ERK Is Regulated by Sodium-Proton Exchanger in Rat Aortic Vascular Smooth Muscle Cells*

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The purposes of this study were to test 1) the relationship between two widely studied mitogenic effector pathways, and 2) the hypothesis that sodium-proton exchanger type 1 (NHE-1) is a regulator of extracellular signal-regulated protein kinase (ERK) activation in rat aortic smooth muscle (RASM) cells. Angiotensin II (Ang II) and 5-hydroxytryptamine (5-HT) stimulated both ERK and NHE-1 activities, with activation of NHE-1 preceding that of ERK. The concentration-response curves for 5-HT and Ang II were superimposable for both processes. Inhibition of NHE-1 with pharmacological agents or by isotonic replacement of sodium in the perfusate with choline or tetrathylammonium greatly attenuated ERK activation by 5-HT or Ang II. Similar maneuvers significantly attenuated 5-HT- or Ang II-mediated activation of Ras but not transphosphorylation of the epidermal growth factor (EGF) receptor. EGF receptor blockade attenuated ERK activation, but not NHE-1 activation by 5-HT and Ang II, suggesting that the EGF receptor and NHE-1 work in parallel to stimulate ERK activity in RASM cells, converging distal to the EGF receptor but at or above the level of Ras in the Ras-MEK-ERK pathway. Receptor-independent activation of NHE-1 by acute acid loading of RASM cells resulted in the rapid phosphorylation of ERK, which could be blocked by pharmacological inhibitors of NHE-1 or by isotonic replacement of sodium, closely linking the proton transport function of NHE-1 to ERK activation. These studies identify NHE as a new regulator of ERK activity in RASM cells.

Activation of several major effectors has been linked to mitogenic stimuli. Two of those effectors are the extracellular signal-regulated protein kinase (ERK) type of mitogen-activated protein kinases and Na⁺/H⁺ exchangers (NHE). ERK is one member of a family of kinases that participate in mitogenic signaling through complex phosphorylation cascades that convert cell surface signals into nuclear transcription programs. In the typical scenario, GTP-bound Ras, a small G protein, activates Raf kinase. In an alternative scenario, Raf is activated by protein kinase C or other signaling molecules. In either case, Raf phosphorylates and activates mitogen and extracellular signal-regulated kinases (MEK), which in turn phosphorylates and activates ERK. Activated ERK translocates to the nucleus, where it activates a number of transcription factors such as Elk-1. Thus, this pathway can be depicted in a linear form as follows: Ras-GTP (or protein kinase C) → Raf-1 kinase → MEK → ERK. NHEs are expressed at the surface of all mammalian cells, serving to regulate cell volume, intracellular pH (pHᵢ), and transepithelial transport of Na⁺ and acid-base equivalents (1, 2). The signal transduction pathways involved in activating NHEs have been more elusive. Although ERK play relatively well defined roles in mitogenesis, those of NHEs are less clear in that NHEs are possibly permissive factors rather than absolute necessities for mitogenesis (3).

Although it has been known for some time that mitogens typically activate both NHE and ERK in concert (2–5), the exact relationships between NHE and ERK have only recently been explored in any great detail. For example, microinjection of activated Ras (6) or transfection of the Ha-Ras oncogene (7–9) stimulates NHE activity in fibroblasts. In those studies, Ras most likely increased NHE activity by an up-regulation of NHE message and protein via ERK-regulated transcriptional processes. Recent work has shown that short-term activation of ERK leads to rapid stimulation of NHE-1 in multiple cell types (platelets, erythrocytes, fibroblasts, MDCK-11 cells, rabbit skeletal muscle, and cultured rat neonatal and adult ventricular cardiomyocytes) when activated by diverse stimuli, including angiotensin II (Ang II), cannabinoid ligands, aldosterone, and H₂O₂ (10–22). ERK also has been shown to activate NHE-3 in kidney proximal tubule cells (23, 24). At least in some cases, the short-term stimulation of NHE by ERK is mediated by phosphorylation of NHE either by ERK itself or by p90rsk, a newly described ERK-activated kinase (16, 25–28). It has been suggested that activation of the Ang II receptor first leads to activation of the MEK-ERK-p90rsk pathway and that activated p90rsk in turn directly phosphorylates and activates NHE-1 in rat aortic vascular smooth muscle (RASM) cells. Subsequent protein kinase; Ang II, angiotensin II; BCFPP, 2',7'-bis[2-carboxyethyl]-5(6)-carboxyfluorescein; CaM, calmodulin; EGFP, epidermal growth factor; ECAR, extracellular acidification rate; EIPA, 5-N-ethyl-N-isopropylamiloride; FLIPR, fluorescent imaging plate reader; 5-HT, 5-hydroxytryptamine; MEK, mitogen- and extracellular signal-regulated kinase kinase; MIA, 5-N-methyl-N-isobutylamiloride; NHE, Na⁺/H⁺ exchanger; NHE-1, type 1 NHE; PFK, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RASM, rat aortic smooth muscle cells.

