Hypertonicity Activates $\text{Na}^+/\text{H}^+$ Exchange through Janus Kinase 2 and Calmodulin*

Maria N. Garnovskaya, Yuri V. Mukhin, Tamara M. Vlasova, and John R. Raymond

The type 1 sodium-hydrogen exchanger (NHE-1) is a ubiquitous electroneutral membrane transporter that is activated by hypertonicity in many cells. NHE-1 may be an important pathway for $\text{Na}^+$ entry during volume restoration, yet the molecular mechanisms underlying the osmotic regulation of NHE-1 are poorly understood. In the present study we conducted a screen for important signaling molecules that could be involved in hypertonicity-induced activation of NHE-1 in CHO-K1 cells. Hypertonicity rapidly activated NHE-1 in a concentration-dependent manner as assessed by proton microphysiometry and by measurements of intracellular pH on a FLIPR™ (fluorometric imaging plate reader). Inhibitors of $\text{Ca}^{2+}$/calmodulin (CaM) and Janus kinase 2 (Jak2) attenuated this activation, whereas neither calcineurin A nor inhibitors of protein kinase C, Ras-ERK1/2 pathway, Src kinase, and Ca$^{2+}$/calmodulin-dependent enzymes had significant effects. Hypertonicity also resulted in the rapid tyrosine phosphorylation of Jak2 and STAT3 (the major substrate of Jak2) and CaM. Phosphorylation of Jak2 and CaM were blocked by AG490, an inhibitor of Jak2. Immunoprecipitation studies showed that hypertonicity stimulates the assembly of a signaling complex that includes CaM, Jak2, and NHE-1. Formation of the complex could be blocked by AG490. Thus, we propose that hypertonicity induces activation of NHE-1 in CHO-K1 cells in large part through the following pathway: hypertonicity $\rightarrow$ Jak2 phosphorylation and activation $\rightarrow$ tyrosine phosphorylation of CaM $\rightarrow$ association of CaM with NHE-1 $\rightarrow$ NHE-1 activation.

The ubiquitous isoform of the Na$^+/\text{H}^+$ exchanger (NHE-1) is essential for the regulation of cellular volume and intracellular pH. NHE-1 is nearly quiescent in resting cells but is activated by a variety of hormones and growth factors (1, 2). NHE-1 is also rapidly activated by hypertonic stress in many cells (3), and this may be an important pathway for Na$^+$ entry during volume restoration. Despite the potential importance of this process, the molecular mechanisms underlying the regulation of NHE-1 by hypertonicity have not been fully elucidated. The rapid activation of NHE-1 is often associated with an increase in its phosphorylation (3). Kinases that have been shown to directly phosphorylate NHE-1 include p90 S6 kinase (4) and the Nck-interacting kinase (5). However, deletion of the major phosphorylation sites contained within residues 636–815 of NHE-1 only reduces its response to growth factors by about 50% (6), suggesting that mechanisms of regulation other than direct phosphorylation of NHE-1 are also important.

Hypertonicity-induced shrinkage of mammalian cells is a powerful stimulant for many protein kinases that could play important direct or indirect roles in activating NHE-1. These include mitogen-activated protein kinases such as extracellular signal-regulated protein kinase (ERK), stress-activated protein kinases (c-Jun N-terminal kinases) (7–10), Src family tyrosine kinases p59$^{fgr}$ and p56$^{Lck}$ (11), protein kinase C (12–14), Janus kinase 15, and phosphatidylinositol 3-kinase (13), although there is not a consensus that any of those kinases mediate hypertonicity-induced activation of NHE-1.

Hypertonicity induces rapid cellular shrinkage and activation of NHE-1 and, concomitantly, extensive tyrosine phosphorylation of members of the Src family of tyrosine kinases p59$^{fgr}$ and p56$^{Lck}$ but not stress-activated protein kinase, p38, or ERK1/2 (11). In that regard, ERK, stress-activated protein kinase, and p38 do not appear to be involved in the activation of NHE-1 in human neutrophils or lung fibroblasts (9, 11). Cell shrinkage associated with hypertonic exposure induces tyrosine phosphorylation of several proteins of ~40, 80–85, and 110–130 kDa in CHO cells (16). The 40-kDa protein appears to be ERK2, although this enzyme was not shown to be involved in the shrinkage-induced stimulation of NHE-1 (16). The 80–85-kDa protein was later identified as cortactin (cortical actin filament cross-linking protein), a major target of the hypertonicity-stimulated Src family kinase Fyn (17). However, Src family kinases did not appear to be responsible for the hypertonic regulation of NHE-1 (17). The calmodulin (CaM)-dependent myosin light chain kinase is involved in shrinkage-induced activation of the NHE in astrocytes (18, 19). There is controversy.
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dictionary evidence about a potential role for protein kinase C (PKC) in hypertonicity-induced activation of NHE. In Ehrlich ascites tumor cells, PKC inhibitors block hypertonicity-induced activation of NHE-1 (12), whereas in lymphocytes (20) and in astrocytes (18), PKC-dependent pathways are not involved in this process.

