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Osmotic Stimulation of the Na^+/H^+ Exchanger NHE1: Relationship to the Activation of Three MAPK Pathways

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Abstract. The Na^+/H^+ exchanger (NHE) becomes activated by hyperosmolar stress, thereby contributing to cell volume regulation. The signaling pathway(s) responsible for the shrinkage-induced activation of NHE, however, remain unknown. A family of mitogen-activated protein kinases (MAPK), encompassing p42/p44 Erk, p38 MAPK and SAPK, has been implicated in a variety of cellular responses to changes in osmolarity. We therefore investigated whether these kinases similarly signal the hyperosmotic activation of NHE. The time course and osmolyte concentration dependence of hypertonic activation of NHE and of the three sub-families of MAPK were compared in U937 cells. The temporal course and dependence on osmolarity of Erk and p38 MAPK activation were found to be similar to that of NHE stimulation. However, while pretreatment of U937 cells with the kinase inhibitors PD98059 and SB203580 abrogated the osmotic activation of Erk and p38 MAPK, respectively, it did not prevent the associated stimulation of NHE. Thus, Erk1/2 and/or p38 MAPK are unlikely to mediate the osmotic regulation of NHE. The kinetics of NHE activation by hyperosmolarity appeared to precede SAPK activation. In addition, hyperosmotic activation of NHE persisted in mouse embryonic fibroblasts lacking SEK1/MKK4, an upstream activator of SAPK. Moreover, shrinkage-induced activation of NHE still occurred in COS-7 cells that were transiently transfected with a dominant-negative form of SEK1/MKK4 (SEK1/MKK4-A/L) that is expected to inhibit other isoforms of SEK as well. Together, these results demonstrate that the stimulation of NHE and the activation of Erk, p38 MAPK and SAPK are parallel but independent events.

Key words: p42/p44 Erk — p38 MAPK/RK — SAPK/JNK — NHE — SEK1/MKK4, U937

Introduction

When exposed to a hyperosmotic medium, mammalian cells undergo a rapid shrinkage followed by a gradual volume recovery, termed regulatory volume increase (RVI). RVI is attributable to net uptake of Na^+, Cl^- and osmotically obliged water. In many cell types this is brought about by the coupled activities of the Na^+/H^+ exchanger (NHE) and the cation independent Cl^-/HCO_3^- exchanger (Grinstein & Furuya, 1988; McManus, Churchwell & Strange, 1995). The cellular trigger responsible for NHE activation by hypertonic stress appears to be the associated change in cell size, rather than the increased osmolarity or ionic strength (Krump, Nikitas & Grinstein, 1997a). To date, however, the intracellular signal that couples volume reduction with NHE activation is still unknown and is the focus of the present study.

Multiple isoforms of NHE have been cloned (Noel & Pouyssegur, 1995; Wakabayashi, Shigekawa & Pouyssegur, 1997), including the ubiquitous amiloride-sensitive isoform, NHE1, which is believed to be the primary effector of regulatory volume increase. The osmotic stimulation of NHE1 requires the presence of in-

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tracellular ATP (Grinstein et al., 1992), suggesting that phosphorylation reactions are involved in the response to hyperosmolarity. However, overall NHE1 phosphorylation levels are unaffected by hyperosmolarity (Grinstein et al., 1992). Moreover, a deletion mutant of NHE1 that lacks the putative phosphorylation sites on its C-terminal (cytosolic) domain is still responsive to hyperosmolarity (Bianchini et al., 1995). This apparent contradiction can be reconciled by assuming that proteins associated with, but distinct from, NHE1 need to be phosphorylated upon cell shrinkage for effective osmotic stimulation of the exchanger.

The possibility that protein phosphorylation mediates the hyperosmotic stimulation of NHE is lent credence by the discovery of a superfamily of kinases that are exquisitely responsive to changes in medium tonicity. This heterogeneous family, known collectively as the mitogen-activated protein kinases (MAPK), includes isoforms of Erk, p38 MAPK and SAPK. While p38 MAPK and SAPK are strongly activated by hyperosmolarity, Erk has been shown to be somewhat less sensitive (Bianchini, L'Allemain & Pouyssegur, 1997; Galcheva-Gargova et al., 1994; Han et al., 1994). Interestingly, p38 MAPK and SAPK also respond to a variety of other cellular stresses, including changes in intracellular pH (pH_i) (Shrode et al., 1997), heat shock (Ludt, Sandvig & Olsnes, 1993), cytokines (Rozanski & Witt, 1994; Lee et al., 1992) and to a lesser extent hormones and growth factors (Kyriakis & Avruch, 1996). Remarkably, all of these stimuli have also been shown to cause NHE activation in a variety of cell lines (Grinstein, Rotin & Mason, 1989; Lee et al., 1992; Ludt et al., 1993).

The stimulation of MAPKs by hyperosmolarity and other agents, together with the ability of the same agents to activate NHE, provides circumstantial evidence that one or more members of the MAPK superfamily might participate in the activation of the exchanger. We evaluated the possible role of Erk, p38 MAPK and SAPK in the stimulation of NHE1 by: a) comparing the activation kinetics and osmotic dependence of each of these kinase pathways with the associated increase in Na^+/H^+ exchange; b) using pharmacological inhibitors of Erk and p38 MAPK; c) utilizing a mouse embryo fibroblast cell line generated from SEK1/MKK4 knockout mice, and d) transiently transfecting a dominant-negative form of SEK1/MKK4 into COS-7 cells. We conclude that the stimulation of NHE and the activation of Erk, p38 MAPK and SAPK are parallel yet independent events.

