Cytosolic Alkalinitation Increases Stress-activated Protein Kinase/c-Jun NH$_2$-terminal Kinase (SAPK/JNK) Activity and p38 Mitogen-activated Protein Kinase Activity by a Calcium-independent Mechanism*

Lamara D. Shrode‡§, Elizabeth A. Rubie®, James R. Woodgett®, and Sergio Grinstein‡

From the Division of Cell Biology, Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8 and the Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Ontario, Canada M5G 2M9

Stress-activated protein kinases (SAPK) are stimulated by a variety of agents and conditions that also activate the Na$^+$/H$^+$ exchanger (NHE). Activation of the exchanger results in a rapid increase in intracellular pH (pH$_i$), raising the possibility that cytosolic alkalinitation may contribute to SAPK activation. This hypothesis was tested by manipulating the pH$_i$ of U937 cells using permeant weak bases. Three different bases increased pH$_i$ and caused a 4–12-fold increase in SAPK activity with a time course that paralleled intracellular alkalinitation. p38, a related stress kinase, was also stimulated by the weak bases. Stimulation of the stress kinases was not accompanied by changes in cytosolic free calcium nor was the activation of SAPK achieved when calcium was elevated by thapsigargin or calcium ionophores. Weak bases not only alter the pH of the cytosol but also alkalinitize endomembrane compartments such as endosomes and lysosomes. However, the latter do not appear to mediate the stimulation of SAPK, since neither bafilomycin A$_1$ nor desipramine, agents that neutralize acidic endomembrane compartments, activated the kinase. Because hyperosmolarity acutely activates the NHE, we considered whether the resulting cytosolic alkalinitation mediates the activation of SAPK upon cell shrinkage. The addition of amiloride or the omission of Na$^+$, which were verified to inhibit NHE, did not prevent the osmotically induced activation of SAPK. We conclude that cytosolic alkalinitation increases the activity of SAPK and p38 by a calcium-independent mechanism that does not involve acidic intracellular organelles. In addition, even though cell shrinkage is accompanied by alkalinitation due to the activation of NHE, the increased pH$_i$ is not the main cause of the observed stimulation of SAPK upon hyperosmotic challenge.

Stress-activated protein kinase/c-Jun NH$_2$-terminal kinase (SAPK/JNK)$^1$ and p38 mitogen-activated protein kinase (p38 MAPK) are members of a family of enzymes that are activated by a variety of agents and conditions that generate cellular stress. These kinases are members of two parallel, yet independent cascades, with distinct upstream activators and downstream targets. SAPK is activated by SEK (MKK4), which is in turn stimulated by MEKK or MLK3 (1–3). Similarly, p38 MAPK is activated by MKK3 and MKK6 (4, 5). The effector pathways triggered by the stress kinases also differ: the preferred substrate of SAPK is c-Jun, a component of the transcription regulator AP-1 (6, 7), whereas MAPKAPK-2, a serine/threonine kinase, and the transcription factor ATF-2 are main targets of p38 MAPK (8, 9).

The agonists and conditions that activate SAPK and/or p38 are remarkably varied. They include hyperosmolarity, inflammatory cytokines, heat shock, ultraviolet light, and protein synthesis inhibitors such as cycloheximide and anisomycin (for review, see Refs. 10 and 11). Several of these stimuli also activate the Na$^+$/H$^+$ exchanger (NHE), a ubiquitous family of transmembrane proteins involved in the regulation of cytosolic pH, cellular volume, and transepithelial ion transport (for review, see Refs. 12 and 13). For example, hyperosmotic exposure, in most cells, leads to the rapid activation of the NHE (14). Heat shock, another activator of SAPK, also increases NHE activity in Vero cells (15). Moreover, cytokines, including interleukin-1 and TNF$_\alpha$, activate NHE in myocytes (16) and fibroblasts (17), respectively. Ischemia/reperfusion, which activates SAPK (18) and enhances binding of ATF-2 and c-Jun to DNA (19), has likewise been associated with increased transport by the NHE (20). Finally, cycloheximide, a protein synthesis inhibitor that stimulates SAPK, has been shown to increase NHE activity as well (21).