The activation of NHE-1 by hypertonic challenge does not appear to involve direct phosphorylation of the exchanger itself (3). This is because NHE-1 is still activated in response to hypertonic stress in A10 cells transfected with truncated mutants of NHE-1 that lack all of its putative phosphorylation sites (21). Thus, it is somewhat difficult to reconcile that finding with other reports supporting a general role for phosphorylation in the activation of NHE-1. A possible explanation that could reconcile the differences in those reports would be phosphorylation of ancillary regulatory proteins involved in the hypertonicity-induced stimulation of NHE-1. One such protein is CaM, which has been shown to be important in the activation of NHE-1 by multiple distinct stimuli (22, 23). Deletion of a high affinity CaM binding domain has been shown to inhibit hypertonicity-induced activation of NHE-1 by up to 80%, suggesting that CaM is a key regulator of shinkage-induced activation of NHE (22). We recently explored the interactions between CaM and phosphorylation reactions in the process of activation of NHE-1 by the G protein- and phospholipase C-linked bradykinin B2 receptor in cultured mIMCD-3 cells. We proposed the existence of a novel pathway through which Jak2 (activated by the B2 receptor) indirectly increases the activity of NHE-1 by inducing tyrosine phosphorylation of CaM, leading to increased binding of CaM to NHE-1 (24). We hypothesized that this new pathway might also mediate hypertonicity-induced activation of NHE-1. This possibility is plausible in that hypertonicity has been shown previously (15) to activate the Jak/STAT pathway. Thus, in the current report, we studied the roles of CaM and Jak2 in the activation of NHE-1 by hypertonicity in CHO-K1 cells. Our results demonstrate that this pathway might be a fundamental mechanism for the rapid regulation of NHE-1 in multiple cell types.

EXPERIMENTAL PROCEDURES

Materials—Fluo-3 and 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) were purchased from Molecular Probes (Eugene, OR). 5-(N-Ethyl-N-isopropyl)-amiloride was purchased from RBI (Natick, MA). ET-18-OCH3, AG490, and D609 were from Biomol (Plymouth Meeting, PA). Tetramethylammonium chloride, probenecid, phorbol 12-myristate 13-acetate, and various salts were from Sigma. 1,2-Bis(2-aminophenoxyethane-N,N,N′,N′-tetraacetic acid ethoxymethyl ester (BAPTA-AM), calmidazolium, fluphenazine, W-7, GF109203X, PD98059, and anti-phosphotyrosine monoclonal antibody were from Calbiochem. Anti-CaM monoclonal antibody, anti-Jak2-agarose-conjugated antibody, and anti-phosphotyrosine polyclonal antibody were from Upstate Biotechnology (Lake Placid, NY). Anti-phosphospecific Jak2 antibody was from BIOSOURCE International (Camarillo, CA) or QCB (Hopkinton, MA). Anti-phosphospecific STAT antibody and immobilized phosphotyrosine monoclonal antibody were from Cell Signaling Technology (Beverly, MA). Anti-NHE-1 polyclonal antibody was from Chemicon International (Temecula, CA). All cell culture media and supplements were from Life Sciences (Grand Island, NY). CHO-K1 cells were purchased from American Type Culture Collection (Manassas, VA). Polycarbonate cell culture inserts for microphysiometry and black 96-well microtiter plates needed for the FLIPR™ were from Corning Costar (Cambridge, MA). Black pipette tips were from Molecular Devices Corporation (Sunnyvale, CA).

DNA Constructs—DNA constructs were obtained from the following sources: minigene encoding the C-terminal residues of β-adrenergic receptor kinase (ßARK-CT) and Csk were from Dr. R. Lefkowitz (Duke University), dominant negative Ras (N17Ras) was from Drs. D. Aultschuler and M. Ostrowski (Columbus, OH), and dominant negative Raf (ΔNRAf) was from Dr. L. T. Williams (San Francisco, CA).

Cell Culture—CHO-K1 cells were maintained in Ham’s F-12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 400 μg/ml G-418 at 37 °C in a 5% CO2-enriched, humidified atmosphere.

Transfection of CHO-K1 cells—Transient expression of the cDNA constructs was achieved in CHO-K1 cells by transfection with 1 μg of the various plasmids in the presence of 10–15 μl of LipofectAMINE™ (2 h in serum- and antibiotic-free medium) as described previously (25). Transfection efficiency (>90%) was evaluated by co-transfecting with 0.2 μg of cDNA encoding a green fluorescent protein (Clontech) as a marker.

Microphysiometry—The microphysiometer uses a light addressable silicon sensor to detect extracellular protons (26). Each of eight channels has two inlet ports for buffers, one of which usually contains a vehicle control, and the other of which carries the test substance. The other buffer is superfused with Hepes-buffered Ringer solution, and valve switches on each port cycle are totally controlled by a programmable computer. Acidification rate data are transformed by a personal computer running CytoSoft™ version 2.0, and are presented as the extracellular acidification rate (ECAR) in μV/s, which roughly correspond to milliH2O (designation for pH unit/1000) units/min (Nernst equation). To facilitate comparison of data between two channels, values are typically expressed as a percentage of a baseline determined by computerized analysis of the five data points prior to exposure of the cell monolayers to a test substance.

For all of the experiments, CHO-K1 cells were plated onto polycarbonate membranes (3-μm pore size, 12-mm size) at a density of 300,000 cells per well the day prior to experiment. After cells were adhered to the membrane they were grown-attached in an equilibrium-free culture medium for 20 h before the experiment. The day of the study, the pump cycle was set to perfuse cells for 60 s, followed by a 30 s “pump-off” phase, during which proton efflux was measured from the eighth through the twenty-eighth seconds. Cells were exposed to the test agent for three or four cycles (270–360 s). Valve switches (to add or remove test agents) were performed at the middle of the pump cycle. Data points were then acquired every 90 s. The peak effect during stimulation was expressed as the percentage increase from baseline.

