The ubiquitously expressed Na\(^+/\)H\(^+\) exchanger NHE1 is the target of multiple signaling pathways, including those activated by tyrosine kinase receptors, G protein-coupled receptors, and integrins. The intracellular pathways leading to activation of NHE1 are poorly understood. To gain more insight into these activation pathways, we examined the role of mitogen-activated protein kinases (MAPKs) as potential mediators of NHE1 activation by extracellular stimuli such as growth factors and hypertonic stress.

Whereas p44 MAPK does not appear to phosphorylate NHE1 in vitro, we found that inhibition of the p42/p44 MAPK signaling by expression of a dominant negative form of p44 MAPK, by expression of the MAP kinase phosphatase MKP-1, or by inhibition of MAP kinase 1 (MKK1) with the PD 98059 compound reduced by 50–60% NHE1 activation in response to growth factors. This inhibitory effect also was observed in C-terminal NHE1 deletion mutants in which the major phosphorylation sites have been deleted. Furthermore, the use of a CCL39-derived cell line expressing an estradiol-regulated form of oncogenic Raf-1 (CCL39-Araf-1:ER) revealed that the exclusive activation of the Raf → MKK1 → p42/p44 MAPK cascade was capable of inducing NHE1 activation to the same extent as potent growth factors like thrombin.

Together, our findings demonstrate that the p42/p44 MAPK cascade plays a predominant role in the regulation of NHE1 by growth factors, an action that is mediated via accessory proteins that remain to be identified. In contrast, we found no evidence in favor of the contribution of any MAPK, p42/p44, p38 MAPKs, and Jun kinase, in NHE1 activation by osmotic stress.

Na\(^+/\)H\(^+\) exchangers are vital membrane transporters involved in multiple cellular functions. NHE1,\(^1\) the first Na\(^+/\)H\(^+\) exchanger isoform to be cloned (1), is ubiquitously expressed and appears to be the predominant species in nonepithelial cells, where it has been shown to play a major role in intracellular pH homeostasis and cell volume regulation. NHE1 activity can be modulated by a remarkably wide variety of stimuli including growth factors, tumor promoters, and hormones as well as physical factors such as changes in cell volume or cell spreading (reviewed in Ref. 2). On the basis of its hydrophobic profile, NHE1 exhibits two distinct domains: a largely hydrophobic NH\(_2\)-terminal region of 500 residues proposed to span the membrane 10–12 times, followed by a hydrophilic C-terminal domain of more than 300 residues. Structure-function relationship studies have demonstrated that the N-terminal transmembrane part of NHE1 is necessary and sufficient to catalyze the amiloride-sensitive ion exchange, whereas the cytoplasmic C-terminal domain is crucial for mediating the activation of NHE1 by growth factors, hormones, and osmotic stress (3).

Extracellular stimuli have been shown to activate NHE1 by increasing its sensitivity to intracellular H\(^+\) (4–7), resulting in cytoplasmic alkalization; the mechanism underlying this shift in pH\(_i\) sensitivity is not yet fully elucidated. NHE1 is constitutively phosphorylated in unstimulated cells, and mitogenic stimulation is accompanied by an increase in phosphorylation of NHE1 on serine residues, with a time course similar to the rise in intracellular pH (8). Moreover, okadaic acid, a serine/threonine protein phosphatase inhibitor can by itself activate NHE1 in correlation with stimulation of its phosphorylation (9, 10). Based on these results, phosphorylation of NHE1 has been hypothesized to directly trigger the activation of the antipporter. Recent evidence, however, shows that phosphorylation of NHE1 cannot fully account for its activation (11). The picture of NHE1 regulation emerging from recent studies is as follows: at least three different mechanisms appear to control NHE1 regulation: 1) the effect of a regulatory protein(s), potentially phosphorylated, interacting with the cytoplasmic domain of NHE1; 2) elevation of [Ca\(^{2+}\)], leading to the binding of Ca\(^{2+}\)/calmodulin complex to the high affinity calmodulin-binding domain (amino acids 636–656); and 3) direct phosphorylation of the cytoplasmic domain of NHE1 (11–13).

The mechanisms by which extracellular signals are propagated from the cell surface to modify membrane targets such as NHE1 have not been completely resolved. The mitogen-activated protein kinases (MAPKs) have been identified as important mediators in a wide array of physiological processes: they constitute a large family of Ser/Thr protein kinases activated in intracellular pH; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5(4-pyridyl)imidazole; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; VSVG, vesicular stomatitis virus glycoprotein.
The p42/p44 MAPK Cascade Activates NHE1

by separate cascades conserved through evolution (14), which regulate multiple processes stimulated by extracellular agents. Transmission of the signal occurs through sequential activation of cytosolic protein kinases; eventually, these phosphorylation cascades activate nuclear and cytosolic regulatory molecules to initiate cellular responses. In mammalian cells, the first and best characterized MAPK cascade is the p42/p44 MAPK cascade (15, 16); it involves activation of p42/p44 MAPK by direct phosphorylation by the dual specificity kinases MKK1 and MKK2 (17) which are themselves phosphorylated and activated by the serine/threonine kinase Raf (18). Raf is recruited to the membrane by Ras upon activation of either G protein-coupled receptors (19) or tyrosine kinase receptors (20). Recently, two other MAPK subtypes, p38 MAPK (21–24) and p46/p54 JNKs (25, 26), have been discovered. The p42/p44 MAPK cascade has been shown to be essential for the propagation of growth factors and differentiating signals (27–29), whereas p38 MAPK and JNK, also named stress-activated protein kinase or SAPK, mediate signals in response to cytokines and environmental stress such as hyperosmolarity (21–26). Although these cascades are initiated by stimuli that induce NHE1 activation and might therefore be involved in the signaling pathways leading to activation of the exchanger, reports establishing a direct link between MAPK cascades and regulation of ion transporters such as NHE1 are scarce (30).

The purpose of the present study was therefore to gain a better understanding of the activation pathways of NHE1 by investigating the putative role of the p42/p44 MAPK, p38 MAPK, and JNK cascades in the regulation of NHE1 by growth factors and osmotic stress. We report that in the Chinese hamster lung fibroblast cell line CCL39 the Raf → MKK1 → p42/p44 MAPK cascade plays an important role in activation of NHE1 by growth factors, whereas neither p42/p44 nor p38 MAPK nor JNK appeared to be involved in NHE1 activation by osmotic stress.