It is unclear whether the activation of the stress kinases and the stimulation of the ion exchanger are related. The kinases may mediate the stimulation of the antiporter, although direct phosphorylation has been ruled out as the mechanism of NHE activation (22). Conversely, the ionic changes initiated by the exchanger may trigger kinase activation. Enhanced NHE activity is expected to increase the intracellular Na$^+$ concentration and elevate the cytosolic pH (pH$_i$). These parameters could in turn activate SAPK and/or p38 MAPK. In this work, we examined the relationship between the cytosolic pH and the kinetic/c-Jun NH$_2$-terminal kinase; TNF$_\alpha$, tumor necrosis factor $\alpha$; NHE, Na/H exchanger; pH$_i$, intracellular pH; BCECF, 2',7'-bis(2-carboxyethyl)-5'-(and 6')-carboxyfluorescein; p38 MAPK, p38 mitogen-activated protein kinase; NHB, Na-HEPES buffer; PIPES, 1,4-piperazinediethanesulfonic acid; [Ca$^{2+}$]$^\text{cyt}$, intracellular calcium concentration; TPA, 12-O-tetradecanoylphorbol-13-acetate; TrEA, triethylammonium chloride/triethylamine; TMA, trimethylammonium chloride/trimethylamine.

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¶ International Scholar of the Howard Hughes Medical Institute and is cross-appointed to the Dept. of Biochemistry at the University of Toronto. To whom correspondence should be addressed: Div. of Cell Biology, Hospital for Sick Children, 555 University Ave., Toronto, Ontario Canada M5G 1X8. Tel.: 416-813-5727; Fax: 416-813-5028; E-mail: sga@sickkids.on.ca.

$^1$ The abbreviations used are: SAPK/JNK, stress-activated protein kinase/c-Jun NH$_2$-terminal kinase; TNF$_\alpha$, tumor necrosis factor $\alpha$; NHE, Na/H exchanger; pH$_i$, intracellular pH; BCECF, 2',7'-bis(2-carboxyethyl)-5'-(and 6')-carboxyfluorescein; p38 MAPK, p38 mitogen-activated protein kinase; NHB, Na-HEPES buffer; PIPES, 1,4-piperazinediethanesulfonic acid; [Ca$^{2+}$]$^\text{cyt}$, intracellular calcium concentration; TPA, 12-O-tetradecanoylphorbol-13-acetate; TrEA, triethylammonium chloride/triethylamine; TMA, trimethylammonium chloride/trimethylamine.

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activation of the stress kinases. In particular, we analyzed the effects of alkalinization on SAPK and p38 MAPK and consid-
ered the possibility that shrinkage-induced activation of NHE is required for activation of the kinases.

EXPERIMENTAL PROCEDURES

Materials—Radiolabeled ATP was purchased from Mende1/Dupont (Guelph, Ontario, Canada). Fetal bovine serum was purchased from Life Technologies, Inc., and the cell culture medium was prepared by the Media Department at Princess Margaret Hospital (Toronto, Onta-
rio, Canada). B€CEF, endo-I, nigericin, and ionomycin were pur-
chased from Molecular Probes (Eugene, OR). All other materials were purchased from Sigma.

Antibodies—Antibodies to SAPK and p38 MAPK were raised in rab-
bit against a pGEX vector containing full-length p54 SAPK or full-
length p38, respectively.

Cell Culture—U937 cells (American Type Culture Collection, Be-
thesda, MD) were grown in Dulbecco’s modified Eagle’s medium sup-
plemented with 10% fetal calf serum in a humidified environment
under 5% CO2. For kinase assays, U937 cells were preincubated for 24 h
in medium supplemented with only 0.5% fetal calf serum.

Cytosolic pH Determinations—To measure pH, U937 cells were sedi-
mented and resuspended in a Na-HEPES-buffered solution (NHB) con-
taining (in mM): 117 NaCl, 25 Na-HEPES, 5.36 KCl, 1.66 MgCl2, 1.36
CaCl2, and 25 glucose, pH 7.4, at 37 °C, at a density of 2 × 107 cells/ml.
This suspension was then incubated with 1 μM acetyloxymethyl ester
form of BECF for 15 min at room temperature. Cells were sedi-
mented, resuspended in fresh NHB, placed into a polystyrene cuvette,
and inserted into the thermally regulated (37 °C) holder of a Perkin-Elmer
650-40 fluorescence spectrophotometer. BECF was continually excited
at 495 nm, and emission was recorded at 525 nm. For each experiment,
fluorescence emission was calibrated internally versus pH, by using the high
KCl/nigericin technique (23).