Materials and Methods

Chemicals

The acetoxymethyl ester form of 2′,7′-*bis*(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) and nigericin were purchased from Molecular Probes (Eugene, OR). Radiolabeled ATP was purchased from Mandel/Dupont (Guelph, Ontario, Canada). Fetal bovine serum (FBS) was purchased from Gibco BRL (Gaithersburg, MD). Smith Kline Beecham (King of Prussia, PA) generously provided SB203580. PD98059 was a gift from Parke-Davis (Ann Arbor, MI). The NHE1-specific inhibitor HOE-694 was a gift from Dr. Wolfgang Scholz from Hoechst (Frankfurt, Germany). All other chemicals were purchased from Sigma Chemical (St. Louis, MO). Rabbit antibodies against the nonphosphorylated and phosphorylated forms of Erk, p38 MAPK and SAPK were purchased from New England Biolabs. Anti-MAPKAPK-2 rabbit antibody was the generous gift of Dr. Steven L. Pelech (Kinetech Pharmaceuticals, Vancouver, B.C., Canada). Secondary goat anti-rabbit antibody conjugated to horseradish peroxidase was either from New England Biolabs (Mississauga, Ontario, Canada) or from Amersham Life Sciences (Little Chalfont, England, U.K.) and was used with the Amersham enhanced chemiluminescence (ECL) detection system.

Solutions

Na-HEPES buffered solution (NHB) contained (in mM): 132 NaCl, 5 KCl, 1 $MgSO_4$, 1 $CaCl_2$, 25 Na-HEPES, and 5.5 glucose, pH 7.4, at 37°C. Na-free HEPES-buffered solution (Na-free HB) was made by iso-osmotically replacing Na^+ with $NMDG^+$. Iso-osmotic solutions were adjusted to 290 ± 5 mOsm by addition of either water or the major salt. For hyperosmolar stimulation, appropriate amounts of a 5 M solution of either NaCl or KCl were added to NHB or Na-free HB, respectively. Gentle soft lysis buffer contained (in mM) 10 NaCl, 20 PIPES (pH = 7.0), 5 EDTA, 50 NaF, 0.5% NP-40 and 0.05% β-mercaptoethanol. All buffers used for cell incubations were nominally bicarbonate-free.

Tissue Culture and Mouse Embryonic Fibroblast Preparation

U937 cells, a line exhibiting monocyte-like characteristics, and COS-7 cells, a line from monkey kidney, were obtained from the American Type Culture Collection (ATCC). These lines, as well as mouse embryonic fibroblasts, were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, NY), with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified environment with 5% CO_2. U937 cells were grown in suspension and experiments were conducted when cell counts were between 5×10^5 and 1×10^6 cells/ml. For pH measurements, COS-7 and mouse embryonic fibroblasts were plated onto glass coverslips and used when cells had reached 70–90% confluence.

A mouse embryonic fibroblast cell line was generated by utilizing the $SEK^{+/-}$ mouse which has been previously described (Nishina et al., 1997). Embryos from SEK1/$MKK4^{+/-}$ × SEK1/$MKK4^{+/-}$ crosses were dissected free from maternal tissues at embryonic day nine. Cells were dissociated and then maintained using an NIH3T3 protocol until spontaneous immortalization occurred (Rittling, 1996). To identify SEK1/$MKK4^{-/-}$ cell lines, DNA was extracted and then digested with Bgl II. Southern blots were probed using a SEK1/MKK4 flanking probe which was generated by PCR using the following oligonucleotides: GGATCCCTGCATTTTCTG (S) and GAATTCACCTCTACAAAT (A). This probe hybridizes to a 3.3 kB fragment in the SEK1/MKK4 knockout, and a 1.7 kB fragment in wild type cells.

Transient Transfection of COS-7 Cells

COS-7 cells were plated onto 60 mm dishes and grown to ~50% confluence. Cells were then co-transfected using the calcium phosphate

method (Chen & Okayama, 1987) with either: 5 μg of HA-SEK1/MKK4 A/L pcDNA3 + 5 μg of Flag-p54β—SAPK-CMV + 0.5 μg of β-galactosidase gene, or as a control, 5 μg of pcDNA3 vector alone + 5 μg of Flag-p54β—SAPK-CMV + 0.5 μg of β-galactosidase gene. Forty-eight hours later the cells were exposed to hypertonic sorbitol (400 mM) in DMEM and were incubated for 30 min at 37°C. The cells were lysed with gentle soft lysis buffer, triturated and spun down to remove the cellular debris. A β-galactosidase colorimetric assay was used to normalize for the amount of transfected protein. Normalized cell lysates were then incubated with anti-Flag antibody for 1 hr. Immunoprecipitation of the Flag-tagged SAPK was then achieved by adding 30 μl of a 50% slurry of Protein-A-sepharose beads for 1 hr. Subsequently, the beads were sedimented and washed 5 times in PBS + 0.1% Tween 20, and then were resuspended in kinase buffer containing (in mM): 50 Tris-Cl, 1 EGTA, 10 $MgCl_2$ and 100 μM [^{32}P-γ]ATP (1 μCi radioactivity per sample), (pH 7.5) for 20 min at 30°C. The reaction was terminated by adding 20 μl of 2× Laemmli's sample buffer.

For pH measurements, the cells were co-transfected with the cDNA of interest and pEGFP (9:1 ratio), to allow visual identification of the transfected cells.

Measurement of Intracellular pH

U937 cells (0.5×10^6/ml) were incubated with 1 μM of the acetoxymethyl ester form of BCECF for 15 min at 37°C, sedimented and resuspended at the same concentration in NHB or Na-free HB. The cell suspension was transferred to a cuvette, which was placed into a thermally controlled cuvette holder in a spectrofluorimeter (Perkin-Elmer Model 650-40). BCECF was excited at 490 nm, and emission was collected at 530 nm. Fluorescence intensity was converted to pH and calibrated to pH_i after addition of both nigericin and monensin at 10 μg/ml and subsequent titration with various amounts of 1 M MES and 1 M Tris solutions, as previously described (Shrode et al., 1997).