Measurement of Intracellular pH Using BCECF—pH. Using BCECF-AM, we used a FLIPR™ fluorometric imaging plate reader system (27) to measure intracellular pH in CHO-K1 cells. Cells were seeded (~50,000 cells/well) in 96-well clear bottom black microplates (Corning Costar Corp., Cambridge, MA) and left overnight in CO2 incubator at 37 °C. On the day of assay, cells were loaded with a dye (5 μM BCECF acetoxymethyl ester) in a loading buffer (Hank’s balanced salt solution, pH 7.4, containing 20 mM HEPES, 2.5 mM probenecid) for 1 h at 37 °C. 20 min before the end of the loading phase, 20 mM NH4Cl was added to each well. Cells were then washed four times with the loading buffer containing 20 mM NH4Cl. In the FLIPR™, cells are acid-loaded by an ammonium chloride prepulse protocol. In this method, the extracellular pH is maintained within a range 7.4–7.8 and NH4Cl, in an equimolar amount, is rapidly recapturilized in the cell interior. When the extracellular medium is changed to a buffer lacking NH4Cl, intracellular NH3 diffuses rapidly out of the cell, causing the cells to become acutely loaded with protons donated from NH4+ (28). Some cells were pretreated with 1 μM EIPA for 10 min. Cells were allowed to recover from the acid load in the presence of sodium and various test substances. Fluorescence excitation was obtained using an Argen laser (488 nm wavelength at 300 mW), and emission (~540 nm) was monitored kinetically (29).

We also performed experiments without the acid load. Cells in 96-well plates were loaded with BCECF for 1 h. Negative control wells were pretreated with 5 μM EIPA for 10–20 min. Plates were placed in the FLIPR™, and fluorescence tracings were received in the presence or absence of different concentrations of sucrose. The tracings from at least six wells under the same conditions were averaged, and tracings from the negative controls were subtracted. The slopes of fluorescence changes were calculated as rate fluorescence change (fluorescence counts per second).

Immunoprecipitation—Quiescent CHO-K1 cell monolayers were treated with hypertonic media or vehicle for 10 min and lysed in 1 ml/100-mm dish of radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% Nonidet P-40, 1 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, aprotinin, leupeptin, and pepstatin at 1 μg/ml of each). Cell lysates were preincubated by incubating with anti-Jak2/protein A-agarose, with anti-NHE-1 antibody or with polyclonal anti-phosphotyrosine antibody overnight at 4 °C. Phosphotyrosine and NHE-1 immunoprecipitates were captured by addition of
protein A-agarose. The agarose beads were collected by centrifugation, washed three times with radioluneimmunoprecipitation assay buffer, re-
suspended in 2× Laemmli sample buffer, boiled for 5 min, and sub-
jected to SDS-PAGE and subsequent immunoblot analysis with mono-
clonal anti-CaM or anti-phosphotyrosine IgG. The same Western blots
were re-probed with the antibody used for immunoprecipitation to
assure that equal amounts of protein were loaded in each lane.

Jak2 Phosphorylation Assay—Phosphorylation of Jak2 in response
to hypertonic medium was assessed using a Jak2 dual phosphospecific
antibody. Quiescent cells were treated with hypertonic medium for 10
min and lysed in radioimmune precipitation assay buffer. The lysates
were subjected to SDS-PAGE under reducing conditions with 4–20%
pre-cast gels (Novex, San Diego, CA). After semi-dry transfer to poly-
vinyldene difluoride membranes, membranes were blocked with a
Blotto buffer and incubated with the phospho-Jak2 antibody (0.5 μg/
ml). After incubation with alkaline phosphatase-linked secondary anti-
bodies immunoreactive bands were visualized by a chemiluminescent
method (CDP Star™, New England Biolabs, Beverly, MA) using pre-
flushed Kodak X-AR film and quantified using a GS-670 densitometer
and Molecular Analyst software (Bio-Rad). Alternatively, bands were
visualized with a Storm™ PhosphorImager (Amersham Biosciences).
To ensure that equal amounts of protein were loaded in each lane, bands
were stripped and re-probed with control Jak2 antibody, which recog-
nizes both phosphorylated and nonphosphorylated Jak2 equally.

STAT3 Assay—STAT3 phosphorylation was assessed using a phos-
pho-specific STAT3 (Tyr705) antibody, which specifically detects STAT3
only when activated by phosphorylation at Tyr705. Cells were treated
with NaCl or sucrose, harvested in Laemmli buffer, and subjected to
SDS-PAGE under reducing conditions with 4–20% pre-cast gels
(Novex). After semi-dry transfer to polyvinylidene difluoride mem-
branes, membranes were blocked with a Blotto buffer and incubated
with the phospho-STAT3 antibody at a 1:1000 dilution. After incubation
with alkaline phosphatase-linked secondary antibodies, immunoreac-
tive bands were visualized using Vistra ECF Western blotting system
(Amersham Biosciences) and a Storm™ PhosphorImager (Amersham Biosciences).
To ensure that equal amounts of protein were loaded in each lane, bands were stripped and re-probed with control STAT3 anti-
body, which recognizes total (phosphorylation-state independent) levels
of STAT3 protein.

Statistical Analysis—Data were analyzed for repeated measures by
Student’s t test for unpaired two-tailed analysis. p values less than 0.05
were considered significant.