Cytosolic Calcium Determinations—U937 cells at a density of 2 × 107
cells/ml were sedimented, resuspended in NHB, and incubated with 2 μM acetyloxymethyl ester precursor of indo-1 for 25 min at room tem-
perature. Cells were once again sedimented, resuspended in NHB, and
incubated for an additional 15 min in NHB at 37 °C. Indo-1 fluorescence
was monitored as described above, with the excitation at 331 nm and
the emission recorded at 410 nm. Free cytosolic calcium ([Ca2+]i) was
measured as described previously (24). Briefly, Fmax and Fauto were
obtained by adding 5 μM ionomycin and 1 mM MnCl2, respectively, and
a dissociation constant of 250 nM for the indo-1-Ca2+ complex (25)
was used to calculated [Ca2+]i.

Immunoprecipitation of SAPK and p38—Following incubation under
the conditions specified in the text, aliquots of 5 × 107 U937 cells
were lysed in hypotonic lysis buffer containing 10 mM NaCl, 20 mM PIPES,
pH 7.0, 5 mM EDTA, 0.5% Nonidet P-40, 0.05% β-mercaptoethanol, 0.1
mM phenylmethylsulfonyl fluoride, 100 μM Na3VO4, 20 μg/ml leupeptin,
50 μg/ml NaFp, and 1 mM benzamidine. After 20 min, the lysate was
sheared through a 23-gauge needle, and insoluble material was re-
moled by centrifugation at 21,000 × g for 10 min. Samples were
normalized for the amount of protein, and SAPK and p38 were immu-
noprecipitated from the lysates by incubating for 1 h at 4 °C with
anti-SAPK (1:500 dilution) or anti-p38 (1:250) antibodies, respectively.
Immune complexes were collected by adding 20 μl of protein A-Sepha-
rose beads to the lysate and incubating for an additional 30 min at 4 °C.
The beads were then washed four times with an ice-cold solution which
contained 150 mM NaCl, 16 mM NaHPO4, 4 mM Na2HPO4, and 0.1%
Triton X-100.

Kinase Assays—After immunoprecipitation, the beads were sedi-
mented in 20 μl of kinase buffer containing 50 mM Tris-Cl, 1 mM EGTA,
10 mM MgCl2, and 100 μM (800 nCi) [32P]ATP, pH 7.5. For SAPK kinase assays we added 5 μg of a fusion protein of glutathione S-transferase with residues 5–89 of c-Jun and incubated the samples at 30 °C for 30 min. The kinase reaction was terminated by adding 40 μl of 2 × Laemmli’s sample buffer. Samples were resolved by SDS-polyacrylamide gel electrophoresis on 10% acrylamide gels, stained with Coomassie Blue, destained, and dried. Autoradiography was performed using Mende1/Dupont Reflection film, and radioactivity was quantified using ImagingQuant (Molecular Dyanmics). Kinase assays for p38 were performed similarly, except that a glutathione S-transferase fusion protein comprising the 178
carboxy-terminal amino acids of the NHE-1 isoform of the Na+/H+
exchanger was used as a substrate. The construct encoding this fusion
protein was the kind gift of Dr. L. Fliegel (University of Alberta, Ed-
dmonton, Alberta, Canada). Equal loading of the immunoprecipitated
kinases was confirmed in some of the experiments by immunoblotting with
either anti-SAPK or anti-p38 antibodies. To resolve SAPK, which
co-migrates with the heavy immunoglobulin chain, the samples were
not reduced to avoid dissociation of the IgG complex.