COS-7 cells and mouse embryonic fibroblasts lacking SEK1/MKK4 were grown on glass coverslips, and mounted into a coverslip holder that was placed in a thermally controlled microincubator (Medical Systems) on the stage of a Nikon TMD-Diaphot microscope attached to an M series dual wavelength illumination system (Photon Technology International, South Brunswick, NJ). The cells were then loaded with BCECF for 10 min, washed and then alternately excited at 440 and 490 nm, while fluorescence emission was collected at 530 nm. Cells were then exposed to a solution made hyperosmotic by addition of 250 mM mannitol. At the end of each experiment, fluorescence intensity was converted to pH_i using the high KCl-nigericin technique (Thomas et al., 1979), as previously described (Shrode et al., 1998).

In transient transfection experiments, the transfected cells were visually identified by expression of EGFP while on the stage of the imaging setup, and then loaded with BCECF as above. Only transfected COS-7 cells (usually ≈40% of the total) were considered for the measurement of pH, while untransfected cells were excluded.

Immunoblotting

U937 cells maintained overnight in 0.5% FBS were sedimented and resuspended in NHB and kept in this medium for 1–2 hr at 37°C. Cells were then sedimented, resuspended in NHB at a concentration of $2–5 \times 10^6$ per ml and then challenged by the addition of the indicated amounts of a 5 M solution of NaCl, for the designated times. Experiments were terminated by rapid sedimentation for 10 sec in a microcentrifuge and resuspension of the pellet in 200 μl of hot Laemmli's sample buffer. Samples were resolved by SDS-PAGE, using a 12% gel and proteins were transferred to polyvinylidene difluoride membranes. After blocking the membranes with 5% skim milk powder, immunoblotting was carried out overnight at 4°C with primary antibody to the phosphorylated forms of either Erk (1:3000) or p38 MAPK (1:800). After application of anti-rabbit HRP-conjugated secondary antibody (1:5000), the blots were developed using the ECL chemiluminescence detection system (Amersham). Membranes were then stripped and reprobed with primary antibody to the nonphosphorylated forms of Erk or p38 MAPK at 1:3000 or 1:2500, respectively. Immunoblotting for MAPKAPK-2 was performed with primary antibody at 1:2000 dilution.

For mouse embryonic fibroblast experiments, both $SEK^{+/+}$ and $SEK^{-/-}$ mouse embryonic fibroblasts were grown until 80% confluence was reached. Cells were then treated with hypertonic sorbitol (400 mM) in serum-free DMEM for 30 min at 37°C. Cells were washed twice with PBS containing (in mM) 137 NaCl, 7.74 Na_2HPO_4, 2.26 NaH_2PO_4, and 2.7 KCl, and lysed using gentle soft lysis buffer. The lysate was incubated on ice for 10 min, triturated, and then centrifuged to remove insoluble material. Samples were resolved by SDS-PAGE, using a 10% gel and then transferred to polyvinylidene difluoride membranes. After blocking the membranes with 5% skim milk powder, blotting was carried out as above with antibody to phospho-specific SAPK (Thr 183/Tyr 185) (1:1000) followed by 1:2000 anti-rabbit HRP-conjugated (Amersham) secondary antibody for 1 hr at room temperature. After application of secondary antibody, the blots were developed using ECL.

Immunofluorescence

COS-7 cells transiently transfected with pEGFP alone or with pEGFP + HA-SEKA/L were washed twice with PBS and fixed with 4% paraformaldehyde in PBS at room temperature for 30 min. After fixation, the cells were washed 3 times with PBS and incubated for 10 min at RT with 100 mM glycine in PBS. The cells were next permeabilized for 10 min in PBS containing 0.1% Triton-X 100 and blocked for 1 hr in PBS containing 5% milk. After blocking, cells were incubated for 1 hr with mouse-anti-HA antibody (1:1000), washed 3 times with PBS, and then incubated for 1 hr with Cy3-conjugated donkey anti-mouse antibody (1:1500). Cells were finally washed 4 times with PBS and then mounted onto glass slides using Dako fluorescence mounting medium.

SAPK Kinase Assays

SAPK activity was assessed by an in vitro kinase assay. U937 cells were serum-starved overnight in DMEM with 0.5% fetal calf serum and were then exposed to media of different osmolarity for various lengths of time. Cells were then lysed in hypotonic lysis buffer containing (in mM): 10 NaCl, 5 EDTA, 1 benzamidine, 5 NaF_2, 20 PIPES, 1 PMSF, 0.1 Na_3VO_4, 20 μg/ml leupeptin, 0.5% NP-40, and 0.05% 2-mercaptoethanol. After 10 min, the lysate was triturated through a 23 gauge needle and insoluble material was removed by centrifugation at 12,000 × g for 10 min at 4°C. Samples were normalized for the amount of protein using the Bio-Rad protein assay and suspended with GST-c-jun-coated Protein-A sepharose beads for 1 hr. Subsequently, the beads were sedimented and washed 5 times in a PBS + 0.1% Tween-20 solution and then resuspended in kinase buffer for 20 min at 30°C. The reaction was terminated by adding 20 μl of 2× Laemmli's sample buffer. Samples were resolved by SDS-PAGE on 12% gels, stained with Coomassie Blue, destained and dried. Autoradiography was performed by PhosphorImager analysis using ImageQuant software (Molecular Dynamics).

Abbreviations

Erk, p42/p44 MAPK/extracellular signal-regulated protein kinase; FBS, fetal bovine serum; GST-c-Jun, glutathione S-transferase-c-Jun