RESULTS

Hypertonicity Activates NHE-1 in CHO-K1 Cells—We used a
Cytosensor™ microphysiometer (Molecular Devices Corporation,
Sunnyvale, CA) (26) to measure proton efflux from intact
monolayers of CHO-K1 cells plated onto polycarbonate mem-
branes. We have used the microphysiometer previously to spe-
cifically study Na+/H+ exchange in fibroblasts (25, 30), enter-
cytes (31), and polarized epithelial mIMCD-3 cells (24). Fig. 1A
shows that cells treated with media made hypertonic (450
mosmol/liter) by addition of NaCl (closed circles) or sucrose
(open circles) to isotonic medium had rapid >70% increases in
ECAR. Fig. 1B shows that the stimulatory effect of sucrose
occurred in sodium-containing balanced salt solution but not in
a solution in which tetramethylammonium was substituted for
sodium. Pretreatment of cells for 30 min with 1 μM of the
NHE-1 inhibitor, EIPA, did not affect the basal rate of extra-
cellular acidification but greatly attenuated the increase in
ECAR caused by hypertonic treatment. Thus, the increased
ECAR induced by exposure to hypertonic media was both so-
dium-dependent and inhabitable by an NHE inhibitor, suggest-
ing the involvement of NHE-1 (CHO-K1 cells only express
NHE-1). In some experiments, cells were perfused with a buffer
in which pyruvate was substituted for glucose to minimize the
effects of glycolysis on ECAR. Under these conditions, medium
made hypertonic by the addition of sucrose increased ECAR by
~85% (Fig. 1B).

To support our microphysiometry data, we used another
method of studying NHE activity by measuring intracellular
pH using BCECF fluorescence on a FLIPR™. Cells were acid-
loaded by the ammonium chloride prepulse method as de-
scribed under “Experimental Procedures” and then were al-
lowed to recover in the presence or absence of different
concentrations of sucrose with or without the addition of EIPA.
Those experiments showed that sucrose increases the rate of
recovery from acid load in a concentration-dependent manner
(not shown). We also monitored NHE-1 activation on a FLIPR™
without an acid load to more closely approximate the measure-
ment conditions used in the microphysiometry experi-
ments. The results presented in Fig. 1C demonstrate a con-
centration-dependent increase in the rate of fluorescence
change after stimulation of cells with media made hypertonic
by addition of sucrose. Thus, media made hypertonic by addi-
tion of NaCl or sucrose activate NHE-1 in CHO-K1 cells as
measured by two independent assays. Further, hypertonicity-
induced changes in ECAR are due nearly completely to the
activation of NHE-1 independent of glycolysis-mediated pro-
duction of protons.

Lack of a Role for Classical Signaling Intermediates in Hy-
pertonicity-induced Activation of NHE-1 in CHO-K1 Cells—
Fig. 2 shows studies in which various inhibitors were examined
for effects on hypertonicity-stimulated ECAR in CHO-K1 cells.

FIG. 1. Hypertonic medium stimulates NHE-1 activity in
CHO-K1 cells. Microphysiometry was performed on quiescent
CHO-K1 monolayers as described under “Experimental Procedures.”
Panel A, medium made hypertonic (450 mosmol/liter) by the addition of
NaCl (closed circles) or by the addition of sucrose (open circles) stimu-
lates ECAR. Shaded area represents time during which cells were
exposed to NaCl or sucrose. Panel B, ECAR stimulated by medium
made hypertonic (450 mosmol/liter) by addition of sucrose in various
buffers, including a balanced salt solution containing glucose and NaCl
without and with 1 μM EIPA, a balanced salt solution containing tet-
ramethylammonium substituted mM per mM for sodium, and sodium-
replete buffer with pyruvate substituted for glucose to minimize the
glycolytic component of the acidification response. All experiments were
performed at least five times. Error bars represent the S.E. (*, p < 0.01).
Panel C, the FLIPR™ was used to monitor NHE-1 activity by meas-
uring pH. Cells were loaded with BCECF as described under “Exper-
imental Procedures.” Negative control wells were pretreated with 5 μM
EIPA for 10–20 min. Plates were placed in the FLIPR™ and fluores-
cence tracings were recorded in the presence or absence of different
concentrations of sucrose. The data were calculated as average fluores-
cence tracing from at least six wells after subtraction of signals from
negative control wells. Rate of fluorescence change represents the
slopes of the fluorescence changes. Experiments were performed at
least three times. Data are presented as mean ± S.E.

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Fig. 2. Comparative effects of inhibitors of candidate molecules upon activation of NHE by sucrose in CHO-K1. ECAR measurements from CHO-K1 cell monolayers were obtained as described under “Experimental Procedures.” Panel A, lack of involvement of pertussis-sensitive G protein α subunits and G protein βγ subunits. Pertussis toxin treatment (200 ng/ml for 18 h) had no effect on sucrose-stimulated activity of NHE-1 but completely blocked 5-HT-increased ECAR (n = 6 for each). βARK-CT, a βγ-sequestering reagent, does not affect sucrose-activated ECAR (n = 3). Panel B, lack of involvement of PKC. Pretreatment of the cells with H-7 (100 μM) for 30 min or prolonged PMA treatment to induce PKC depletion (160 nM PMA for 18 h) does not affect sucrose-activated ECAR; both treatments abrogate PMA-increased ECAR (n = 4). Panel C, lack of role for the classical Ras-mitogen-activated protein kinase pathway. Expression of inhibitory negative constructs of Ras (N17Ras) and Raf (ΔN Raf) and a specific mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-1 inhibitor (50 μM PD98059) do not affect NHE activation by sucrose (n = 3). Panel D, lack of involvement of Src. Expression of Csk and treatment with PP1 (10 μM) had no effect on sucrose-stimulated ECAR (n = 3). Transfections were performed as described under “Experimental Procedures.” Chemical inhibitors were added 30 min prior to addition of sucrose (400 mosmol/liter). Error bars represent the mean ± S.E. *p < 0.01 versus vehicle-treated samples.

 Typically, cells were perfused for 15–30 min with media containing chemical inhibitors of signaling molecules and then exposed to hypertonic medium (400 mosmol/liter by addition of sucrose or NaCl to isotonic medium) in the presence of inhibitors. Control cells were pretreated only with vehicle. We also used cells transiently transfected with inhibitory negative constructs of potential signaling molecules, whereas cells transfected with an empty vector served as a control.