Statistical Analyses—All values are reported as the mean ± S.E. of
the number of experiments specified. Statistical differences between
the control and individual experimental conditions were evaluated us-

Incorporating the Control Experiment into the final step of this experience

RESULTS

Increasing Intracellular pH Activates SAPK—Many of the
substances and conditions that increase SAPK activity, can
also lead to an increase in pHi by activating NHE (see the
introduction). It was therefore of interest to establish whether
these events are causally related. To this end, we studied the
effects of cytosolic alkalinization on SAPK activity. pHi was
manipulated by means of weak electrolytes, and the imposed
changes were monitored fluorometrically using B€CEF. As illu-
strated in Fig. 1A, the pH of suspended U937 cells increased
rapidly upon addition of 30 mM NH4Cl. In six similar experi-
ments, pHi rose from a resting value of 7.36 ± 0.04 to 7.76 ± 0.03
within 10 s. The alkalinization was transient with pHi
returning to near-basal levels within 10 min 7.42 ± 0.03 (Fig.
1, A and B). This recovery likely reflects gradual entry of NHi
via K+ channels and/or the Na’/K+ pump. We next determined
the effect of this alkalinization on SAPK activity. A comparable
exposure of the cells to 30 mM NH4Cl resulted in a reproducible
4-fold increase in SAPK activity (Fig. 1C), which was noticeable
as early as 5 min and was sustained for up to 30 min (Fig. 1, C
and D). Treatment with the weak base did not alter the effi-
ciency of SAPK immunoprecipitation (Fig. 1C, lower panel).

The data described above cannot discern whether NH4Cl
exerts its stimulatory effect on SAPK by alkalinizing the cy-
tosol or by other means. To ascertain the mechanism of activa-
tion, we compared the effects of two other weak bases, namely
trimethylamine (TMA) and triethylamine (TrEA). As shown in
Fig. 1, A and B, these bases also induced a brisk increase in
pHi from 7.43 ± 0.01 to 7.85 ± 0.06 and from 7.41 ± 0.01 to 7.86 ±
0.04 (n = 3), respectively. Unlike the effects of NH4Cl, however,
the alkalinization induced by TMA and TrEA persisted after 10
min (Fig. 1, A and B). The much slower decay of the alkalin-
zation reflects the slower permeation of the protonated forms
of the organic amines. Both TMA and TrEA also activated
SAPK (Fig. 1C). In fact, the stimulation of the kinase was
greater and progressed over the course of the experiment. After
a 5-min incubation in either TMA or TrEA, SAPK activity
increased 6-fold (Fig. 1D). By 10 min, the stimulation was
8-fold, and it was nearly 10-fold by 30 min. The more pro-
nounced activation of the kinase parallels the sustained alka-
linization of the cytosol. Jointly, these results suggest that
SAPK is responsive to changes in the cytosolic pH, regardless
of the agent used to impose them.

The effect of the weak bases was not osmotic in nature, since
care was taken to maintain the total osmolarity constant by
reducing the content of NaCl when the bases were added. In
fact, the cell volume (measured electronically using the Coulter
Channelizer) was not detectably reduced even when the bases
were added on top of the normal osmotic complement of the
medium, likely because the permeation of the weak bases
increased the osmotic content of the cells, which compensated at
least in part for the increased extracellular osmolarity.

Role of Calcium in the Alkalization-induced Increase In

SAPK Activity—Changes in intracellular pH are often accom-
panied by a change in cytosolic calcium concentration
([Ca2+]i). Because it has been previously shown that cytosolic MAPK

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cation in SAPK activation by alkalosis. For this purpose, we compared the effects of NH4Cl to those of calcium ionophores and of thapsigargin, an inhibitor of endomembrane Ca2+-ATPases. By inhibiting pumping into the endoplasmic reticulum, thapsigargin unmasks an endogenous calcium “leak” which results in a transient elevation of [Ca2+]i. The capacitative coupling between depleted stores and the plasmalemmal calcium channels facilitates calcium influx, inducing a sustained elevation of [Ca2+]i, when cells are suspended in calcium-containing media (27). These phenomena were readily reproduced in U937, as shown in Fig. 2A. When exposed to 30 mM thapsigargin, U937 cells responded with a large and sustained rise in [Ca2+]i, from a steady-state level of 375 ± 30 (n = 10) to 1040 ± 114 nM (n = 5) (Fig. 2, A and B). Even higher levels of [Ca2+]i, were attained by exposure to 1 mM ionomycin, a non-fluorescent calcium ionophore. The precise [Ca2+]i levels attained with ionomycin could not be defined, because they exceeded the dynamic range of the probe used (indo-1, $K_I = 250$ nM). By contrast, [Ca2+]i, did not significantly increase at any time after addition of 30 mM NH4Cl (after 5 min [Ca2+]i, was 238 ± 8 nM (n = 5) (Fig. 2, A and B)). In fact, exposure to NH4Cl, after [Ca2+]i, had been elevated by thapsigargin resulted in a sizable decrease in the cytosolic concentration of the cation. After 5 min of incubation with TMA or TrEA [Ca2+]i, averaged 360 ± 1 and 343 ± 4 nM, respectively. It therefore appears unlikely that alkalization-induced activation of SAPK is mediated by an increase in [Ca2+]i. This notion was confirmed by comparing the effects of the weak base on SAPK with those elicited by thapsigargin or the calcium ionophores. Thapsigargin, ionomycin, and A23187 produced only a modest stimulation of SAPK up to 10 min after addition, much smaller than that induced by anisomycin (Fig. 1, C and D). The effects of these calcium mobilizing agents are considerably smaller than those of the weak bases (cf. Fig. 1), despite the much greater effects of the former on [Ca2+]i. Thus, an increase in [Ca2+]i, cannot explain the stimulatory effects of weak bases on SAPK.