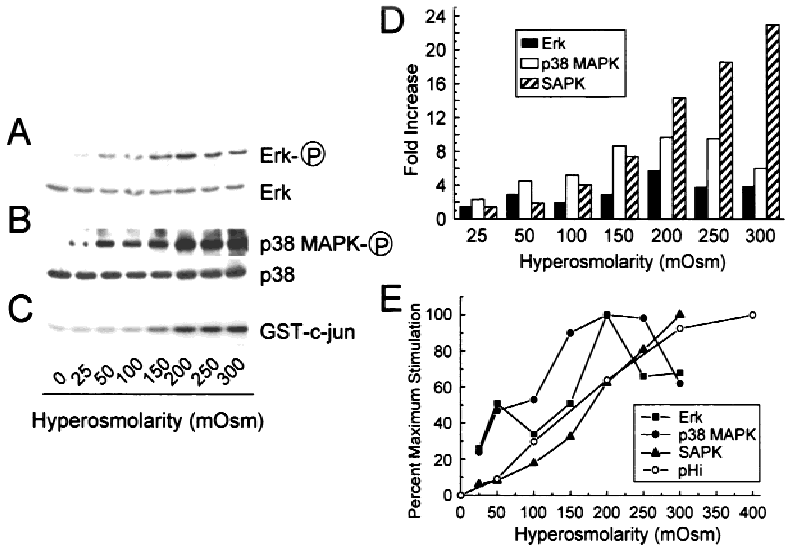

Fig. 1. Response of NHE, Erk, p38 MAPK and SAPK to increasing hyperosmolar stress. U937 cells were suspended in NHB and stimulated for 30 min by addition of concentrated NaCl to increase the osmolarity of the medium by the amount indicated (iso-osmolar medium = 0 hyperosmolarity). (*A–B*) after the hypertonic challenge whole-cell lysates were subjected to SDS-PAGE and immunoblotting with antibodies to phospho-Erk (*A*) or phospho-p38 MAPK. (*B*). The blots were next stripped and reprobed with anti-Erk or anti p38 MAPK, respectively, to ensure equal loading (lower lanes in *A* and *B*). (*C*) SAPK activity was measured by an in vitro kinase assay using GST-c-jun as the substrate, as described in Materials and Methods. (*D*) Quantification of the osmolyte concentration dependence of the activation of Erk (black bars; $n = 2$), p38 MAPK (white bars; $n = 2$) and SAPK (hatched bars; $n = 3$). Data are increases relative to baseline, quantified by densitometric integration of experiments like those in *A–C*. (*E*) Normalized concentration dependence of the activation of Erk (■), p38 MAPK (●), SAPK (▲) and the Na^+-dependent change in pH_i (○). The latter was measured by fluoimetry as described in Methods. Data were normalized to the maximal stimulation (100%) observed for each parameter.

fusion protein; MAPK, mitogen-activated protein kinase; MAPKAPK-2, MAPK-activated protein kinase 2; MEK, MAPK/Erk kinase; pH_i, intracellular pH; NHE, Na/H exchanger; NMDG, *N*-methyl-D-glucamine; SAPK, stress-activated protein kinase/c-Jun *N*-terminal kinase; SDS-PAGE, polyacrylamide gel electrophoresis using sodium dodecyl sulfate; SEK1/MKK4, MAPK-kinase 4.

Results

Osmolyte Concentration Dependence of the Activation of NHE, Erk, p38 MAPK and SAPK

To be a viable candidate effector of NHE1, the MAPK family members must be activated at osmolarities similar to those that activate the exchanger. In most studies, MAPK activity is measured at osmolarities that greatly exceed those used to activate NHE1. We therefore carried out a detailed study of the dependence of kinase and NHE1 activities on medium osmolarity. The results are summarized in Fig. 1, where U937 cells were used. Erk, p38 MAPK and SAPK become activated when they are phosphorylated by upstream kinases. Therefore, we used phospho-specific antibodies to quantify the phosphorylation of Erk and that of p38 MAPK as an index of their activation (Tibbles & Woodgett, 1999). Equal sample loading was ensured by stripping and reprobing the same blots with antibodies to the kinases. After 30 min, only modest activation of Erk was detectable when the basal osmolarity was increased by up to 100–150 mOsm. A considerable stimulation (4- to 6-fold over baseline) was observed with an increase of ~200 mOsm (Figs. 1*A* and *D*). p38 MAPK phosphorylation, and thus activity, increased 4.5-fold with as little as 50 mOsm above iso-osmotic levels (Fig. 1*B* and *D*). The activity of this kinase continued to increase as the osmolarity was elevated, reaching a maximum 9.7-fold over baseline at 200 mOsm above physiological levels.

SAPK activity was measured with an in vitro kinase assay, using GST-c-jun as the substrate (Fig. 1*C*). SAPK stimulation was clearly detectable at 150 mOsm above iso-osmolarity and continued to increase throughout the range of osmolarities tested, reaching a 23-fold enhancement at 300 mOsm (Fig. 1*D*).

Activation of NHE1 was monitored measuring pH_i in the presence and absence of Na^+ (e.g., Fig. 2*A*). Although Na^+-dependent pH_i changes were occasionally detected in U937 cells when osmolarity was increased by 50 mOsm, significant and reproducible increases were not observed until ~100 mOsm. The maximal ΔpH attained was 0.25–0.3 units. The concentration dependence of NHE1 activation is summarized in Fig. 1*E*, where the data are expressed as a percent of maximal stimulation, to facilitate direct comparison with the activation of the kinases (Fig. 1*E*). As shown in the figure, all the kinases displayed an osmotic sensitivity that resembled or exceeded that of NHE1. On this basis, one or more of the MAPK family members could mediate the activation of the antiporter.