Our previous work has shown a role for Gαi2 and Gαq in the G protein-coupled receptor stimulation of NHE-1 in CHO-K1 cells (30). A role for G protein βγ subunits in the activation of an endogenous NHE in Xenopus laevis oocytes has also been demonstrated by others (32). Therefore, we tested a role for G protein βγ subunits and Gα subunits in hypertonicity-induced activation of NHE. Pertussis toxin (200 ng/ml for 18 h) failed to inhibit NHE under hypertonic conditions, suggesting that pertussis toxin-sensitive G protein α subunits are not involved in hypertonicity-induced activation of NHE (Fig. 2A). Such treatment completely blocked the increase in NHE activity induced by recombinant 5-hydroxytryptamine1A receptor, which activates NHE-1 through pertussis toxin-sensitive Gαq Subunits (30). Likewise, sequestration of G protein βγ subunits by transfecting cells with a minigene construct that encodes βARK-CT does not inhibit the NHE activation by hypertonicity, showing a lack of involvement of G protein βγ subunits in this process (Fig. 2A). The expression of βARK-CT in CHO-K1 cells was demonstrated by performing Western blots on cell lysates with antibody specific for βARK-1 (not shown). This expressed protein was also functionally active, because it completely prevented activation of ERK1/2 by the recombinant 5-hydroxytryptamine1A receptor in CHO-K1 cells (33).

To test the possible involvement of PKC in NHE activation by hypertonicity we used a PKC inhibitor (100 μM H-7), as well as PKC depletion by prolonged exposure of cells to 160 nM phorbol 12-myristate 13-acetate (PMA). Those treatments did not affect NHE activation by hypertonic solutions but were able to inhibit PMA (1 μM)-elicited proton efflux, showing that those maneuvers blocked PKC in our conditions (Fig. 2B). Those results are consistent with the non-involvement of PKC in hypertonicity-induced activation of NHE in CHO-K1 cells.

We next tested the hypothesis that Ras and/or its downstream effectors are involved in hypertonicity-induced activation of NHE in CHO-K1 cells. Expression of inhibitory negative constructs of Ras (N17Ras) and Raf (ΔN Raf) and a selective mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-1 inhibitor (50 μM PD98059) had no effect on NHE activation by hypertonicity (Fig. 2C). As a positive control, to ensure that functional constructs were expressed, we measured the activity of ERK1/2. All of those treatments blocked activation of ERK1/2 by the recombinant 5-hydroxytryptamine1A receptor in CHO-K1 cells (25, 33). Thus, these studies do not support a role for the Ras signaling pathway as an immediate convoyer of hypertonicity-induced activation of NHE in CHO-K1 cells.

Because of evidence that Src family kinases are volume-sensitive enzymes (11), we tested the effects of Src inhibition on hypertonicity-induced activation of NHE. Neither transient transfection with Csk (a kinase that inactivates Src family tyrosine kinases) nor incubation with PP1 (10 μM; a chemical inhibitor of Src) had any effect on the activation of NHE by hypertonic medium (Fig. 2D) suggesting that Src family kinases are not involved in the osmotic activation of NHE-1, which is in agreement with the study by Kapus et al. (17). Thus, many classical signaling molecules do not appear to play a significant role in the hypertonicity-induced activation of NHE-1 in CHO-K1 cells.

Inhibitor Studies Suggest the Involvement of CaM but Not CaM-dependent Enzymes in Hypertonicity-induced Increases in ECAR—Taking into consideration that NHE-1 possesses CaM-binding sites that are critical for its activity, we tested the effects of five structurally distinct CaM inhibitors on hypertonicity-induced increases in ECAR. All of the inhibitors were shown to significantly attenuate the hypertonicity-induced activation of NHE (Fig. 3A). The inhibitors included 100 μM W-7 (−60% inhibition), 5 μM calmidazolium (−90% inhibition), 10 μM fluphenazine (−65% inhibition), 50 μM trifluoperazine (−55% inhibition), and 1 μM ophiohelin A (−94% inhibition). We also considered the possibility that the inhibitory effect was because of a CaM-dependent enzyme. Thus, we tested specific inhibitors of CaM kinases and myosin light chain kinase (10 μM each of KN-93, KT-5926, K-252a, and 50 μM ML-9), CaM-dependent cyclic nucleotide phosphodiesterase (50 μM VIP-cycloheximide and 20 μM 8-methoxy-isobutylmethylxanthine), and calcineurin (1 μM cyclosporin A). None of those inhibitors had a significant effect on the activation of NHE by medium made hypertonic (450 mosmol/liter) by addition of sucrose (Fig. 3B). Thus, these studies support a role for CaM (but not for CaM-dependent enzymes) in the stimulatory effect of hypertonicity on NHE-1 in CHO-K1 cells.

Role for Janus Kinase 2 in Hypertonicity-induced Activation of NHE-1—Because the Jak/STAT pathway is activated by hypertonicity (15), and Jak2 is an indirect regulator of bradykinin-stimulated NHE-1 activity in mIMCD-3 cells (24), we
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Effects of inhibitors of CaM and CaM-dependent enzymes on sucrose-induced ECAR. ECAR was measured by microphysiometry as described under "Experimental Procedures." Cells were preincubated with inhibitors for 30 min prior to stimulation with medium made hypertonic (450 mosmol/liter) by addition of sucrose. W-7, fluphenazine, trifluoperazine, calmidazolium, and ophiobolin A are inhibitors of CaM. KN-93 and KT-5926 are inhibitors of CaM kinase. K-252a and ML-9 inhibit myosin light chain kinase, vinpocetine and inhibitors of CaM. KN-93 and KT-5926 are inhibitors of CaM kinase, fluphenazine, trifluoperazine, calmidazolium, and ophiobolin A are inhibitors of CaM-dependent cyclic nucleotide phosphodiesterase, and cyclosporin A inhibits calcineurin. All experiments were performed at least four times. Error bars represent the mean ± S.E.; *p < 0.05; †p < 0.01; ‡p < 0.005 versus vehicle-treated samples.

