replacement of bath Cl$^{-}$ with the impermeable anion gluconate reduced the magnitude of the shrinkage-induced current without accompanying changes in the reversal potential. Lowering the intracellular Cl$^{-}$ concentration by changing the pipette solutions failed to affect the reversal potential or the amplitude of shrinkage-induced currents. It is likely that there is an external anion binding site, which may act as a modulator for the shrinkage-induced cation conductance. The shrinkage-induced NSC channel in human nasal epithelial cells (4) also showed the same characteristic of [Cl$^{-}$]i dependence.

MAP kinases are usually thought to mediate the effects of growth factors and hormones on long-lasting cellular effects such as proliferation and differentiation (6, 7). This study and our previous study (15) address the role that MAP kinase can play as an important modulator of membrane proteins and is involved in the fast cellular adaptation to extracellular osmotic stress. The present study shows that cell shrinkage triggers
MAP kinase cascades leading to the activation of p38 MAPK and MEK1/2. Furthermore, the hypertonicity-induced p38 kinase activity links with the activation of shrinkage-induced NSC channel. SB202190, the specific membrane-permeable inhibitor of p38 MAPK, blocked the activation of shrinkage-induced NSC channel with a low IC50 value of 0.5 μM. There is a good correlation between the inhibitory effects of SB202190 on the p38 kinase activity, the activation of shrinkage-induced NSC channel, and RVI. For example, 1 μM SB202190 abolished the hypertonicity-induced phosphorylation of p38 MAPK, completely blocked the shrinkage-induced currents and inhibited significantly the RVI. In complementary transfection experiments, more than 90% of shrinkage-induced NSC channel activity is suppressed in the p38 kinase-deficient mutants. MEK1/2, another important member of the MAP kinase family, is also activated by the osmotic shrinkage of cervical cancer cells. Both pharmacological and genetic evidence however indicates that the signal pathways of MEK1/2 are not involved in the activation of shrinkage-induced NSC channel. Similar observations on volume regulation have been described in cells from rat medullary thick ascending limb of Henle’s loop: extracellular hypertonicity triggers the activation of both MEK1/2 and p38 kinase, but the RVI process only depends on p38 kinase activation (35). However, the profile is different with hypotonic swelling. Our previous study demonstrated that osmotic swelling of cervical cancer cells induced MAP kinase cascades leading to the activation of MEK1/2 and p38 kinase (15). The hypertonicity-induced MEK1/2 signaling pathway strongly linked with the volume-regulatory ion transport mechanisms of RVD. On the other hand, the mechanism for RVD was independent of the activation of p38 MAP kinase. Thus, p38 MAPK and MEK1/2 seem to have complementary functions in the volume regulation of human cervical cancer cells.

In addition to involvement in volume regulation, what other possible functional significance is there for the activation of MAP kinase cascades? In MDCK cells, hypertonicity activates p38 kinase activity, which is essential for osmotic induction of mRNAs for heat shock proteins and the transporter for the organic solute betaine (36). Hypertonicity could augment degranulation of human neutrophils by enhancing p38 signaling (37). In COS-7 cells, p38 MAPK is critically involved in the hypertonicity-induced activation of the signal transducer and activator of transcription (STAT) which is a key system in the signal transduction pathways for numerous interleukins and interferons (11). MAP kinase could also mediate the hypertonicity-stimulated cyclooxygenase-2 expression in renal medullary collecting duct cells (38). All these studies suggest a possible role for MAP kinase in transcription regulation, maintaining cellular homeostasis and/or long-term survival. However, for human cervical cancer cells, a definite long-term physiological function for hypertonicity-provoked MAP kinase activation remains to be established.

The present study shows that p38 MAPK has a novel function to regulate the activation of shrinkage-induced NSC channel and the process of RVI in human cervical cancer cells. The present data will not discriminate between a direct effect of p38 MAPK to phosphorylate the channel or acting via phosphorylation of downstream substrates in the shrinkage of cervical cancer cells. Until the molecular identities of shrinkage-induced NSC channels are established, we are limited to indirect methods to show this unusual function of MAP kinases in human cervical cancer cells. Nevertheless, the use of specific inhibitors and dominant-negative mutants allows us to make some significant progress in demonstrating the novel role of MAP kinases in cell volume regulation.

Acknowledgments—We thank Dr. H. S. Liu at National Cheng Kung University Taiwan and Dr. P. Stambrook at University of Cincinnati for kindly providing plasmids for dominant-negative mutants of Raf-1 and p38 kinase, respectively. We also thank Saprina P. H. Wang at the National Cheng Kung University, Taiwan for skillful technical assistance and help with the cell cultures.