Role of Acidic Endomembrane Compartments in SAPK Activation by NH4Cl—Exposure of cells to permeating weak bases will alkalinate not only the cytoplasm but also intracellular compartments, particularly those that are maintained at an acidic pH by vacuolar H+-ATPases. It is therefore possible that the stimulatory effect of the weak bases on SAPK is mediated by a pH change within an endomembrane compartment. This is particularly relevant since Verheij et al. (28) showed that TNFα activates SAPK via ceramide, which is generated by hydrolysis of sphingomyelin within both neutral and acidic compartments. Therefore, it was conceivable that NH4Cl induced activation of SAPK through modulation of sphingomyelinase activity in an acidic compartment. Two approaches were used to test this possibility. First, we preincubated cells with desipramine, which has previously been shown to inhibit acidic sphingomyelinase by neutralizing the acidic compartment (29). As illustrated in Fig. 3, A and B, preincubation with 10 μM desipramine for 1 h had no effect on SAPK activity, but obliterated the ability of TNFα to activate SAPK. In contrast, desipramine had no effect on the ability of NH4Cl to activate SAPK.

The role of endomembrane acidic compartments was also evaluated using bafilomycin A1, a potent and very selective inhibitor of vacuolar-type H+-ATPases. This inhibitor permeates into the cells, reaches the ATPases of intracellular compartments, and thereby dissipates their pH gradients, while affecting the cytosolic pH minimally. Unlike the weak bases, treatment with 100 nM bafilomycin for up to 1 h had only a marginal statistically insignificant ($p > 0.1$) effect on SAPK.
activity (Fig. 3, C and D). In addition, pretreatment with bafilomycin did not preclude the ability of NH₄Cl to activate SAPK. Therefore, neutralization of acidic endomembrane compartments is not likely the mechanism whereby weak bases activate SAPK.

Changes in the pH of intracellular compartments are similarly unlikely to play a role in the activation of SAPK effected osmotically or by anisomycin. This conclusion was derived from the experiments in Fig. 4. Neither desipramine nor bafilomycin, at concentrations known to inhibit acidic sphingomyelinase and the H⁺ pump, respectively, had a significant inhibitory effect on the activation of SAPK by anisomycin or by hypertonic sorbitol (Fig. 4, A–D).

Cytosolic Alkalinization Also Activates p38 MAPK—p38 MAPK, a homolog of the yeast Hog1 protein, is also a member of the stress-activated protein kinase family (30). Like SAPK, p38 MAPK is activated by anisomycin, hyperosmolarity, and the cytokines interleukin-1 and TNFα (10). We therefore considered the possibility that, like SAPK, p38 MAPK could also be activated by changes in cytosolic pH. Thus, U937 cells were exposed to either NH₄Cl or TMA, and the activity of immunoprecipitated p38 MAPK was assessed in vitro using as a substrate the carboxyl-terminal domain of NHE-1, which we had found earlier to be effectively phosphorylated by this kinase.2

As illustrated in Fig. 5, exposure to NH₄Cl resulted in a 5-fold increase in p38 MAPK activity detectable within 5 min and maintained through 30 min. Similar results were obtained with the organic base TMA, suggesting that, like SAPK, p38 MAPK is responsive to changes in pH. As in the case of SAPK, the

2 L. D. Shrode, E. A. Rubie, J. R. Woodgett, and S. Grinstein, unpublished observations.
weak bases did not affect the efficiency of p38 immunoprecipitation (lower panel in Fig. 5A).