**EXPERIMENTAL PROCEDURES**

**Materials**—Estradiol, horseradish peroxidase-conjugated anti-rabbit IgG, myelin basic protein, insulin, and anisomycin were obtained from Sigma; IL1-α, myelin basic protein, insulin, and anisomycin were obtained from Sigma; IgG, myelin basic protein, insulin, and anisomycin were obtained from Sigma. Rabbit anti-NHE1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-rabbit p38 MAPK, anti-rabbit p42/p44 MAPK, and anti-rabbit JNK antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); and anti-rabbit serum was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Rabbit polyclonal anti-NHE1 antibody (RP-c28) raised against a 12–18 amino acid sequence containing the Myc epitope (31) was provided by Dr. B. Gould (Institut Pasteur, Paris); P5D4 monoclonal antibody against the VSVG epitope (203580) was provided by SmithKline Beecham Pharmaceuticals (King of Prussia, PA). The specific MKK1 inhibitor PD 98059 was provided by BioLabs. 12CA5 monoclonal antibody that recognizes the HA epitope was from BABCO (Emeryville, CA); FDS4 monoclonal antibody against the VSVG epitope (51) was provided by Dr. B. Gould (Institut Pasteur, Paris); 9E10 monoclonal antibody against the Myc epitope was provided by Dr. G. Evan (Imperial Cancer Research Fund, London.). The affinity-purified polyclonal rabbit anti-NHE1 antibody (RP-28) raised against a β-galactosidase fusion protein containing the last 157 residues (amino acids 658–815) of the human NHE1, has already been described (8).

**Cell Lines and Culture**—The parental Chinese hamster lung fibroblast cell line CCL39, the Na+/H+ antiporter-deficient cell line PS120, and the corresponding transfecteds were maintained in Dulbecco’s modified Eagle’s medium (H21 catalog number 52100; Life Technologies, Inc.) containing 25 mM NaHCO3. The CCL39-Dαraf-1:ER clone derived from the CCL39 cell line was maintained in H21 medium without phenol red and supplemented with glutamine and glucose to reach the concentrations of normal H21 (H21 without phenol red; catalog number 11880). Both culture media were supplemented with 7.5% fetal calf serum (Life Technologies, Inc.), penicillin (50 units/ml), and streptomycin (50 µg/ml). Cells were maintained at 37 °C in the presence of 5% CO2. The CCL39-Dαraf-1:ER clonal cell line was obtained by transfection of CCL39 cells with the plasmid pLNCaRaf-1:ER (32) and selection of clones resistant to Geneticin (G418). The clone that displayed the highest stimulation of MAPK activity upon estradiol addition was selected and recloned (33).

**Expression Vectors and Transfection Procedures**—PS120 cells are Na+/H+ antiporter-deficient mutants derived from the CCL39 cell line by the H+ suicide method (34). A series of stable transfectants was obtained by expression of different constructs of human NHE1 cDNA in PS120 cells. These included the full-length cDNA deleted from the 5′-untranslated region (pEAP construct), and C-terminal truncations at positions 698 and 635 (constructs Δ698 and Δ635, respectively) (35). pEAP–plasmid was NH2-terminally-tagged with a Myc epitope (NHE1-Myc) by Dr. J. Noéil; the Myc epitope was subcloned in the N terminus of Δ698 (Δ698-Myc) and Δ635 (Δ635-Myc). pEAP–was also NH2-terminally-tagged with a double VSVG epitope (NHE1-VSVG) by Dr. R. C. Poole.

Other Plasmids—The plasmids p44 MAPK and p44 MAPK-T192A are derived from the hamster cDNA of p44 MAPK as described previously (27). The human cDNA of the phosphatase MKP-1 kindly provided by Dr. S. Keyse (35) was subcloned in the expression vector pcDNAneo (Invitrogen, San Diego, CA) by J.-M. Brondello. The plasmid p38 MAPK is derived from murine cDNA of p38 MAPK (36) and was kindly provided by Dr. R. J. Ulevitch; the plasmid JNK was kindly provided by Dr. R. J. Davis.

For stable transfections, PS120 cells were transfected with each plasmid construct by using the calcium phosphate co-precipitation technique. The transfected cells were selected after three consecutive tests of pH recovery by cytoplasmic acidification as described previously (3).

For transient expression, a H+−killing selection was also used. PS120 cells (3 × 106/10-cm plate) were cotransfected by the calcium phosphate technique with 7 µg of pEAP expression vector encoding the Na+/H+ antiporter (1, 3) and 43 µg of the relevant construct. Forty-eight hours after transfection, cells were subjected to an acid load selection that killed nontransfected cells, usually >90% of the cell population (1, 3). pH determination experiments were done the next day in surviving cells.

**Measurement of Intracellular pH Changes**—Changes in the intracellular pH were estimated from the distribution of [7-32P]benzoic acid (37) as described previously (8).

**[32P]Orthophosphate Cell Labeling and Immunoprecipitation**—The cells expressing wild type or mutant exchangers were grown to confluence in 10-cm dishes and labeled for 5 h at 37 °C in phosphate-free, serum-free medium containing [32P]orthophosphate (100–300 µCi/ml). The cells were then stimulated in the same medium with 10% FCS for 15 min.

Immunoprecipitation of NHE1 was carried out as described previously (11). For immunoprecipitation with the monoclonal 9E10 antibody, a suspension of protein G-Sepharose beads was used instead of protein A-Sepharose beads. Immunoprecipitated proteins were solubilized by boiling in Laemmlli sample buffer. Samples were analyzed by SDS-PAGE on 7.5% polyacrylamide gels. Phosphoproteins were visualized by autoradiography.

**Immune Complex Kinase Assays**—Cells were serum-deprived overnight or for 5 h and stimulated with various agonists. Kinase assays were performed as described previously (40). Briefly, the cells were lysed in Triton X-100 lysis buffer. Equal amounts of proteins from cell lysates were immunoprecipitated on protein A-Sepharose beads coupled with the 12CA5 anti-HA antibody. Activity of the kinases was assayed in 40 µl of kinase buffer with various substrates: glutathione S-transferase-ATF2(1–109) for HA-p38MAPK and HA-JNK, MBP for HA-p44MAPK; and 50 µmol, 1–3 µCi [γ-32P]ATP for 20 min at 30 °C. For the immune complex kinase assays using immunoprecipitated NHE1 as substrate, after washing with the Triton X-100 lysis buffer, the beads coming from HA-p44MAPK and pEAP− or NHE1-VSVG immune complex were mixed and then washed with kinase buffer. HA-p44 MAPK activity was assayed by incubating the mixture of beads in 40 µl of kinase buffer with various substrates: glutathione S-transferase-ATF2(1–109) for HA-p38MAPK and HA-JNK, MBP for HA-p44MAPK; and 50 µmol, 1–3 µCi [γ-32P]ATP for 20 min at 30 °C. The kinase reactions were stopped by boiling Laemmlli sample buffer. The samples were heated at 95 °C for 5 min, protein was separated by SDS-PAGE, and phosphoproteins were detected by autoradiography.