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We hypothesized that the Jak/STAT pathway could be involved in the hypertonicity-induced activation of NHE in CHO-K1 cells. We tested this hypothesis in three separate sets of experiments. First, pretreatment of cells for 30 min with 40 μM AG-490, a specific Jak2 inhibitor, effectively blocked the stimulation of NHE by medium made hypertonic by addition of sucrose or NaCl, suggesting the involvement of Jak2 in the hypertonicity-induced activation of NHE in CHO-K1 cells (Fig. 4A). To support the microphysiometry data, we also monitored hypertonicity-induced NHE activation in cells pretreated with AG-490 by measuring intracellular pH using BCECF fluorescence on a FLIPRTM without an acid load. The results presented in Fig. 4B show that pretreatment of cells with 40 μM AG-490 clearly blocked the hypertonicity-induced increase in the rate of fluorescence change measured on a FLIPRTM. Thus, a Jak2 inhibitor was able to block hypertonicity-induced activation of NHE-1 in CHO-K1 cells as measured by two independent assays.

Second, we used Western blotting with a phosphospecific anti-Jak2 antibody to assess the phosphorylation state of Jak2. Exposure of cells to medium made hypertonic (500 mosmol/liter by addition of sucrose) for 10 min induced an ~3-fold increase in the level of phosphorylation of Jak2 (Fig. 4C). Those increases were because of increased phosphorylation of Jak2, because we normalized the results by re-probing the same blots with a control Jak2 antibody (which recognizes Jak2 independent of its phosphorylation state) to confirm that we had loaded equal amounts of Jak2 in the samples (Fig. 4D). Third, we tested the ability of hypertonicity to induce the phosphorylation of the STAT3 isoform (one direct target of Jak2). Time- and concentration-dependent increases in STAT3 phosphorylation after stimulation cells with NaCl and sucrose were assessed by Western blotting of cell lysates with a phosphospecific STAT3 antibody, which specifically detects STAT3 only when activated by phosphorylation at Tyr705. Fig. 5 demonstrates that treatment of cells with both media made hypertonic by addition of either NaCl (Fig. 5, A and B) or sucrose (Fig. 5, C and D) caused time- and concentration-dependent increase in Tyr705 phosphorylation of STAT3. The increased phosphorylation of STAT3 was inhibited by pretreatment of the cells with AG-490 (40 μM for 30 min), supporting the involvement of Jak2 in the activation of STAT3 by hypertonicity and importantly confirming that hypertonicity activates Jak2. The results were normalized by stripping and re-probing the same blots with a control STAT3 antibody that detects total STAT3 independent of its phosphorylation state (Panel D). The experiment was repeated at least four times. Error bars represent the mean ± S.E.

Involvement of Janus kinase 2 in hypertonicity-induced NHE-1 activation. Panel A, ECAR was measured by microphysiometry as described under "Experimental Procedures." Cells were preincubated with 40 μM AG-490, a selective Jak2 inhibitor, for 30 min prior to stimulation with different concentrations of sucrose (330–600 mosmol/liter) or with NaCl (450 mosmol/liter). Experiments were performed at least three times in duplicate. Error bars represent the mean ± S.E.; *p < 0.05; †p < 0.01 versus vehicle-treated samples. Panel B, NHE-1 activity was assessed by measuring pH, using the FLIPRTM. Cells were loaded with BCECF as described under "Experimental Procedures" and preincubated with 40 μM AG-490 for the last 30 min of the loading phase. Negative control wells were preincubated with 5 μM EIPA, an NHE-1 inhibitor, in the presence or absence of AG-490 for 15 min. Plates were placed in the FLIPRTM, and fluorescence tracings were recorded in the presence or absence of sucrose. The data were calculated as average fluorescence tracing from at least six wells after subtraction of signals from negative control wells. Rate of fluorescence change represents the slopes of the fluorescence changes. Experiments were performed at least three times. Data are presented as mean ± S.E.; *p < 0.05; †p < 0.01 versus vehicle-treated samples. Panel C, sucrose induces tyrosine phosphorylation of Jak2. The phosphorylation state of Jak2 was determined in whole cell lysates from CHO-K1 cells using a phosphorylation state-specific antibody for Jak2 in an immunoblot as described under "Experimental Procedures." Cells were treated with medium made hypertonic (500 mosmol/liter) by addition of sucrose or isotonic medium for 10 min, lysed, and subjected to immunoblot. The inset is a representative immunoblot. The same blot was stripped and re-probed with antibodies for total Jak2 that recognize Jak2 independent of phosphorylation state (Panel D). The experiment was repeated at least four times. Error bars represent the mean ± S.E.
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A23187 (1 μM), caused an ~60% increase in Jak2 phosphorylation in CHO-K1 cells (not shown), similar to the effect in mIMCD-3 cells. Exposure of CHO-K1 cells to A23187 for 5 min also increased the amount of CaM in NHE-1 immunoprecipitates by ~70% (Fig. 7B). Pretreatment of CHO-K1 cells for 30 min with 50 μM BAPTA-AM, a cell-permeable Ca²⁺ sequestrant, did

During oxidative stress (34) we studied the possible involvement of Src family kinases in hypertonicity-induced Jak2 phosphorylation. We pretreated cells for 30 min with 10 μM PP1 (Src family kinases inhibitor) before stimulation for 10 min with medium made hypertonic (500 mosmol/liter) by addition of sucrose to isotonic medium. Cell lysates were immunoprecipitated with an immobilized phosphotyrosine monoclonal antibody followed by Western blotting with a polyclonal anti-Jak2 antibody. Experiments were performed as described under “Experimental Procedures.” Experiments were repeated at least four times. Error bars represent the mean ± S.E. *p < 0.05, **p < 0.01 versus vehicle-treated samples.