This work was supported by grants from the Department of Veterans Affairs (Merit Awards and a Research Enhancement Award program to M. N. G. and J. R. R.), the National Institutes of Health (DK52448 and DK52448-02 to J. R. R. and DK02694 to Y. V. M.), the American Heart Association (to M. E. U. and Y. V. M.), and a laboratory endowment DK52448-02 to J. R. R. and DK02694 to Y. V. M.), the American Heart Association (to M. E. U. and Y. V. M.), and a laboratory endowment DK52448-02 to J. R. R. and DK02694 to Y. V. M.), the American Heart Association (to M. E. U. and Y. V. M.), and a laboratory endowment DK52448-02 to J. R. R. and DK02694 to Y. V. M.), the American Heart Association (to M. E. U. and Y. V. M.) (250623, Charleston, S.C. 29425-2227. Tel.: 843-789-6776 or 843-876-
experiments that have been performed in fibroblasts resulted in identification of NHE-1 serine 703 as a major target of serum-stimulated p90rsk (28). However, it is not still clear whether Ang II-induced phosphorylation of NHE-1 takes place in RASM cells in vivo and whether this phosphorylation is physiologically significant. Nonetheless, there is clear evidence that ERK can increase the activity of NHE by increasing its expression and/or by stimulating the activity of existing NHE molecules.

On the other hand, several groups have been unable to show any role for ERK in activating NHE in multiple cell types, including U937 cells (29), human PMNs (5), Xenopus oocytes (30), proximal tubule cells (23), Ehrlich ascites cells (31), or Chinese hamster ovary cells (32). Moreover, there is one report in which ERK was shown to mediate inhibition of NHE activity in MTAL cells (33). Therefore, the ability of ERK to stimulate NHE activity has not been a universal finding.

Despite the increasing interest in potential roles for ERK in the activation of NHE, surprisingly little is known regarding the role of NHE in regulating ERK. NHE is not involved in ERK activation in hepatic stellate cells (34) or Chinese hamster ovary cells (32). In contrast, there have been two reports that suggest that NHE might play a role in regulating ERK activation. Takewaki et al. (35) presented some evidence that a potential antagonist of NHE-1 could partially inhibit stretch-induced activation of ERK in the cultured cardiomyocytes. In human gut cells, NHE inhibition suppressed both activation of the p42/p44 mitogen-activated protein kinase and NF-κB (36). Although it is clear that NHE and ERK can be activated in concert, the relationship between these two key mitogenic cellular proteins varies between cell types.

5-HT and Ang II have previously been shown to activate both NHE and ERK in cultured vascular smooth muscle cells (37, 38). Recently we conducted a screen for signaling molecules that could be involved in activation of NHE in RASM cells and demonstrated that the serotonin 5-HT$_2A$ receptor and the angiotensin AT$_1$ receptor activate NHE through a pathway that involves phospholipase C, elevated intracellular Ca$^{2+}$, calmodulin (CaM), and Janus kinase 2 (38).