**Western Blotting and SDS-PAGE Analysis of Proteins**—Western blotting analysis of NHE1 was performed as described previously (3).

**Other Methods**—Protein concentration was measured using the bicinchoninic acid protein assay kit (Pierce) with bovine serum albumin as standard.

**Autoradiograms are representative of three separate experiments.** Data are presented as the mean ± S.E. of at least three independent experiments performed in quadruplicate.
RESULTS

In Vivo Phosphorylation of NHE1 and NHE1 Deletion Mutants: Effect of Serum

The cytoplasmic domain of NHE1, which has been determined by mutagenesis studies to be crucial for activation by growth factors (3), has also been found to contain both the basal and stimulus-induced phosphorylation sites (11). To clarify the contribution of direct phosphorylation of NHE1 in the response to growth factors, we expressed in PS120 cells (a Na+/H+ exchanger-deficient derivative of the CCL39 cell line (34)) wild type and C-terminally truncated forms of the human NHE1. The behavior of these truncated mutants has been previously characterized as follows (38): deletion of 117 amino acids (698–815) of the cytosolic tail was found to largely preserve the cytoplasmic alkalinization induced by the potent mitogenic combination of α-thrombin and insulin (ΔpH1 = 0.15 ± 0.01, n = 10, for the Δ698 cells compared with ΔpH1 = 0.22 ± 0.03, n = 9, for the pEAP– cells). A larger deletion of the cytosolic tail of 180 amino acids (635–815) still preserved the alkalinization induced by α-thrombin and insulin, although the extent of the pH increase was reduced by 50% by the deletion (ΔpH1 = 0.10 ± 0.02, n = 6).

We investigated the phosphorylation status of these NHE1 truncation mutants both in quiescent cells and upon stimulation with growth factors. The specific anti-NHE1 polyclonal antibody RP-c28 raised against a β-galactosidase fusion protein containing the last C-terminal 157 amino acids of the human NHE1 (658–815) (8), was found to recognize only the wild type exchanger pEAP+ and not the Δ698 and Δ635 deletions (38). To allow immunoprecipitation of the NHE1 deletions, a Myc epitope tag recognized by the anti-Myc monoclonal antibody 9E10 was introduced at the N terminus of these cytoplasmic truncated forms of NHE1 (see “Experimental Procedures”). PS120 cells were stably transfected with cDNAs encoding the Myc-tagged full-length (NHE1-Myc) or truncated versions of NHE1 (Δ698-Myc and Δ635-Myc). Immunoprecipitation of NHE1-Myc, Δ698-Myc and Δ635-Myc from 32P-labeled cells is shown in Fig. 1. In NHE1-Myc quiescent cells (lane 1), the RP-c28 antibody recognized a protein running as a diffuse 32P-labeled band at a molecular mass of about 110 kDa as reported for NHE1 (8); the anti-Myc 9E10 antibody was found to recognize the same protein (data not shown). As described previously for NHE1 (8), phosphorylation of NHE1-Myc was found to increase upon stimulation with FCS (lane 2). The Δ698-Myc deletion mutant was shown to run as a diffuse 32P-labeled protein at a lower molecular mass than NHE1-Myc, consistent with the molecular mass expected from the deletion of 117 amino acids (lane 3). Interestingly, the Δ698-Myc deletion mutant did not demonstrate any increase in phosphorylation upon serum stimulation but rather a decrease in total phosphorylation. No 32P-labeled protein at the expected molecular mass for the Δ635-Myc deletion could be detected when Δ635-Myc was immunoprecipitated from resting cells (lane 5) or cells stimulated with 10% FCS (lane 6).

These results indicate that the Δ635-Myc mutant is no longer phosphorylated, therefore allowing the mapping of NHE1 phosphorylation sites to the cytoplasmic tail (amino acids 636–815).

It is noteworthy that these results confirm the mapping that had already been obtained by indirect means, by comparison of phosphopeptide maps of four NHE1 variants: the wild type NHE1, two internal deletion mutants (Δ515–566 and Δ567–635), and the expressed NHE1 cytoplasmic domain (11). The Δ698-Myc mutant was phosphorylated in unstimulated cells, allowing the localization of basal phosphorylation site(s) for NHE1 in the 635–698 domain. More importantly, no increase in the total phosphorylation of the Δ698-Myc mutant could be detected upon addition of serum, suggesting that the growth factor-sensitive phosphorylation site(s) are distal to the 698 residue. These results should however be taken with caution, since considering the total phosphorylation of the NHE1 deletion mutant could be misleading; an increase in phosphorylation in one region could be masked by a decrease in phosphorylation on a distinct site in another region. Phosphopeptide mapping would be necessary to resolve this uncertainty, but unfortunately the signals obtained from 32P-immunoprecipitates with the 9E10 antibody were too weak to allow phosphopeptide mapping for the Myc-tagged NHE1 deletions. It has been established that two classes of mitogens initiating their signals either through receptor tyrosine kinases (i.e. epidermal growth factor) or through G protein-coupled receptors and protein kinase C (i.e. thrombin) both stimulated NHE1 phosphorylation exclusively on serine residues. Moreover, the patterns of NHE1 phosphorylation in response to epidermal growth factor and thrombin were found to be identical. These findings led us to postulate that MAPKs that can integrate signals from multiple transmembrane receptors might play an important role in the cascade leading to NHE1 activation and presumably phosphorylation (10). As stated above, truncation of the NHE1 cytoplasmic tail from residue 635 eliminated all major sites of phosphorylation, yet the resulting truncated mutant was still activable (although to a lesser extent) by all growth factors tested (11). In contrast, deletion of the 566–635 domain abolished NHE1 activation by growth factors, raising the possibility of the existence of one or multiple regulatory protein(s) potentially interacting with the cytoplasmic domain of NHE1. This regulatory protein could itself be the target of a MAPK cascade. In this respect, it is worth noting that the Δ635 transfectant was found to still respond to the serine/threonine phosphatase inhibitor okadaic acid (data not shown; Ref. 11), suggesting that phosphorylation reactions upstream of NHE1 play a role in the exchanger activation pathway. We therefore investigated the potential involvement of the various MAPK cascades in this mechanism of activation. To this end, we first compared the effect of various stimuli on activation of these kinase cascades with their effects on NHE1 regulation in CCL39 fibroblasts.