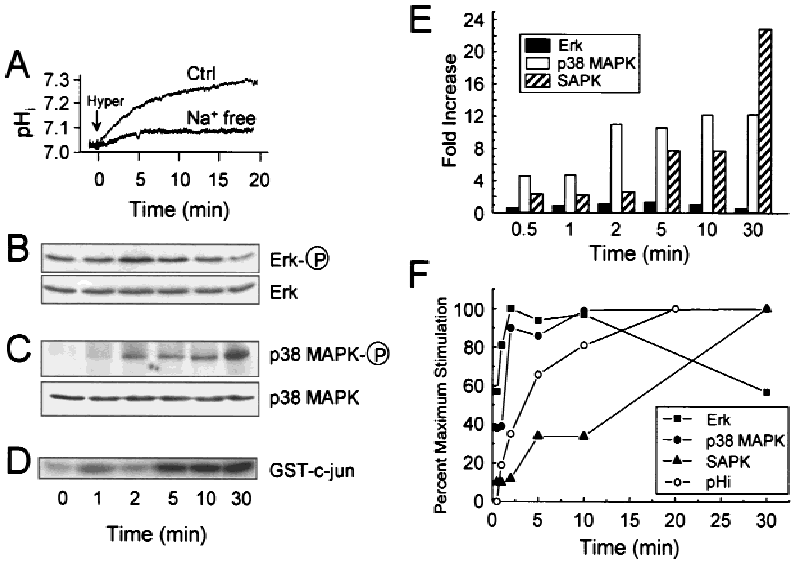

Fig. 2. Time course of NHE, p42 Erk, p38 MAPK and SAPK responses to hyperosmolarity. U937 cells were suspended in iso-osmotic medium and stimulated by addition of an extra 250 mOsm at t = 0. (*A*) pH_i determinations. Cells loaded with BCECF were suspended in either NHB or Na-free NHB and stimulated at t = 0 by addition of 125 NaCl or KCl, respectively. (*B–C*) samples were taken at the indicated times following hypertonic challenge and whole-cell lysates were subjected to SDS-PAGE and immunoblotting with antibodies to phospho-Erk (*B*) or phospho-p38 MAPK (*C*). The blots were next stripped and reprobed with anti-Erk or anti p38 MAPK, respectively, to ensure equal loading (lower lanes in *B*) and *C*). (*D*) SAPK activity was measured by an in vitro kinase assay using GST-c-jun as the substrate. (*E*) Quantification of the time course of activation of Erk (black bars; $n = 2$), p38 MAPK (white bars; $n = 2$) and SAPK (hatched bars; $n = 3$). Data are increases relative to baseline, quantified by densitometric integration of experiments like those in *B–C*. (*F*): Normalized concentration dependence of the activation of Erk (■), p38 MAPK (●), SAPK (▲) and the Na^+-dependent change in pH_i (○). Data were normalized to the maximal stimulation (100%).

Time Course of Activation of pH_i, Erk, p38 MAPK and SAPK

We next investigated the time course of shrinkage-induced stimulation of NHE and compared it to the kinetics of hyperosmotic activation of the MAPKs. As shown previously (Shrode et al., 1998), U937 cells display a detectable alkalinization as early as 1 min after exposure to hypertonic solution (normal + 125 mM NaCl). The alkalosis progresses with time, reaching a steady-state after 10–20 min (Fig. 2*A*). This increase in pH_i is largely Na-dependent (Fig. 2*A*) and is inhibited by HOE-694 (5 μM), a specific inhibitor of NHE1 (*not shown*).

Exposure to the hyperosmotic solution used to activate NHE1 in Fig. 2*A* resulted in a rapid, but modest and transient stimulation of Erk phosphorylation (Fig. 2*B* and *E*). The increase in phosphorylation was maximal between 2–5 min and declined thereafter. Like Erk, p38 MAPK was also activated rapidly: a 4-fold increase in p38 MAPK phosphorylation was observed within 30 sec of hyperosmotic exposure (Fig. 2*C* and *E*). In contrast to Erk, however, the stimulation of p38 MAPK was sustained, persisting for at least 30 min, the longest time point measured.

In contrast to Erk and p38 MAPK, activation of SAPK was somewhat delayed. Even though increases of over 20-fold were recorded after 30 min, the stimulation after 2 min was marginal (Fig. 2*D* and *E*). As before, to compare the activation of the kinases to each other and to the change in pH_i, we expressed the data as percent of the maximal effect (Fig. 2*F*). While the early activation of Erk and p38 MAPK is consistent with a possible role in the stimulation of NHE1, a role for SAPK is less likely, since the increase in pH_i appears to precede significant activation of the latter kinase.

Role of p38 MAPK and Erk in Osmotic Activation of NHE

The preceding data are consistent with a causal relationship between activation of Erk and/or p38 MAPK and the stimulation of NHE1. To test this possibility we investigated the effect of potent and relatively specific kinase inhibitors on the pH_i changes upon cell shrinkage. In the first series of experiments, U937 cells were pretreated with PD98059, an inhibitor of MEK1 (Dudley et al., 1995), an upstream activator of Erk. As shown in Fig. 3*A*, no significant difference in the degree of shrinkage-induced alkalinization was observed between control and PD98059-treated cells (Fig. 3*A*). By contrast, the osmotically-induced increase in Erk phosphorylation was totally suppressed in cells pretreated with PD98059 (Fig. 3*B*). These results argue strongly against a role for Erk activity in the shrinkage-induced activation of NHE.

In the second set of experiments, we tested the p38 MAPK-specific inhibitor, SB203580 (Cuedna et al., 1995). As in the previous case, the shrinkage-induced alkalinization was not altered by treatment of the cells with SB203580. To assess the potency of SB203580 in suppressing p38 MAPK activity, we examined the phos-