NHE and ERK have each been proposed as key therapeutic targets for vascular illnesses, including myocardial infarction and reperfusion injury (41), ventricular fibrillation (42), and congestive heart failure (43), and ventricular hypertrophy (44). Thus, in the current study we investigated the role of NHE in ERK activation in vascular smooth muscle cells derived from rat aorta using 5-HT and Ang II as our primary test agents.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture supplies were obtained from Invitrogen or Corning Costar (Cambridge, MA). Ethylisopropylamidolactide (EIPA) and metilisobutylamidolactide (MIA) were from RBI (Natick, MA). Angiotensin II, 5-HT, and probenecid were from Sigma. Phospho-ERK and NF-κB were from Cell Signaling Technology (Beverly, MA). The Ras activation kit was from Upstate Biotechnology (Lake Placid, NY). GF109203X, PD98059, and ophiobolin were from Calbiochem. BCECF was purchased from Molecular Probes (Eugene, OR). Black 96-well microtiter plates needed for the FLIPR were from Corning Costar. Black pipette tips were from Molecular Devices Corp. (Sunnyvale, CA). Measurement of Extracellular Acidification Rate (ECAR) by Microphysiometer—For all of the experiments, RASM cells were plated onto polycarbonate membranes (3-µm pore size, 12-mm size) at a density of 300,000 cells/insert the night prior to experimentation. After cells were attached to the membranes, they were grown in serum-free culture medium for 20 h before the experiment. The day of the experiment, cells were washed with serum-free, bicarbonate-free Ham’s F-12 medium, placed into chambers of a microphysiometer (Molecular Devices Corp.) (45), and perfused at 37°C with the same medium or balanced salt solutions. For most studies, 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 6th through the 28th seconds. Cells were never 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.

ERK and MEK Assays—ERK and MEK phosphorylations were assessed by immunoblot using phosphorylation state-specific antibodies as previously described (46, 47) in RASM cells treated for various times with varying concentrations of 5-HT, Ang II, or vehicle.

Cell Culture—RASM cells were obtained as described previously (48) by terminal harvesting of aortas from male Sprague-Dawley rats (150–275 g, Charles River Laboratories, Wilmington, MA) using anesthesia approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina. A 2-cm segment of artery cleaned of fat and adventitia was incubated in 1 mg/ml collagenase for 3 h at room temperature. The artery was then cut into small sections and fixed to a control flask for explantation in minimal essential medium containing 10% fetal calf serum, 1% nonessential amino acids, 100 milliunit/ml penicillin, and 100 µg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 95% air–5% CO$_2$. Medium was changed every 3–4 days, and cells were passaged every 6–8 days by harvesting with trypsin-EDTA. We have previously characterized these cultures by immunostaining as positive for intracellular cytoskeletal fibrils and smooth muscle cell-specific myosin and negative for factor VIII antigens. RASM cells isolated by this procedure were homogeneous and were used in all studies between passages 3 and 7.

Ras Assay—Ras activation was assessed by a nonradioactive Ras assay kit (Upstate Biotechnology, Inc) as previously described (49). Quiescent RASM cell monolayers were pretreated with 10 µM MIA or vehicle for 30 min, stimulated with 1 µM 5-HT, 100 µM Ang II, or vehicle for 5 min, and lysed in a 1 ml/100 mm-dish of Mg$^{2+}$-lysiss buffer (MLB) (150 mM NaCl, 25 mM HEPES, pH 7.5, 1 mM EDTA, 10 mM MgCl$_2$, 1% Igepal CA-630, 25 mM sodium fluoride, 1 mM Na$_2$VO$_4$, 10 µM ß-mercaptoethanol, and leupeptin and pepstatin, each, 10 µg/ml). Cell lysates were precleared by incubating with glutathione-agarose for 10 min at 4°C. Precleared lysates (50 µg total cell protein) were incubated with Ras binding domain (glutathione S-transferase fusion protein, corresponding to the Ras binding domain of Raf-1), and bound to glutathione-agarose for 30 min at 4°C. The agarose beads were collected by centrifugation, washed three times with MLB buffer, resuspended in 2× Laemmli sample buffer, boiled for 5 min, and subjected to SDS-PAGE and subsequent immunoblot analysis with monoclonal anti-Ras IgG.