Differential Effects of Growth Factors and Stress Signals on p44 and p38 MAPK Activities

HA-p44 MAPK was immunoprecipitated from CCL39 stable transfectants submitted to various stimuli, and activation of HA-p44 MAPK was measured by the capacity to phosphorylate its preferential substrate MBP. As shown in Fig. 2 and as reported previously (39), mitogenic stimuli such as FCS and

FIG. 1. In vivo phosphorylation of NHE1 and NHE1 deletion mutants: effect of serum. Cells stably expressing the Myc epitope-tagged wild-type (NHE1-Myc), or the Myc epitope-tagged NHE1 COOH-terminally truncated mutants (Δ698-Myc and Δ635-Myc) were labeled with 32P and stimulated for 15 min with 10% FCS. Each exchanger variant was then immunoprecipitated (NHE1-Myc with the anti-NHE1 RP-c28 antibody; Δ698-Myc and Δ635-Myc with the 9E10 (anti-Myc) antibody) and analyzed on a 7.5% acrylamide SDS-PAGE gel.
The activity of p44 MAPK and p38 MAPK was measured in immune complex protein kinase assays using, respectively, MBP and ATF2 as the substrates. CCL39 cells stably expressing HA-p44 MAPK and PS120 cells stably expressing HA-p38 MAPK were serum-deprived, respectively, overnight and for 5 h and stimulated with 20% FCS (5 min) (lane 2), anisomycin (50 ng/ml; 20 min) (lane 3), α-thrombin (1 unit/ml) plus insulin (10 μg/ml) (15 min) (lane 4), 300 mM sorbitol (15 min) (lane 5), or 100 mM sucrose (15 min) (lane 6). Cell extracts were prepared as described under “Experimental Procedures”; HA-p44 MAPK and HA-p38 MAPK were immunoprecipitated with the anti-HA antibody, and their activity was determined by their ability to phosphorylate, respectively, MBP and ATF2. The phosphorylated MBP and ATF2 were detected after SDS-PAGE (12.5% acrylamide) by autoradiography. The rate of phosphorylation was quantitated by PhosphorImager analysis and is presented as the p44 MAPK and p38 MAPK activity relative to control cells treated without agonist (1.0).

α-thrombin plus insulin were demonstrated to induce a very large stimulation of p44 MAPK activity. In contrast, two stress agents, anisomycin and sorbitol (300 mM) were found to only weakly stimulate p44 MAPK.

In addition to p42/p44 MAPKs, novel members of the family of the MAPKs (p38 MAPK and JNK) have recently been identified. These kinases are selectively activated by proinflammatory cytokines such as IL1β but also by environmental stresses such as UV radiation and hyperosmolality. HA-p38 MAPK was immunoprecipitated from PS120 stable transfectants submitted to various stimuli, and activation of p38 MAPK was measured by the capacity to phosphorylate its preferential substrate ATF2. As reported for other cell lines (36, 41), growth-promoting agents such as FCS or α-thrombin plus insulin that maximally activated p44 MAPK only had a small effect on p38 MAPK activation in hamster fibroblasts. The best stimulus for p38 MAPK was found to be anisomycin (10-fold stimulation); p38 MAPK was also stimulated by an osmotic shock of 300 mM sorbitol but to a lesser extent than by anisomycin, whereas an osmotic shock of 100 mM sucrose was totally ineffective on both p38 and p44 MAPKs regardless of the time of stimulation.

We can conclude from these experiments that, in CCL39 fibroblasts, p44 MAPK is preferentially activated by growth factors (FCS, α-thrombin plus insulin), whereas p38 MAPK is selectively stimulated by stress (sorbitol, anisomycin).

FIG. 2. Growth factors and stress signals differentially stimulate p44 and p38 MAPKs. The activity of p44 MAPK and p38 MAPK was measured in immune complex protein kinase assays using, respectively, MBP and ATF2 as the substrates. CCL39 cells stably expressing HA-p44 MAPK and PS120 cells stably expressing HA-p38 MAPK were serum-deprived, respectively, overnight and for 5 h and stimulated with 20% FCS (5 min) (lane 2), anisomycin (50 ng/ml; 20 min) (lane 3), α-thrombin (1 unit/ml) plus insulin (10 μg/ml) (15 min) (lane 4), 300 mM sorbitol (15 min) (lane 5), or 100 mM sucrose (15 min) (lane 6). Cell extracts were prepared as described under “Experimental Procedures”; HA-p44 MAPK and HA-p38 MAPK were immunoprecipitated with the anti-HA antibody, and their activity was determined by their ability to phosphorylate, respectively, MBP and ATF2. The phosphorylated MBP and ATF2 were detected after SDS-PAGE (12.5% acrylamide) by autoradiography. The rate of phosphorylation was quantitated by PhosphorImager analysis and is presented as the p44 MAPK and p38 MAPK activity relative to control cells treated without agonist (1.0).

FIG. 3. Effect of p44 and p38 MAPKs activators on cytosolic pH. Cells stably expressing wild type NHE1 (pEAP) were depleted of serum for 5 h and then equilibrated for 1 h in bicarbonate-free and Hepes-buffered saline (pH 7) as described previously (38). Then the cells were incubated for 15 min in the same medium containing 0.3 μCi/ml [14C]benzoic acid in the absence or presence of stimulus: anisomycin (50 ng/ml), sorbitol (100 mM), sorbitol (300 mM), IL1β (20 ng/ml), IL1β (20 ng/ml) plus insulin (10 μg/ml), insulin (10 μg/ml), α-thrombin (1 unit/ml) plus insulin (10 μg/ml). The variation of pH was measured at the end of this 15-min incubation as described previously (37). ΔpH represents the variation of pH, relative to control (unstimulated) cells. Error bars (±S.E.) are based on triplicate determinations.

Effect of p44 and p38 MAPK Activators on NHE1 Regulation

We next investigated the effect of p44 and p38 MAPK activators on NHE1 regulation. As shown in Fig. 3, anisomycin and IL1β, which were shown to be specific activators of p38 MAPK in hamster fibroblasts (36), failed to activate NHE1 in these cells. The pH experiments were done in PS120 fibroblasts stably transfected with wild type NHE1 (pEAP), therefore expressing only their endogenous level of p38 MAPK, but similar results were obtained on HA-p38 MAPK transfectants (data not shown). In contrast, as reported previously (12, 38), an osmotic shock of 100 mM sucrose that had no effect on p38 MAPK activation (Fig. 2) had the capacity to induce a marked stimulation of NHE1, arguing against the involvement of p38 MAPK in the osmotic induced activation of NHE1.