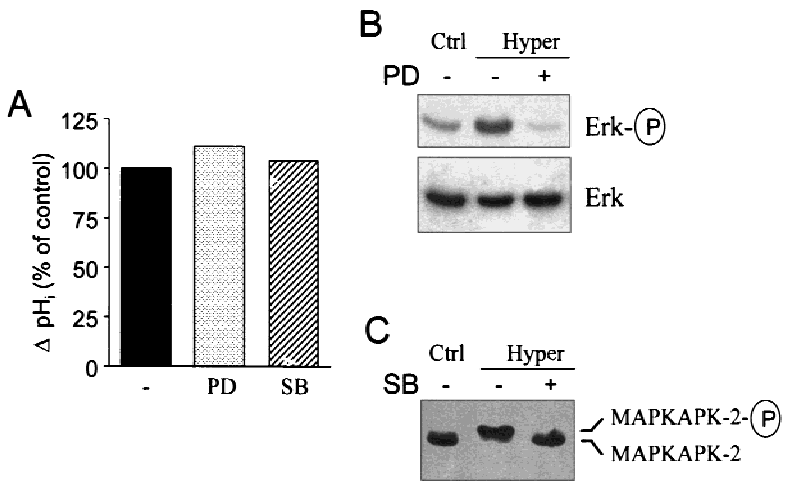

Fig. 3. Effects of inhibitors of MEK-1 and p38 MAPK. (*A*) U937 cells were pretreated with either vehicle (0.1% DMSO; black bar; $n = 3$), 50 μM PD98059 for 1 hr (stippled bar labeled PD; $n = 3$), or 10 μM SB203580 for 15 min (hatched bar labeled SB; $n = 3$) and loaded with BCECF. Cells were then subjected to hyperosmotic stress as in Fig. 2 and the maximal ΔpH_i was recorded when the new steady-state was attained. Data are expressed as percent of the control. (*B*) Cells were pretreated with or without PD98059 as in *A*. The cells were challenged by an additional 250 mOsm NaCl and after 30 min cell lysates were analyzed by SDS-PAGE and immunoblotting with anti-phospho-Erk (top lanes) and reprobed with anti-Erk (lower lanes). (*D*) Cells were pretreated with or without SB203580 as in *A*. The cells were challenged by an additional 250 mOsm NaCl and after 30 min cells lysates were analyzed by SDS-PAGE and immunoblotting with anti-MAPKAPK-2. Phosphorylation of MAPKAPK-2 is indicated by a decrease in its electrophoretic mobility.

phorylation status of MAPKAPK-2, a substrate and downstream effector of p38 MAPK. As shown in Fig. 3*C*, p38 MAPK-induced phosphorylation results in a change in the electrophoretic mobility of MAPKAPK-2, which can be used to monitor the activity of the former kinase (Krump et al., 1997b). Treatment with SB203580 completely suppressed the MAPKAPK-2 mobility shift induced by hyperosmolarity (Fig. 3C). Thus, under conditions where p38 MAPK is inactivated, the osmotic activation of NHE1 persisted, ruling out this kinase as a likely mediator of the stimulation of transport.

Role of SAPK in NHE Activation

The above data suggest that Erk and p38 MAPK are not essential for shrinkage-induced activation of NHE. SAPK, which is strongly activated by shrinkage, remained a viable candidate. Unlike Erk and p38 MAPK, however, the involvement of SAPK could not be tested pharmacologically, since selective inhibitors are not available. We therefore used two alternative strategies. First, SAPK activation was prevented or minimized by utilizing mouse embryonic fibroblasts from SEK1/MKK4$^{-/-}$ mice. SEK1/MKK4 can directly phosphorylate and activate SAPK (Nishina et al., 1997). Therefore, in the knockout cells, activation of SAPK by stimuli that normally utilize an SEK1/MKK4-dependent pathway will be defective. Mouse embryonic fibroblasts were obtained as described in Materials and Methods. Figure 4*A* illustrates a representative Southern blot, which shows that the cDNA probe designed to identify the knockouts recognizes the predicted 1.7 kB fragment in wild-type cells and a 3.3 kB fragment in the putative SEK$^{-/-}$ cells, confirming that the SAPK gene had been ablated.

To determine if hyperosmotic activation of SAPK was inhibited in SEK1/MKK4$^{-/-}$ mouse embryonic fibroblasts, we incubated the cells with or without addition of 400 mM sorbitol for 30 min. Cell lysates were separated using SDS-PAGE, transferred to a membrane and probed with a phospho-specific anti-SAPK antibody. An increase in SAPK phosphorylation, and thus activity, was observed in SEK1/MKK4$^{+/+}$ fibroblasts (Fig. 4*B*). However, hyperosmotic activation of SAPK was greatly inhibited in SEK1/MKK4$^{-/-}$ fibroblasts. Having established that hyperosmotic activation of SAPK is inhibited in SEK1/MKK4$^{-/-}$ fibroblasts, we asked whether the hyperosmotic activation of NHE was also inhibited. Typical results are shown in Fig. 3*C*. Exposure to a hyperosmotic medium resulted in a rapid shrinkage-induced alkalinization in both the SEK1/MKK4$^{+/+}$ cells and SEK1/MKK4$^{-/-}$ cells (Fig. 4*C* and *D*). The kinetics and magnitude of the responses were similar in both cell types (Figs. 4*C* and *D*). Therefore, hyperosmotic activation of NHE is independent of SAPK activation via SEK1/MKK4.

It was originally reported that in embryonic stem cells from SEK1/MKK4$^{-/-}$ mice hyperosmotic activation of SAPK occurred via a SEK1/MKK4-independent pathway (Nishina et al., 1997). It was therefore suggested that, at least in embryonic stem cells, the osmotic activation of SAPK occurred via a MKK7-dependent pathway. Though our results suggest that virtually all of the hyperosmotic activation of SAPK is inhibited in SEK1/MKK4$^{-/-}$ fibroblasts, we were concerned that hyperosmotic activation of SAPK by another SEK isoform may be responsible for the activation of NHE. We therefore used a second approach, namely the overexpression of a dominant-negative protein that would inhibit SAPK ac-