Is the Alkalization Resulting from Shrinkage-induced Activation of the Na\(^+\)/H\(^+\) Exchanger Responsible for the Shrinkage-induced Activation of SAPK?—In addition to activating SAPK and p38 MAPK, hyperosmotic treatment also increased pH\(_i\). This cytosolic alkalinization in most cells is mediated by the shrinkage-induced activation of the Na\(^+\)/H\(^+\) exchanger (NHE). Since our present data show that alkalinization suffices to activate SAPK as well as p38 MAPK, we entertained the possibility that shrinkage-induced alkalinization, mediated by the NHE, is responsible for the observed activation of the kinases. When exposed to a hyperosmotic solution, U937 cells underwent a rapid intracellular alkalinization (Fig. 6A). pH increased at a rate of 0.05 ± 0.007 pH unit/min to a new steady-state pH\(_i\) of 7.58 ± 0.05 (n = 6). As reported for other cell types, this shrinkage-induced alkalinization in U937 cells was Na\(^+\)-dependent and inhibited by amiloride (Fig. 6A). Indeed, in the absence of external Na\(^+\) or in the presence of the diuretic, pH\(_i\) became more acidic, at a rate of −0.02 ± 0.005 (n = 3) and −0.06 ± 0.05 pH units/min (n = 3), respectively. These findings confirm that cell shrinkage activates the NHE in U937 cells.

We then tested whether the alkalinization generated by the antiporter is responsible for stimulation of SAPK. Cells were stimulated with hypertonic sorbitol, and the extent of SAPK activation was tested in otherwise untreated cells or under conditions shown above to preclude antiporter-mediated alkalinization of the cytosol. As shown in Fig. 6B, SAPK was comparably activated in Na\(^+\)-containing and Na\(^+\)-free media, in the presence and absence of amiloride. Summarized data from multiple experiments are presented in Fig. 6C. We conclude that, while alkalinization alone can activate SAPK and p38 MAPK, osmotic stimulation of the NHE is not responsible for the activation of SAPK.

DISCUSSION

Because a variety of stimuli concomitantly activate SAPK and the NHE, we considered the possibility that these events are related. Our data indicate that a cytosolic alkalinization of a magnitude comparable to that attained by stimulating the antiporter suffices to activate SAPK and p38 MAPK. While these data are suggestive of a causal relationship, subsequent exper-

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**Fig. 4. Role of endomembrane compartments in the activation of SAPK induced by anisomycin and hyperosmolarity.** U937 cells were pre-equilibrated for 1 h in NHB, then exposed to the agents indicated. SAPK was subsequently immunoprecipitated, and its activity was determined using c-Jun as a substrate, as in Fig. 1. Representative radiograms are shown in A and C, while the average of multiple experiments, quantified by phosphorimaging, is presented in B and D. Data in B and D are means ± S.E. of the number of experiments indicated. A and B, cells were preincubated for 1 h with or without 10 μM desipramine (Des). SAPK was next stimulated with either anisomycin (10 μg/ml) or sorbitol (400 mM) for 30 min. C and D, cells were preincubated for 1 h with or without 100 nM bafilomycin A₁ (Baf). SAPK was next stimulated with anisomycin or sorbitol as in A.

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**Fig. 5. NH\(_4\)Cl and TMA activate p38 in U937 cells.** U937 cells were pre-equilibrated for 1 h in NHB and then exposed to sorbitol (NHB + 400 mM for 30 min) or to 30 mM either NH\(_4\)Cl and TMA for the indicated times. p38 was subsequently immunoprecipitated, and its activity was determined using a fusion of glutathione S-transferase with the carboxyl-terminal domain of NHE-1 (fpNHE) as a substrate. A representative radiogram is shown in the top panel of A, while the average of three experiments, quantified by phosphorimaging, is presented in B. The bottom panel in A is a p38 immunoblot, confirming that comparable amounts of the kinase were immunoprecipitated in each instance. The data in B are means ± S.E.
Alkalination Activates SAPK and p38 MAPK

Figure 6. Role of the Na+/H+ exchanger in the osmotic activation of SAPK. A, cytosolic pH measurements. U937 cells were suspended in NHB with or without 1 mM amiloride (Amil) or in Na+-free medium. Where indicated, the osmolarity of the medium was increased by addition of 250 mM NaCl. B and C, SAPK activity determinations. Cells were pre-equilibrated in NHB with or without amiloride or in Na+-free medium, then challenged osmotically by addition of 400 mM sorbitol. SAPK was subsequently immunoprecipitated, and its activity was determined using c-Jun as a substrate, as shown in B, and the average of multiple experiments, quantified by phosphorimaging, is presented in C. The data are means ± S.E. of the number of experiments indicated.