Effect of Osmotic Shocks of Varying Intensities on Activation of p38 MAPK and JNK

The stress-activated protein kinase JNK has also been reported to be activated by osmotic stress in a number of cell lines (41, 42). We therefore compared the effect of osmotic stress ranging from 100 to 500 mM on the activity of p38 MAPK and JNK. A 15-min stimulation time was chosen in accordance with the time course of NHE1 activation by osmotic stress, which peaked at around 15 min (data not shown). As shown in Fig. 4, activation of both kinases was found to be osmolarity-dependent. No stimulation of p38 MAPK could be detected when the intensity of the osmotic stress was lower than 300 mM sorbitol. An osmotic stress of 300 mM was found to produce a 2–3-fold activation of p38 MAPK, whereas a 4-fold stimulation was obtained with 400 and 500 mM sorbitol. The response of JNK to hyperosmolality was more pronounced, but an intensity of 400 mM sorbitol was required to produce activation of the kinase.

Inhibition of p38 MAPK (SB203580) Does Not Affect Stimulation of NHE1 by Sorbitol (or Thrombin plus Insulin)

The role of the p38 MAPK cascade in NHE1 activation by growth factors or osmotic stress was finally assessed using a chemical compound, pyridinylimidazole (SB203580). This com-
p42 and p44 MAPKs have been shown to phosphorylate the sequence P-X-S/T-P in their target proteins (44). Interestingly, such a consensus site (two adjacent serine residues, 722 and 725) can be identified in the region of the cytoplasmic domain of NHE1 where the potential phosphorylation sites have been localized (amino acids 700–746). To examine whether p44 MAPK can regulate NHE1 directly, we tested the ability of the kinase to phosphorylate NHE1 in vitro. For this purpose, we performed kinase assay experiments where, as a substrate for the kinase reaction, we used NHE1, immunoprecipitated from pHK2 transfected fibroblasts. As shown in Fig. 6, upper panel, stimulation of the cells with 20% FCS induced the phosphorylation of several proteins immunoprecipitated with the monoclonal anti-HA antibody, therefore commounprecipitating with HA-p44 MAPK. In particular, a protein migrating just under the 112-kDa molecular mass marker could be detected: this protein has been identified as p90 rsk, which was reported to modify the pattern of phosphorylation; NHE1-VSVG could be detected after SDS-PAGE (10% acrylamide) by autoradiography. In a pretreatment of several hours in the presence of 5 μM SB203580 that reduces p38 MAPK activity by more than 90%2 had absolutely no effect on the stimulation of NHE1 either by osmotic stress (300 mM sorbitol) or by growth factors (α-thrombin plus insulin).

Taken together, these findings argue strongly against a role of p38 MAPK in NHE1 activation pathway by osmotic stress or α-thrombin plus insulin. NHE1 is not an in vitro substrate for p44 and p38 MAPKs.

The activity of HA-p44 MAPK and HA-JNK were immunoprecipitated with the anti-HA antibody, and their activity was determined by their ability to phosphorylate ATF2. The phosphorylated ATF2 was detected after SDS-PAGE (12.5% acrylamide) by autoradiography.

The p42/p44 MAPK Cascade Activates NHE1

The p38 MAPK inhibitor was measured as described in the legend to Fig. 3. The changes in pH (pH 7.4, 5) were serum-deprived for 5 h and stimulated with sorbitol (300 mM) or α-thrombin (1 unit/ml) plus insulin (10 μg/ml) in the absence or presence of the p38 MAPK inhibitor (SB 203580, 5 μM; 5-h pretreatment). The changes in pH, induced by these agents with or without the p38 MAPK inhibitor were measured as described in the legend to Fig. 3.

**Fig. 4. Effect of varying intensities of osmotic stress on activation of p38 MAPK and JNK.**

The activity of HA-p38 MAPK and HA-JNK was measured in immune complex protein kinase assays using ATP2 as substrate. PS120 cells stably expressing HA-p38 MAPK or HA-JNK were serum-deprived for 5 h and stimulated for 15 min with varying concentrations of sorbitol from 100 to 500 mM. Cell extracts were prepared as described under “Experimental Procedures”, HA-p38 MAPK and HA-JNK were immunoprecipitated with the anti-HA antibody, and their activity was determined by their ability to phosphorylate ATF2. The phosphorylated ATF2 was detected after SDS-PAGE (12.5% acrylamide) by autoradiography.

**Fig. 5. Inhibition of p38 MAPK does not affect NHE1 activation by growth factors or sorbitol.**

Cells stably expressing NHE1 were serum-deprived for 5 h and stimulated with sorbitol (300 mM) or α-thrombin (1 unit/ml) plus insulin (10 μg/ml). Cells were either unstimulated (-) or stimulated (20% FCS for 5 min) cells (+). The capacity of HA-p44 MAPK to phosphorylate NHE1 was tested in immune complex protein kinase assays using NHE1 as a substrate. NHE1 was immunoprecipitated as described under “Experimental Procedures” with the RP-c28 antibody from PS120 cells stably expressing HA-p44 MAPK (p38 MAPK) instead of HA-p44 MAPK. As shown in Fig. 5, a pretreatment of several hours in the presence of 5 μM SB203580 that reduces p38 MAPK activity by more than 90%2 had absolutely no effect on the stimulation of NHE1 either by osmotic stress (300 mM sorbitol) or by growth factors (α-thrombin plus insulin).

Taken together, these findings argue strongly against a role of p38 MAPK in NHE1 activation pathway by osmotic stress or α-thrombin plus insulin.

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p42 and p54 MAPKs have been shown to phosphorylate the sequence P-X-S/T-P in their target proteins (44). Interestingly, such a consensus site (two adjacent serine residues, 722 and 725) can be identified in the region of the cytoplasmic domain of NHE1 where the potential phosphorylation sites have been localized (amino acids 700–746). To examine whether p44 MAPK can regulate NHE1 directly, we tested the ability of the kinase to phosphorylate NHE1 in vitro. For this purpose, we performed kinase assay experiments where, as a substrate for the kinase reaction, we used NHE1, immunoprecipitated from pHK2 transfected fibroblasts. As shown in Fig. 6, upper panel, stimulation of the cells with 20% FCS induced the phosphorylation of several proteins immunoprecipitated with the monoclonal anti-HA antibody, therefore commounprecipitating with HA-p44 MAPK. In particular, a protein migrating just under the 112-kDa molecular mass marker could be detected: this protein has been identified as p90 rsk, which was reported to modify the pattern of phosphorylation; NHE1-VSVG could be detected after SDS-PAGE (10% acrylamide) by autoradiography. In a pretreatment of several hours in the presence of 5 μM SB203580 that reduces p38 MAPK activity by more than 90%2 had absolutely no effect on the stimulation of NHE1 either by osmotic stress (300 mM sorbitol) or by growth factors (α-thrombin plus insulin).

Taken together, these findings argue strongly against a role of p38 MAPK in NHE1 activation pathway by osmotic stress or α-thrombin plus insulin.