REFERENCES
1. Lang, F., Busch, G. L., Ritter, M., Voldl, H., Waldegger, S., Gulbins, E., and Hausninger, D. (1998) Physiol. Rev. 78, 247–306
2. Hoffmann, E. K., and Dunham, P. B. (1995) Int. Rev. Cyt. 161, 173–262
3. Okada, Y. (1997) Am. J. Physiol. 273, C755–C759
4. Chan, H. C., and Nelson, D. J. (1992) Science 257, 669–671
5. Valler, F. T., Fremont, E., and Korbmacher, C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8478–8482
6. Seger, R., and Krebs, E. G. (1995) FASEB J. 9, 726–735
7. Cobb, M. H. (1999) Prog. Biophys. Mol. Biol. 71, 479–500
8. Brewster, J. L., de Valoir, T., Dwyer, N. D., Winter, E., and Gustin, M. C. (1993) Science 259, 1769–1763
9. Han, J., Richter, B., Li, Z., Kravechenko, V., and Ulevitch, R. J. (1995) Biochem. Biophys. Acta 1265, 224–227
10. Paul, A., Wilson, S., Belham, C. M., Robinson, C. J., Scott, P. H., Gould, G. W., and Plevin, R. (1997) Cell Signal. 9, 403–410
11. Bode, J. G., Gatsios, P., Ludwig, S., Rapp, U. R., Hausninger, D., Heinrich, P. C., and Greve, L. (1999) J. Biol. Chem. 274, 30222–30227
12. Nadkarni, V., Gabbay, K. H., Bohren, K. M., and Sheikh-Hamad, D. (1999) J. Biol. Chem. 274, 20185–20190
13. Shen, M. R., Furka, P., Chen, C. Y., and Ellory, J. C. (2002) Pflügers Arch. 444, 276–285
14. Shen, M. R., Droogmans, G., Gergermond, J., Voets, T., Ellory, J. C., and Nilius, B. (2000) J. Physiol. (Lond.) 529, 385–394
15. Shen, M. R., Chou, C. Y., Browning, J. A., Wilkins, R. J., and Ellory, J. C. (2001) J. Physiol. (Lond.) 537, 347–362
16. Koch, J. P., and Korbmacher, C. (2000) J. Membr. Biol. 177, 231–242
17. Chen, C., and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752
18. Chang, M. Y., Won, S. J., Yang, B. C., Jan, M. S., and Liu, H. S. (1999) Exp. Cell Res. 248, 589–598
19. Shen, M. R., Yang, T. P., and Tang, M. J. (2002) J. Biol. Chem. 277, 15592–15599
20. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
21. Ross, P. E., and Cahalan, M. D. (1995) J. Gen. Physiol. 106, 415–444
22. Urbach, V., Leguen, I., O’Kelli, I., and Harvey, B. J. (1999) J. Membr. Biol. 168, 29–37
23. Legge-Wilshaus, A., Schae, I., Laun, T., Kaha, N. K., Gulbins, E., and Lang, F. (1998) J. Cell Biol. 141, 281–286
24. Ji, H. L., Fuller, C. M., and Benos, D. J. (1998) Am. J. Physiol. 275, C1182-C1190
25. Bohnen, C., and Wehner, F. (2001) FEBS Lett. 495, 125–128
26. Kawahara, K., Ogawa, A., and Suzuki, M. (1991) J. Membr. Biol. 120, 1037–1045
27. Junger, H., Loomis, W., and Altman, A. (1998) J. Cell Biol. 141, 286–297
28. Fisher, A., Bohnen, C., Barakat, A. I., and Nemer, R. M. (2001) Am. J. Physiol. 281, L529–L535
29. Reifarth, F. W., Claus, W., and Weber, W. M. (1999) Biochem. Biophys. Acta 1417, 63–76
30. Niggl, J., Sigurdson, W., and Sachs, F. (2000) J. Membr. Biol. 174, 121–134
31. Nielsen, B., and Droogmans, G. (2001) Physiol. Rev. 81, 1415–1459
32. McCarty, N. A., and O’Neill, R. G. (1992) Physiol. Rev. 72, 1057–1061
33. Gray, M. A., and Argent, E. B. (1990) Biochem. Biophys. Acta 1028, 33–42
34. Muraki, K., and Imaiizumi, Y. (2001) J. Physiol. (Lond.) 537, 451–444
35. Nelson, D. J., Tien, Y. X., Xie, W., Braitsus, T. A., Kaetzel, M. A., and Dedman, J. R. (1996) Am. J. Physiol. 270, C179-C191
36. Sheikh-Hamad, D., Dziersak, C., Suki, W. N., Safarstein, R., Watts, B. A., 3rd, and Dziersak, D. (1998) J. Cell Biol. 141, 1833–1837
37. Jung, W. G., Hoyt, D. B., Davis, R. E., Herdon-Renemelius, C., Namiki, S., Junger, H., Loomis, W., and Altman, A. (1998) J. Clin. Invest. 101, 2768–2779
38. Yang, T., Huang, Y., Heasley, L. E., Berl, T., Schermermann, J., and Briggs, J. P. (2000) J. Biol. Chem. 275, 23281–23286
Osmotic Shrinkage of Human Cervical Cancer Cells Induces an Extracellular Cl⁻-dependent Nonselective Cation Channel, Which Requires p38 MAPK
Meng-Ru Shen, Cheng-Yang Chou, Keng-Fu Hsu and J. Clive Ellory

J. Biol. Chem. 2002, 277:45776-45784.
doi: 10.1074/jbc.M207993200 originally published online September 10, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M207993200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 13 of which can be accessed free at http://www.jbc.org/content/277/48/45776.full.html#ref-list-1