...ments demonstrated that activation of the kinases occurs even when NHE-induced alkalization is precluded pharmacologically or by ionic substitution. These findings rule out that the activation of SAPK and p38 MAPK is secondary to the activation of Na+/H+ exchange. The converse relationship, namely that the NHE is stimulated by a pathway involving the stress kinases, remains a viable possibility. Alternatively, the two events may lie on parallel pathways, which could conceivably share common upstream elements. In this regard, independent studies have shown that members of the Rho family of small GTP-binding proteins can stimulate SAPK (2, 31) as well as NHE activity (32). Cdc42 has been found to activate Rac which in turn can activate Rho (33). These GTP-binding proteins regulate the formation of filopodia, lamellipodia, and stress fibers, and it is noteworthy that NHE-1, the “housekeeping” isoform of the antiporter, has been reported to accumulate at or near these structures (34). Hence, it is possible that activation of Cdc42, Rac, and/or Rho promotes the interaction between the NHE and the cytoskeleton, thereby increasing antiport activity, as well as activating SAPK pathways.

Alternatively, heterotrimeric G proteins could be the common step leading to the parallel activation of NHE and the stress kinases. Prasad et al. (35) demonstrated that constitutively active GTPase-deficient mutants of Gq12 and Gq13 promote the activation of SAPK. Interestingly, Gq13 has also been shown to activate the NHE (36), seemingly via pathways involving small GTP-binding proteins of the Rho family and MEKK1 (32). Thus, Gq13 may give rise to the coordinate, yet independent activation of SAPK and NHE by cell shrinkage or other stimuli.

Parallel yet independent activation of the stress kinases and of the NHE is also suggested by the diverging time courses of these events in cells challenged with hypertonic solutions: cation exchange is noticeable and attains maximal rate within seconds, while full osmotic activation of SAPK or p38 MAPK is delayed, reaching maximal level tens of minutes after cell shrinkage (see Ref. 37). This temporal disparity suggests that the two responses may have different functional roles in cell volume homeostasis. We speculate that the early response of the antiporter is intended to accomplish the acute phase of regulatory volume increase, an immediate defense against osmotic perturbation. In addition, however, chronic exposure of cells to hyperosmolality is known to be counterbalanced by a slower accumulation of organic osmolytes (38). The latter process depends on an increase in biosynthetic enzymes (39) and in the abundance of organic osmolyte transporters (40), which are in turn associated with elevated mRNA levels (41). Therefore, it is conceivable that activation of the stress kinases signals an increase in transcription via activation of c-Jun or ATF-2, to prepare the cell for a prolonged period of hyperosmotic exposure.

In summary, stressful situations seemingly activate both NHE as well as SAPK and p38 MAPK. While alkalination such as that generated by Na+/H+ exchange is capable of stimulating the stress kinases, neither chemical (anisomycin) nor physical stresses (hypertonicity) require a pH change to exert their stimulatory effect on the kinases. Nevertheless, it is conceivable that other situations leading to stimulation of Na+/H+ exchange may secondarily lead to activation of the stress kinases. Stimulation of the exchanger can be induced by integrin engagement, activation of mitogenic receptors, and by some hormones, and some of these treatments also result in activation of stress kinases.

Because osmotic cell shrinkage stimulation of NHE precedes activation of the kinases, we find it unlikely that SAPK and/or p38 MAPK mediate the stimulation of the antiporter. Instead, we favor the hypothesis that the two events are parallel yet independent responses, perhaps triggered by a common early event such as activation of small or heterotrimeric G proteins. The divergent activation of these pathways may provide the cell with separate complementary responses to the early and sustained phases of stressful perturbations.

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Lamara D. Shrode, Elizabeth A. Rubie, James R. Woodgett and Sergio Grinstein

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