EGF Receptor Phosphorylation Assay—The phosphorylation state of EGFR was assessed by immunoprecipitation/Western blotting studies as previously described (49). Quiescent RASM cells, grown in 100-mm dishes, were pretreated with vehicle or 10 µM MIA for 30 min. RASM cells were subsequently treated with 1 µM 5-HT, 100 µM Ang II, or vehicle for 5 min, and lysed in a 1 ml/100 mm-dish of Mg$^{2+}$-lysiss buffer (MLB) (150 mM NaCl, 25 mM HEPES, pH 7.5, 1 mM EDTA, 10 mM MgCl$_2$, 1% Igepal CA-630, 25 mM sodium fluoride, 1 mM Na$_2$VO$_4$, 10 µM ß-mercaptoethanol, and leupeptin, and pepstatin, each). Cell lysates were precleared by incubating with a protein A-agarose bead slurry for 30 min at 4°C. Precleared lysates (1 µg/ml total cell protein) were incubated with 4 µg of anti-EGFR polyclonal IgG (Upstate Biotechnology) overnight at 4°C. The immunocomplexes were captured by the addition of protein A-agarose bead slurry and incubation for 2 h more at 4°C. The agarose beads were collected by centrifugation, washed three times with RIPA buffer, resuspended in 2× Laemmli sample buffer, boiled for 5 min, and subjected to SDS-PAGE. After semidy transfer to polyvinylidene difluoride membranes, the immunoproteins were captured by the addition of monoclonal anti-EGFR antibody (Upstate Biotechnology). Measurement of Intracellular pH (pH$i$) In Intracellular pH (pH$i$) was measured in 96-well microtiter plates using a FLIPR fluorescent imaging plate reader (50). The night prior to experimentation, RASM cells were plated onto polycarbonate cellwells of black 96-well plates. The following morning, cells were washed with Hank’s balanced salt solution and then loaded with 5 µM BCECF for one hour. Cells were washed four times with Hank’s balanced salt solution containing 2.5 mM probenecid, 1% fetal bovine serum, and 20 mM Hepes (pH 7.4) with an automated platewasher (Labsystems, Helsinki, Finland). 20 min before end of the loading phase, 20 mM NH$_4$Cl was added well. Cells were then washed four times with the loading buffer containing 20 mM NH$_4$Cl. In the FLIPR, cells were acid loaded by an ammonium chloride prepulse protocol. In this method, the extracellular buffer contains NH$_4$Cl and NH$_3$ in an equilibrium that is essentially
5-HT and Ang II induce rapid activation of NHE in RASM. A, microphysiometry was performed on cultured RASM cells as described under “Experimental Procedures.” 5-HT (1 μM) and Ang II (100 nM) were applied to the cells for three measurement cycles (gray background), and ECAR was measured. The plots are representative of at least six different experiments for each condition. B, 5-HT- and Ang II-induced increases in ECAR could be blocked by incubation in sodium-free isotonic medium. Data are means ± S.E. for at least four different assays performed in duplicate for each agonist. * indicates p < 0.01 when compared with vehicle. † indicates p < 0.05 when compared with either Ang II or 5-HT alone. C, 5-HT- and Ang II-induced increases in ECAR could be blocked by incubation for 30 min with EIPA or MIA. Data are means ± S.E. for at least four different assays performed in duplicate or triplicate for each agonist. ‡ indicates p < 0.05 when compared with either Ang II or 5-HT.