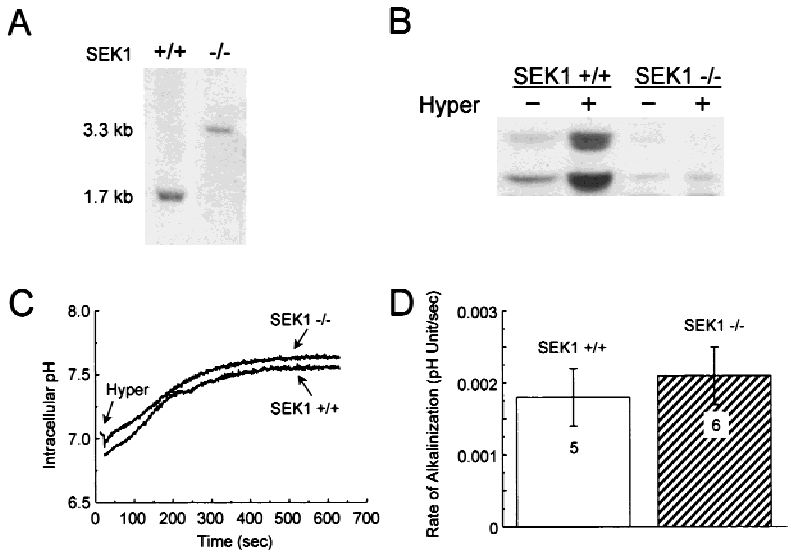

Fig. 4. Effects of hyperosmotic stress in SEK1/MKK4$^{-/-}$ fibroblasts. (*A*) Representative Southern blot probing the integrity of the SEK1/MKK4 gene in SEK1/MKK4$^{+/+}$ fibroblasts and its modification in SEK1/MKK4$^{-/-}$ cells. *See* Methods for details. (*B*) SEK1/MKK4$^{+/+}$ and SEK1/MKK4$^{-/-}$ fibroblasts were left untreated (−) or challenged with 400 mM sorbitol (+). Lysates were then analyzed by SDS-PAGE followed by immunoblotting with anti-phospho-SAPK antibody. (*C–D*) SEK1/MKK4$^{+/+}$ and SEK1/MKK4$^{-/-}$ fibroblasts were loaded with BCECF and their pH_i measured by fluorimetry. Traces start upon addition of an extra 250 mOsm NaCl to the isotonic NHB medium. Representative traces (*C*) and means ± SEM of the maximal rate of alkalinization (*D*) are shown. Numbers indicate number of experiments.

tivation by all SEK isoforms. To this end, we transfected COS-7 cells with SEK1/MKK4-A/L. Overexpression of this inactive mutant will not only prevent activation of SAPK via SEK1/MKK4, but will also reduce, and likely eliminate activation by other SEK isoforms. This generic inhibition results from scavenging of the limited amount of SAPK by the inactive, ectopically expressed SEK1/MKK4-A/L.

We cotransfected the mutant kinase with pEGFP in order to identify the transfectants visually. Forty-eight hr after transfection, some coverslips were used for immunofluorescence staining to ensure that all EGFP-positive cells also expressed SEK1/MKK4-A/L. As shown in Figure 5*A*, essentially all COS-7 cells that expressed SEK1/MKK4-A/L were also positive for EGFP. In addition, the cells were also cotransfected with a vector encoding Flag-tagged SAPK, to enable us to measure the activity of SAPK in the transfected cells only. Exclusion of the untransfected cells was accomplished by measuring SAPK activity in immunoprecipitates obtained with anti-Flag antibodies. Representative results are shown in Fig. 5*B*. The heterologously expressed Flag-tagged SAPK was markedly stimulated by hyperosmotic stress, as found for the endogenous enzyme. More importantly, the activation of SAPK was prevented by co-expression of SEK1/MKK4-A/L.

In parallel, cells transfected with either EGFP alone or EGFP plus SEK1/MKK4-A/L were used to monitor intracellular pH. Expression of the fluorescent protein was first used to identify transfected cells. A neutral density filter was then placed interposed in the light path, greatly attenuating fluorescence emitted by EGFP. Cells were then loaded with BCECF, and the effects of osmolarity on pH_i were monitored. Figs. 5*C* and *D* demonstrate that shrinkage induced a comparable alkalinization in cells expressing EGFP alone or EGFP plus SEK1/MKK4-A/L. Together with the results obtained with knockout cells, these findings imply that SAPK is not essential for activation of NHE1.

Discussion

When cells are exposed to a hyperosmotic environment, activation of Erk, p38 MAPK and SAPK occur. In addition, hyperosmotic exposure also causes an increase in pH_i, mediated in most cells by NHE1. We have previously shown that hyperosmotic activation of SAPK in U937 cells occurred even when NHE-mediated shrinkage-induced alkalinization was inhibited (Shrode et al., 1997). These results strongly suggested that the activation of SAPK is not secondary to the activation of the Na^+/H^+ exchanger. We therefore wanted to determine if the opposite was true, that is, whether hyperosmotic activation of Erk, p38 MAPK or SAPK is required for the stimulation of NHE. Indeed, the dependence of these events on the osmolarity was comparable (Fig. 1), consistent with a cause-effect relationship.

In most cells, hyperosmotic activation of NHE occurs within 30 sec, and intracellular pH reaches a new steady-state within 5 min. Likely due to their larger size, and therefore smaller surface-to-volume ratio, U937 cells displayed a slower alkalinization. Nonetheless, the maximal rate of alkalosis was established within 1–2 min. Maximal activation of Erk and p38 MAPK was also very rapid. In contrast, activation of SAPK was slower, seemingly lagging behind the activation of the exchanger. This would seem to rule out SAPK as an effector of the osmotic activation of NHE1. However, this argument is not entirely compelling, since only partial activation of SAPK may be required for full stimulation of NHE1. Thus, analysis of the time course per se