**Fig. 5. Inhibition of p38 MAPK does not affect NHE1 activation by growth factors or sorbitol.**

Cells stably expressing NHE1 were serum-deprived for 5 h and stimulated with sorbitol (300 mM) or α-thrombin (1 unit/ml) plus insulin (10 μg/ml). Cells were either unstimulated (-) or stimulated (20% FCS for 5 min) cells (+). The capacity of HA-p44 MAPK to phosphorylate NHE1 was tested in immune complex protein kinase assays using NHE1 as a substrate. NHE1 was immunoprecipitated as described under “Experimental Procedures” with the RP-c28 antibody from PS120 cells stably expressing HA-p44 MAPK (p38 MAPK) instead of HA-p44 MAPK. As shown in Fig. 5, a pretreatment of several hours in the presence of 5 μM SB203580 that reduces p38 MAPK activity by more than 90%2 had absolutely no effect on the stimulation of NHE1 either by osmotic stress (300 mM sorbitol) or by growth factors (α-thrombin plus insulin).

Taken together, these findings argue strongly against a role of p38 MAPK in NHE1 activation pathway by osmotic stress or α-thrombin plus insulin.

**Fig. 6. p44 MAPK does not phosphorylate directly NHE1 in vitro.**

Upper panel, CCL39 cells stably expressing HA-p44 MAPK were serum-deprived overnight. Extracts were prepared as described under “Experimental Procedures,” and HA-p44 MAPK was immunoprecipitated with the anti-HA antibody from either unstimulated (-) or stimulated (20% FCS for 5 min) cells (+). The capacity of HA-p44 MAPK to phosphorylate NHE1 was tested in immune complex protein kinase assays using NHE1 as a substrate. NHE1 was immunoprecipitated as described under “Experimental Procedures” with the RP-c28 antibody from PS120 cells stably expressing HA-p44 MAPK. As shown in Fig. 6, upper panel, phosphoproteins were detected after SDS-PAGE (10% acrylamide) by autoradiography. Lower panel, immunoblotting of the same samples as in upper panel, with the anti-NHE1 RP-c28 antibody, showed that NHE1 (lanes 3 and 4) and NHE1-VSVG (lanes 5 and 6) were indeed immunoprecipitated.

difficulty, we performed similar experiments using fibroblasts expressing an N terminus VSVG epitope-tagged NHE1. Insertion of the VSVG epitope removed the N-glycosylation of NHE1, and NHE1-VSVG was shown to migrate at the level of the 84-kDa molecular mass marker (Fig. 6, lower panel; lanes 5 and 6), therefore precluding potential interference of the migration of NHE1-VSVG with the migration of p90rsk. As shown in Fig. 6, upper panel, lanes 5 and 6, immunoprecipitation of NHE1-VSVG with the monoclonal anti-VSVG antibody did not modify the pattern of phosphorylation; NHE1-VSVG could be detected by Western blotting with the anti-NHE1 antibody but did not appear as a 32P-labeled protein.

We can conclude from these experiments that p44 MAPK does not appear to phosphorylate NHE1 directly.

It is noteworthy that similar experiments using HA-p38 MAPK instead of HA-p44 MAPK allowed us to show that, as
Inhibitors of the p42/p44 MAPK Cascade Inhibit Growth Factor Activation of NHE1

p44 MAPK Dominant Negative Mutant Inhibits Growth Factor-mediated Activation of NHE1—To investigate the role of the p42/p44 MAPK signaling pathway in the regulation of NHE1 by growth factors, we first examined the effects of blocking the MAPK cascade at the level of MAPK itself. As reported previously, inhibition of endogenous p42/p44 MAPK activity can be achieved by several approaches including transient overexpression of the inactive p44 MAPK-T192A form (27). In Fig. 7A, we have assessed the effect of the expression of the vector p44 MAPK-T192A compared with wild type p44 MAPK or empty vector on the pH response of NHE1 to growth factors (a-thrombin plus insulin or serum). Expression of the dominant negative mutant was found to reduce by 50% the response of NHE1 to growth factors (ΔpH = 0.14 ± 0.04, n = 7 for the cells expressing the p44 MAPK-T192A compared with ΔpH = 0.27 ± 0.02, n = 7 for the cells transfected with the control vector), suggesting the involvement of p42/p44 MAPK in the NHE1 activation pathway initiated by a-thrombin plus insulin or serum.

Expression of MKP-1, a MAPK Phosphatase, Inhibits Growth Factor-mediated Activation of NHE1—As an alternative approach to reduce endogenous MAPK, we used the same transient transfection assay to overexpress MKP-1, a dual specificity phosphatase shown, in vivo and in vitro, to dephosphorylate and inactivate MAPKs (46). As shown in Fig. 7B, in the cells transfected with the control vector, the combination a-thrombin plus insulin induced a cytoplasmic alkalization of 0.16 ± 0.02, n = 4, and expression of MKP-1 was found to significantly inhibit the pH response of NHE1 to a-thrombin + insulin (ΔpH = 0.09 ± 0.02, n = 4). This inhibition is of the same order of magnitude as the inhibition obtained with the dominant negative form of p44 MAPK.

Specific Inhibition of MKK1 Greatly Reduces the Response of NHE1 to Growth Factors but Not to Osmotic Shock—To confirm the involvement of the p42/p44 MAPK cascade in the activation pathway of NHE1, we took advantage of the recent discovery of a specific MKK1 inhibitor, PD 98059, and investigated the effect of this compound on the activation of NHE1 by various stimuli. The MKK1 inhibitor has been shown to specifically inhibit the activation of p42/p44 MAPK without affecting the activity of other MAPKs such as p38 and JNK (47, 48). In CCL39 cells, PD 98059 at 10 μM was demonstrated to inhibit by 70% the activation of p44 MAPK by growth factors (data not shown). As shown in Fig. 7C, PD 98059 (10 μM) was found to significantly inhibit the response of NHE1 to a-thrombin + insulin (65% inhibition); this inhibitory effect could also be detected and was even more pronounced when the cells were stimulated with sorbitol alone. In contrast, stimulation of NHE1 with sorbitol (300 mM) remained unaffected by the same pretreatment with PD 98059.

These results demonstrate that blocking the MKK1 inhibits the activation of NHE1 by a-thrombin plus insulin but not by sorbitol, reinforcing the notion that the p42/p44 MAPK cascade plays an important role in the activation of NHE1 by growth factors and suggesting that, in contrast, this cascade does not seem to be involved in the osmotic activation of NHE1.