Convergence of the CaM and Jak2 Signals—In the next series of experiments, we examined the possibility that hypertonicity induces a physical interaction between Jak2 and CaM. This was accomplished by using immunoprecipitation of lysates from cells treated with vehicle or hypertonic medium with anti-Jak2/protein A-agarose, followed by Western blotting with a CaM antibody. Fig. 7A shows that CaM and Jak2 are co-immunoprecipitated and that this association can be increased ~2.5 times by treatment of CHO-K1 cells for 10 min with medium made hypertonic (500 mosmol/liter) by addition of sucrose. Pretreatment of the cells with AG490 (50 μM for 30 min) significantly decreased the amount of CaM in Jak2 immunoprecipitates, suggesting that hypertonicity-induced Jak2 activity is necessary for the formation of the complex that includes CaM and Jak2.

Because of our previous work on mIMCD-3 cells stimulated with bradykinin, we considered the possibility that elevated Ca²⁺ contributes to the activation of Jak2 (24). Therefore, we studied a potential role for Ca²⁺ in the hypertonicity-induced interaction between Jak2 and CaM. Elevation of intracellular Ca²⁺ by incubation of CHO-K1 cells with a calcium ionophore,
not decrease the amount of CaM in Jak2 immunoprecipitates from cells stimulated with medium made hypertonic (500 mosmol/liter) by addition of sucrose, whereas it completely abolished the increase of CaM in Jak2 immunoprecipitates caused by 1 µM A23187. Thus, although increased intracellular Ca\(^{2+}\) increases Jak2 phosphorylation and induces formation of a complex between CaM and Jak2, hypertonicity employs a Ca\(^{2+}\)-dependent pathway of Jak2 activation, distinct from the one used by the G protein-coupled bradykinin B\(_2\) receptor in mIMCD-3 cells (24). This notion is also supported by the fact that medium made hypertonic (500 mosmol/liter) by addition of sucrose causes only a very slight elevation of intracellular Ca\(^{2+}\), as measured by Fluo-3 fluorescence in FLIPR™ experiments (data not shown).

**Hypertonicity Increases Tyrosine Phosphorylation of CaM in a Jak2-dependent Manner**—We next tested the hypothesis that hypertonicity stimulates tyrosine phosphorylation of CaM. Cells were treated with medium made hypertonic (500 mosmol/liter) by addition of sucrose or isotonic vehicle for 10 min. Cells were then lysed, and the lysates were immunoprecipitated with a polyclonal phosphotyrosine antibody as described under “Experimental Procedures.” Subsequent immunoblots were performed with monoclonal anti-CaM antibodies. The data presented in Fig. 7C demonstrate that CaM becomes tyrosine-phosphorylated (~3.5-fold increase) in response to hypertonicity and that pretreatment of the cells with AG490 (50 µM for 30 min) significantly decreases hypertonicity-induced tyrosine phosphorylation of CaM, suggesting that the increased phosphorylation is induced by Jak2.

**Presence of CaM in NHE-1 Immunoprecipitates**—We proposed previously (24) that tyrosine phosphorylation of CaM induced by bradykinin increases the binding of CaM to NHE-1 in mIMCD-3 cells. To establish the presence of NHE-1 in the hypertonicity-induced CaM signaling complex in CHO-K1 cells, we performed co-immunoprecipitation experiments in which NHE-1 was isolated from lysates of cells treated with isotonic medium or medium made hypertonic (500 mosmol/liter) by addition of sucrose. Immunoprecipitates were next probed with CaM antibodies. Fig. 7D shows that CaM is present in NHE-1 immunoprecipitates and further that the amount of CaM complexed with NHE-1 increases after treatment of CHO-K1 cells with hypertonic medium. Pretreatment of the cells with AG490 (50 µM for 30 min) significantly decreases the amount of CaM complexed with NHE-1 suggesting that Jak2-induced phosphorylation of CaM is essential for the complex formation between NHE-1 and CaM. When the same blots were stripped and re-probed with NHE-1 antibody, equal amounts of NHE-1 appeared in the samples from cells exposed to isotonic or hypertonic media (data not shown).

**DISCUSSION**

In the current report we studied the regulation of NHE-1 activity by hypertonicity in CHO-K1 cells. What is new about this work is that we have shown for the first time that CaM and Jak2, working in concert, are involved in hypertonicity-induced activation of NHE-1. Specifically, we have shown that exposure of CHO-K1 cells to hypertonic media phosphorylates and stimulates Jak2, increases complexation of Jak2 with CaM, increases the Jak2-dependent tyrosine phosphorylation of CaM, and increases the activity of NHE-1 by increasing complexation of CaM to NHE-1. These findings are important, because in our previous work (24) we showed that Jak2 is involved in the regulation of NHE-1 by bradykinin B\(_2\) receptors in mIMCD-3 cells in a similar manner. Thus, this pathway of activating NHE-1 is not limited to G protein-coupled receptors or to polarized epithelial cells. We propose that it is a new fundamental mechanism for the rapid regulation of NHE-1 by varied stimuli in multiple cell types.

**FIG. 8. Proposed pathway of hypertonicity-induced activation of NHE-1 in CHO-K1 cells.** This scheme is described under “Discussion.” PY indicates tyrosine phosphorylation.