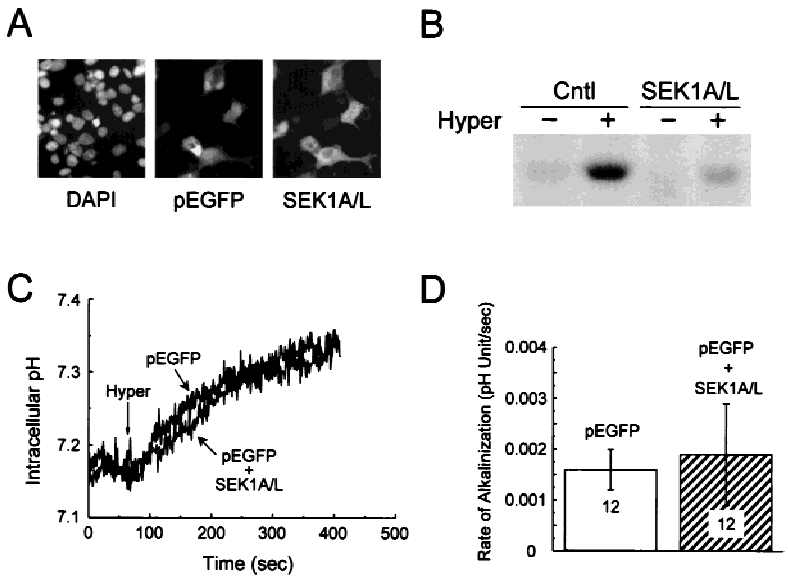

Fig. 5. Expression of a dominant-negative form of SEK1/MKK4 (*A*) COS-7 cells were transiently transfected with pEGFP and HA-tagged SEK1/MKK4-A/L, as described in Materials and Methods. Cells were then fixed, permeabilized and stained with anti-HA antibodies. Left panel: nuclear staining with DAPI. Middle panel: EGFP fluorescence. Right panel: immunostaining of SEK1/MKK4-A/L. (*B*) COS-7 cells were transiently transfected with Flag-tagged SAPK + β-galactosidase (control) or with Flag-tagged SAPK + β-galactosidase + HA-SEK1/MKK4-A/L (*SEK1/A/L*). Cells were treated with 400 mM sorbitol (+) or left untreated (−) for 30 min, lysed and SAPK was immunoprecipitated using anti-Flag antibody. SAPK kinase assay was assayed in vitro as described in Methods. (*C–D*) Cells were transfected as in *A* and after identification of EGFP-positive cells, a neutral density filter was added to the light path and the cells were loaded with BCECF. pH_i measurements were initiated and where noted, the medium was made hypertonic by addition of 125 mM NaCl. Representative traces (*C*) and means ± SEM of the maximal rate of alkalinization (*D*) are shown. Numbers indicate number of experiments.

was insufficient to eliminate any of the kinases as possible mediators.

Erk seemed least likely to mediate the activation of NHE1, since it is only modestly and transiently activated by hypertonicity, whereas NHE1 remains stimulated upon sustained cell shrinkage. This impression was confirmed using PD98059, an inhibitor of MEK1. In agreement with results published by Bianchini et al. (Bianchini et al., 1997), this blocker failed to prevent the osmotic stimulation of NHE1, despite demonstrably preventing Erk activation (Fig. 3).

We previously showed that p38 MAPK could phosphorylate a recombinant form of the C-terminal domain of NHE1 in vitro (Shrode et al., 1997). Together with the activation of this kinase by cell shrinkage, this observation pointed to a role of p38 MAPK in the stimulation of ion transport. The availability of a pharmacological inhibitor allowed us to directly test the potential role of p38 MAPK in the activation of NHE in vivo. In shrunken cells, compound SB203580 obliterated the phosphorylation of MAPKAPK-2 by p38 MAPK, without interfering with the stimulation of NHE1. This suggests that, like Erk isoforms, p38 MAPK activation is not required for NHE activation, but rather occurs as a separate and parallel event.

Recently, it has been reported that SAPK phosphorylates in vitro a fusion protein of the N-terminus of the Na-K-2Cl cotransporter, another transporter involved in regulatory volume increase (Klein, Lamitina and O'Neill, 1999). Therefore, it was possible that SAPK might similarly phosphorylate proteins involved in the activation of NHE1. The absence of selective pharmacological inhibitors of SAPK compelled us to use alternative methods to determine the role of this kinase. To this end, we utilized mouse embryonic fibroblasts that lack SEK1/MKK4, a kinase directly upstream of SAPK. Exposing the SEK1/MKK4$^{-/-}$ fibroblasts to hyperosmotic media nevertheless resulted in seemingly normal activation of NHE. In addition, transfection of a dominant-negative form of SEK1/MKK4 into COS-7 cells precluded SAPK activation, but had no effect on the shrinkage-induced alkalinization. By scavenging SAPK, overexpression of the dominant-negative form of SEK1/MKK4 likely inhibits activation by MKK7 as well. Therefore, our data indicate that SAPK is not responsible for shrinkage-induced activation of NHE.

The aggregate results of our pharmacological, genetic and molecular studies suggest that Erk, p38 MAPK and SAPK are not responsible for shrinkage-induced activation of NHE. Therefore, the signaling pathway responsible for this activation remains elusive. In an earlier study it was shown that the tyrosine phosphorylation of several proteins occurred as a consequence of hyperosmolarity-induced shrinkage in neutrophils and that this phosphorylation was required for the activation of NHE1 (Krump et al., 1997). Tyrosine kinases are involved in activation of RhoA (Nobes et al., 1995), which has been shown to activate NHE1 in fibroblasts (Hooley et al., 1996). Whether RhoA is similarly involved in the hyperosmolar activation of the antiporter remains to be determined. It is noteworthy, however, that tyrosine phosphorylation does not seen to be a universal requirement for the hyperosmotic activation of NHE. In this regard, drastic differences have been reported between sus-

pended and attached cells (Douglas et al., 1999). Thus, if RhoA is involved in the osmotic response, alternate routes may lead to its stimulation in different cell types.

Other families of small GTPases may equally contribute to the response. H-Ras has also been shown to activate NHE (Kaplan & Boron, 1994; Maly et al., 1989) and the cross-linking of growth factor receptors that accompanies shrinkage (Rosette & Karin, 1996) would be expected to stimulate Ras. The possible participation of Ras or Rho-family GTPases needs to be addressed experimentally using inhibitory toxins or dominant-negative constructs.

NHE can also be stimulated by heterotrimeric G proteins, particularly Gα12 (Hooley et al., 1996) and Gα13 (Kitamura et al., 1995). These G proteins are also linked to volume-sensitive pathways, inasmuch as they can promote stimulation of SAPK (Coso et al., 1995). It is tempting to speculate that hyperosmolarity-induced shrinkage promotes the activation of the aforementioned G proteins, which in turn may activate NHE.

In conclusion, it appears that the cellular response to hyperosmolarity involves parallel pathways that likely share several upstream events. Such parallel events include the immediate activation of NHE to protect the cell from immediate volume changes, and the activation of p38 MAPK and SAPK presumably to prepare the cell for prolonged hyperosmotic exposure, perhaps via slower regulation of gene transcription.

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