Specific Activation of the p42/p44 MAPK Cascade Stimulates NHE1

MKK1 and MKK2 constitute the preferential targets of Raf-1 (18). We therefore finally assessed the contribution of the p42/
on the pH$_i$ of quiescent CCL39-ΔRaf-1:ER cells. Treatment of these cells with 1 μM estradiol resulted in a marked cytoplasmic alkalinization. This change in pH$_i$ is due to activation of NHE1, since it was completely abolished in presence of the NHE1-specific inhibitor HOE 694 (49, 50) (data not shown). The addition of the same concentration of estradiol to the parental cell line CCL39 had no effect on pH$_i$, demonstrating that the estradiol-induced activation of NHE1 resulted from Raf activation. The effect of estradiol on pH$_i$ is comparable with that observed with growth factors like α-thrombin in CCL39-ΔRaf-1:ER or CCL39 cells.

The Time Course of Activation of NHE1 by Estradiol—Fig. 8 shows a time course of activation of NHE1 by estradiol and thrombin. After a lag of 2 min, thrombin induced a rise in pH$_i$ that peaked around 5 min and declined after 15 min but nevertheless persisted for 1 h. The effect of estradiol on pH$_i$ was barely detectable 5 min after stimulation but reached a maximal value within 15 min to remain at a maximum level for 1 h. The time course of activation of NHE1 by estradiol is perfectly compatible with the time course of its stimulation of p42/p44 MAPK. As described previously (33), activation of ΔRaf-1:ER induced the mobility shift-up of both p42 and p44 MAPKs in a time-dependent manner. This retardation of MAPK mobility in SDS-PAGE results from phosphorylation of the two activation sites of MAPK and strictly correlates with MAPK activation (51). The activation of MAPK was detected 5 min after the addition of estradiol and reached its maximum 15–30 min later.

It is important to note that in the case of α-thrombin, the time course of activation of p44 MAPK in hamster fibroblasts, readily detectable after 2 min and reaching a maximum within 5 min (data not shown; Ref. 52), is similarly compatible with an involvement of p44 MAPK in the pathway of NHE1 activation (Fig. 8B).

MKK1 Inhibition Reduces Activation of NHE1 by Estradiol—We next investigated the effect of MKK1 inhibition on estradiol-induced activation of NHE1 (Fig. 8C). The inhibitory effect of PD 98059 on the α-thrombin-induced stimulation of NHE1 in CCL39ΔRaf-1:ER cells was comparable with that observed in the parental cell line CCL39. The effect of PD 98059 on the stimulation of NHE1 induced by estradiol was more pronounced, suggesting that the effects of estradiol are mediated by the only substrates of Raf identified so far, MKK1 and MKK2. We also investigated whether the estradiol- and α-thrombin-induced alkalinizations are cumulative. Combination of estradiol and α-thrombin produced a cytosolic alkalinization similar to that obtained with each agent alone, suggesting that the NHE1 activation pathway initiated by α-thrombin is mainly mediated by the Raf → MAPK cascade.

In conclusion, these results demonstrate that exclusive activation of the Raf → MKK1 → p42/p44 MAPK cascade is able to induce NHE1 activation.

Together, these results strongly suggest an important contribution of the p42/p44 MAPK signal transduction pathway in the regulation of NHE1 in response to growth signals.

Inhibition of MKK1 Also Reduces the Growth Factor Response of Truncated Forms of NHE1 (Δ698 and Δ635)

We next investigated the effect of the MKK1 inhibitor on the activation of the NHE1 deletions Δ698 and Δ635 by α-thrombin. As shown in Fig. 9, the inhibitory effect of PD 98059 on activation by α-thrombin plus insulin is preserved in the Δ698 and Δ635 cells (although the effect was found to be less pronounced for the Δ635 mutant), thus suggesting that the alkalinization observed in the deletion mutants upon stimulation with α-thrombin plus insulin is mediated, in part, by a MKK1-dependent pathway. Since the Δ698 deletion removes the con-
The p42/p44 MAPK Cascade Activates NHE1

**Fig. 9. Effect of the MKK1 inhibitor on activation of NHE1 deletion mutants (Δ698 and Δ635) by α-thrombin plus insulin.** Cells expressing NHE1 or the truncated forms, Δ698 or Δ635, were serum-deprived for 5 h and stimulated with α-thrombin (1 unit/ml) plus insulin (10 μg/ml) in the absence or presence of the PD 98059 inhibitor (10 μM; 1-h pretreatment). The changes in pH, induced by α-thrombin plus insulin with or without the PD 98059 inhibitor were measured as described in the legend to Fig. 3.

**DISCUSSION**

The ubiquitously expressed form of Na+/H+ exchanger, NHE1, is involved in a variety of physiological functions by virtue of its ability to govern intracellular pH. One of the most remarkable features of NHE1 is its capacity to respond to multiple extracellular stimuli such as hormones, growth factors, vasoactive peptides, and integrins as well as to hyperosmotic stress. Because many of these extracellular stimuli share the ability to activate a common protein kinase signaling cascade, we previously proposed (8, 53) that this pathway known as the p42/p44 MAPK cascade could be involved in the activation of NHE1. However, although attractive, this notion remained purely hypothetical until the very recent report by Barber and colleagues (30) and the present study. By using selective molecular tools that positively or negatively interfere at each level of the signaling cascade (Raf-1, MKK1 (MEK1), and p44 MAPK (extracellular regulated kinase 1)), we have provided strong evidence for the contribution of the p42/p44 MAPK cascade in the activation of NHE1.

Perhaps the most persuasive results pointing to the role of this cascade in NHE1 activation were derived from the cell line expressing an estradiol-inducible form of oncogenic Raf-1. There, by the ability to bypass multiple upstream signals known to be generated by many growth factors (phosphatidylinositol 3-kinase, phospholipase Cγ, Ca2+ rise, Rho/Rac-activated pathways), we showed that the rapid and exclusive activation of the p42/p44 MAPK cascade, at the level of Raf-1, was able to activate NHE1 to the same extent and with a comparable time course as that induced by α-thrombin in these cells. Raf-1 activation was found to be sufficient to produce maximal activation of NHE1, and this effect was not cumulative with the effect of α-thrombin. In addition, NHE1 activation mediated either by estradiol or by α-thrombin was inhibited by the specific MKK1 inhibitor PD 98059. These results, therefore, support the notion that α-thrombin action is mediated via the MAPK cascade. The α-thrombin-mediated NHE1 activation, however, was incompletely inhibited by the MKK1 inhibitor. Two explanations can account for this result. First, the concentration of PD 98059 that we used in our experiments (10 μM) was shown to inhibit by 70% the activation of p44 MAPK by α-thrombin; the residual p44 MAPK activity could therefore be sufficient to produce NHE1 activation. Note that even the activation of NHE1 mediated by direct stimulation of Raf, which is acting directly upstream of MKK, was not fully abolished by PD 98059 (Fig. 8C). Second, thrombin-mediated activation of NHE1 might involve, in addition to the p42/p44 MAPK cascade, another pathway, in particular the Ca2+/calmodulin activating step triggered by the rapid and sustained elevation of Ca2+ (12). On the other hand, thrombin has been reported to stimulate p38 MAPK in human platelets (54). In CCL39 fibroblasts, our results show that the effect of the combination α-thrombin plus insulin on p38 MAPK is much less pronounced than its effect on p44 MAPK (Fig. 2); in addition, inhibition of p38 MAPK was found to have no effect on the activation of NHE1 by α-thrombin plus insulin (Fig. 5). Taken together, these results argue against an involvement of p38 MAPK in the thrombin-mediated activation of NHE1.