The involvement of CaM in the hypertonicity-induced activation of NHE-1 is supported by the ability of five chemically distinct CaM inhibitors to attenuate NHE-1 activation, the tyrosine phosphorylation of CaM, and increased complexation of CaM to Jak2 and NHE-1 in cells exposed to hypertonic media. Moreover, it appears that CaM itself, rather than a CaM-dependent enzyme, is involved in the activation of NHE-1 by hypertonicity in that multiple inhibitors of CaM-dependent protein kinases, myosin light chain kinase, CaM-dependent phosphodiesterase, and the CaM-dependent phosphatase, calcineurin, are ineffective in blocking the hypertonicity-induced activation of NHE-1. It is not surprising that CaM plays a role in activating NHE in these cells, as NHE-1 has two CaM binding sites that regulate its function (22). These findings suggest that CaM can be induced to activate NHE-1 through several upstream pathways, including increased intracellular calcium and/or tyrosine phosphorylation of CaM. Our results should be contrasted with two studies supporting a role for CaM-dependent myosin light chain kinase activation and phosphorylation of myosin light chains in shrinkage-induced activation of NHE in astrocytes (18, 19). Thus, there remains the possibility that CaM-dependent enzymes (as well as CaM itself) might also play roles in activating NHE-1 in some cases.

CaM has been shown to be phosphorylated by both tyrosine and serine-threonine kinases. Casein kinase II phosphorylates CaM on multiple serine and threonine residues (Thr\(^{79}\), Ser\(^{81}\), Ser\(^{101}\), and Thr\(^{117}\)) (35, 36). Tyr\(^{99}\) is phosphorylated after activation of the insulin receptor (37, 38) and the epidermal growth factor receptor (39, 40) and Tyr\(^{138}\) after activation of the insulin receptor (37, 38). There is also significant precedent for the idea that tyrosine phosphorylation of CaM can alter its ability to interact with and activate its downstream targets. Tyrosine phosphorylation of Tyr\(^{99}\) of CaM generally increases the activity of its various targets by either decreasing the concentration at which half-maximal activation is attained (3′,5′-cyclic nucleotide phosphodiesterase, plasma membrane Ca\(^{2+}\)-ATPase, and type II Ca\(^{2+}\)-dependent protein kinase) or by increasing the V\(_{\text{max}}\) (calcineurin and neuronal nicotinic acetylcholine) (41, 42). However, phosphorylation of Tyr\(^{99}\) of CaM has been variably reported to diminish its activity (43) or to have no effect on the ability of CaM to activate myosin light chain kinase (42) or type I cyclic nucleotide phosphodiesterase (44). The current work supports the hypothesis that tyrosine phosphorylation of CaM by Jak2, when induced by hypertonicity, results in activation of NHE-1.
The involvement of Jak2 in hypertonicity-induced activation of NHE-1 is supported by the inhibition of hypertonicity-induced NHE-1 activity by the Jak2 inhibitor AG-490, the tyrosine phosphorylation of CaM induced by hypertonicity, the Jak2-dependent phosphorylation of STAT3, and the disruption by AG-490 of the hypertonicity-induced tyrosine phosphorylation of CaM, the complexation of CaM with Jak2, and the complexation of CaM with NHE-1. Because of incomplete inhibition of hypertonicity-induced NHE-1 activity by the Jak2 inhibitor (Fig. 4, A and B) we cannot rule out the existence of additional Jak2-dependent mechanisms of hypertonicity-induced NHE-1 activation, such as a recently proposed mechanism of NHE-1 activation through dynamic changes in the actin-based cytoskeleton (1). The role for Jak2 in the regulation of NHE-1 activity is a relatively new finding.

The current results expand on our results (24) showing that G protein-coupled receptors for bradykinin activate NHE-1 through a pathway that involves Jak2 and CaM and Ca2+ mobilization. In the current report, we have shown evidence that chelation of Ca2+ with BAPTA-AM had little effect on the complex formation between Jak2 and CaM and activation of NHE-1 by hypertonicity (Fig. 7B). This supports an alternative mechanism that does not require increases in intracellular calcium levels, such as the tyrosine phosphorylation of CaM by Src family kinases (25). Although it has been shown that members of the Src family are volume-sensitive enzymes (11, 16, 17), our results do not support their role in the osmotic activation of NHE-1, which is in agreement with the study by Kapus et al. (17). Although Fyn (a member of Src family kinases) is required for activation of Jak2 by oxidative stress (34), our data suggest that hypertonicity-induced phosphorylation of Jak2 does not depend on Src family kinases (Fig. 6). Thus, it is not clear at this time how Jak2 is activated when CHO-K1 cells are exposed to hypertonicity.

In summary, we have shown for the first time that CaM and Jak2, working in concert, are involved in hypertonicity-induced activation of NHE-1. In CHO-K1 cells, exposure to hypertonic medium stimulates Jak2, increases Jak2-dependent tyrosine phosphorylation of CaM, increases complexation of Jak2 with CaM, as well as CaM with NHE-1, and increases the activity of NHE-1 in a Jak2- and CaM-dependent manner. Moreover, this process does not require the involvement of many classical signaling molecules. Thus, we propose that hypertonicity induces activation of NHE-1 in CHO-K1 cells in large part through the following pathway: hypertonicity → Jak2 phosphorylation and activation → tyrosine phosphorylation of CaM → association of CaM with NHE-1 → NHE-1 activation (Fig. 8). Further, we suggest that this pathway is a fundamental mechanism for the rapid regulation of NHE-1 by varied stimuli in multiple cell types.

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Maria N. Garnovskaya, Yurii V. Mukhin, Tamara M. Vlasova and John R. Raymond

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