Finally, our findings demonstrating that reducing p44 MAPK activity by expression of a dominant negative form of p44 MAPK or by expression of the dual specificity phosphatase MKP-1 inhibited NHE1 response to growth factors provided additional evidence for a p42/p44 MAPK-dependent regulation of NHE1. It is noteworthy that these results are in agreement with a recently published report by Barber and colleagues (30). Using a different approach, transient expression of constitutively active kinases and GTPases and dominant negative kinases in CCL39 fibroblasts, they reported that constitutively active Ras V12 stimulates NHE1 through a Raf-1- and MKK-dependent mechanism.

In the context of these two independent studies stressing the contribution of the p42/p44 MAPK cascade in the activation of NHE1, it is important to note that NHE1 does not appear to be a direct target for p42/p44 MAPK. We have demonstrated that p44 MAPK does not phosphorylate NHE1 *in vitro* (Fig. 6); in addition, deletion of the consensus sites for phosphorylation by p44 MAPK (Δ698 mutant) did not prevent the inhibitory effect of PD 98059 on growth factor-mediated activation of NHE1 (Fig. 9). These results allow the mapping of the site sensitive to activation by the p42/p44 MAPK pathway proximal to the 698 residue, presumably even proximal to the 635 residue, since an effect of the MKK1 inhibitor could still be detected for the Δ635 mutant.

Our results therefore reinforce the hypothesis formulated by Wakabayashi *et al.* (11), postulating the existence of one or multiple proteins that would regulate NHE1 activity presumably through interaction with its cytoplasmic domain (566–635 region). Identification of these regulatory proteins should undoubtedly result in valuable information concerning the molecular mechanism of activation of NHE1. Exploitation of the two-hybrid system led P. Fafournoux in our group to isolate several cDNA clones encoding distinct proteins that specifically interact with NHE1 cytoplasmic domain. These putative regulatory proteins as targets of the p42/p44 MAPK cascade are currently under investigation.

A second aim of the present study was to gain better understanding of the molecular mechanism underlying regulation of NHE1 by osmotic stress. Like growth factors, osmotic stress has been shown to activate NHE1 by increasing its sensitivity for H+ (7). Despite these kinetic similarities suggesting a common mode of stimulation, activation of NHE1 during volume regulation was not found to be associated with an increase in NHE1 phosphorylation (55). The Raf → MKK → MAPK cascade was shown to be activated by hyperosmolarity in the renal cell line, Madin-Darby canine kidney cells (56), and in 3Y1 fibroblasts (42). Our results show that, in CCL39 fibroblasts, although the osmotic stress was capable of activating p44 MAPK (Fig. 2), inhibition of MKK1 did not prevent NHE1 stimulation by hyperosmolarity, arguing against the involve-
ment of the p42/p44 MAPK cascade in the osmotically induced activation of NHE1. In this context, it is important to note that the effects observed by Terada and colleagues (56) were strictly dependent upon the intensity of the osmotic stress applied to the cells. In the experiments reported there (56), a minimum of 500 mM raffinose was required to get a stimulatory effect on the MAPK cascade. Here, we show that, in hamster fibroblasts, whereas we could detect a strong stimulation of NHE1 with only 100 mM sucrose (Fig. 3), this osmotic stress was not found to stimulate p44 MAPK (Fig. 2). In view of the results summarized above, it appears that the activation pathways initiated by growth factors and osmotic stress to stimulate NHE1 are distinct. In this regard, it is noteworthy that in a recent study, McSwine and colleagues (57) reported that serum, but not hypertonicity, activates NHE1 by a Ca\(^{2+}\)-dependent process: these results support the notion of two distinct pathways for both stimuli.

New members of the MAPK family, namely p38 MAPK and JNK, have recently been cloned. Among the stimuli that selectively activate these kinases, environmental stresses such as osmotic shock have been identified. For instance, hyperosmolarity was reported to induce a strong stimulation of both p38 MAPK and JNK in HeLa cells (41), phosphorylation of p38 MAPK in a murine pro-B cell line (21), and activation of JNK in Chinese hamster ovary cells (58). In addition, Barber and colleagues (30) have recently reported that a GTPase described to stimulate the Jun kinase cascade, Go13, activates NHE1 (through a Cdc42- and MEK kinase-dependent mechanism). It was therefore of particular interest to analyze the potential involvement of p38 MAPK and JNK in the osmotically induced activation of NHE1. Several lines of evidence argue against this hypothesis. First, as already observed in the case of p44 MAPK, an osmotic shock of 100 mM sucrose susceptible to activate NHE1 was unable to stimulate neither p38 MAPK nor JNK (Fig. 4). Second, activation of NHE1 by hyperosmolarity remained unaffected by inhibition of p38 MAPK (Fig. 5). Taken together, our results do not argue in favor of the contribution of any of the MAPK p44, p38, or JNK cascades in the regulation of NHE1 by osmotic stress.

In conclusion, we have demonstrated that in addition to the well characterized Ca\(^{2+}\)/calmodulin activating pathway (12), NHE1 is activable by the Raf \(\rightarrow\) M KK \(\rightarrow\) p42/p44 MAPK and that this pathway contributes primarily to NHE1 activation by potent growth factors such as α-thrombin. In addition, we demonstrated that this action does not directly involve phosphorylation of NHE1, indicating the role of NHE1 accessory proteins in this activation process. Finally, we found no evidence in favor of the involvement of p38 MAPK and JNK cascades in the osmotically induced activation of NHE1.

Acknowledgments—P. Lenormand is grateful for providing us the cCCL3-ΔRaf-1ER cell line. We thank J. Noël and R. C. Poole, respectively, for the Myc- and VSVG-tagged NHE1 constructions. We thank J. Lavoie, A. Brunet, and F. R. McKenzie for providing unpublished results. J.-M. Brondello, P. Fafournoux, and J. Noël are acknowledged for fruitful discussions.

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