Recapitulated 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 NH₃ - (51). The 96 wells were simultaneously illuminated for 0.3 s by an Argon laser (488 nm) set at −0.3 watt. Non-ratiometric fluorescence emission readings were obtained using a 540-nm bandpass filter at 1-s intervals. Calibration of the BCECF fluorescence response to changes in intracellular pH was performed on a separate 96-well plate of cells using the electroneutral H⁺/K⁺ ionophore, nigericin (52). Using this strategy, when the K⁺ concentration is equal on both sides of the plasma membrane, the intracellular pH is clamped to the same value as in the extracellular medium. For calibration, cells were washed and equilibrated with buffers of varied pH and 130 mM KCl (the estimated intracellular concentration of K⁺). Calibration plots were generated by equilibrating intracellular to extracellular pH by adding 20 μM nigericin (K⁺/H⁺ ionophore) to cells in various pH buffers.

RESULTS

5-HT and Ang II Activate Na⁺/H⁺ in RASM—We have recently shown that the 5-HT₂A and AT₁A receptors activate NHE in kidney mesangial cells and RASM cells, respectively (53, 54). In Fig. 1, we used proton microphysiometry to verify that 5-HT and Ang II each stimulate time-dependent increases in ECAR from monolayers of RASM cells (Fig. 1A). The increase in ECAR is because of proton efflux (and not bicarbonate uptake) because the cells were superfused with nominally bicarbonate-free media. Moreover, the increase in ECAR could be blocked by removal of sodium from the perfusate (Fig. 1B) or by preincubation with 5 μM EIPA or 10 μM MIA, inhibitors of NHE-1 and -2 (Fig. 1C). Thus, both 5-HT and Ang II stimulate sodium-dependent and EIPA/MIA-inhibitable proton efflux from RASM cells. Because NHE-1 is the only NHE expressed in vascular smooth muscle cells (55), 5-HT and Ang II most likely activate NHE-1 in RASM cells.

We next used microphysiometry to establish that the activation of NHE was dependent upon the concentration of Ang II or 5-HT to which the RASM cells were exposed (Fig. 2). We also established that both ligands could activate ERK in our RASM cells by using a phosphorylation state-specific antibody that recognizes phosphorylated ERK1 and -2. Further, we compared the concentration-response relationships for the ability of Ang II and 5-HT to activate ERK and NHE (Fig. 2). Those experiments demonstrated that the concentration-response relationships for each agonist to activate NHE and to induce phosphorylation of ERK were virtually superimposable. This suggested to us that there might be a relationship between the two pathways in RASM cells. This was not surprising in that a number of previous studies have demonstrated a role for ERK in activating NHE in various cell types (10–24).

5-HT and Ang II Activate ERK through a Pathway That Requires Na⁺/H⁺ Exchanger in RASM Cells—We investigated potential regulation of NHE by 5-HT₂A receptor- and angiotensin AT₁A receptor-stimulated ERK in RASM cells by studying the effect of a MEK inhibitor (PD98059) on the activation of NHE induced by 5-HT and Ang II. Those studies showed that the MEK inhibitor blocked ERK activation without affecting NHE activity (Fig. 3, A and B). Similar results were obtained when the Ras-MEK-ERK pathway was inhibited using apigenin. Thus, it appears that inhibition of MEK does not affect the abilities of Ang II or 5-HT to activate NHE in RASM. In contrast, removal of sodium from isotonic media (by substitution with tetramethylammonium) significantly attenuated ERK phosphorylation induced by both 5-HT and Ang II in RASM (Fig. 3C). This maneuver blocks NHE activity by depriving it of its extracellular ionic substrate. Similar results were obtained when sodium was substituted mM/mM with choline (not shown).

Nearly identical results were obtained when cells were preincubated with 5 μM EIPA. Thus, blockade of NHE activation by removal of extracellular Na⁺ or with the chemical inhibitor EIPA prevents ERK phosphorylation induced by two distinct G protein-coupled receptor agonists (Ang II and 5-HT). To confirm that these results were not simply because of alterations in the kinetics of activation of ERK, we compared the kinetics of activation of ERK by Ang II and 5-HT in the presence and absence of 5 μM EIPA (Fig. 4, A and B). Those studies showed...
induced activation of NHE-1 as well as a PKC inhibitor (that have been previously shown to block 5-HT- and Ang II-
for 30 min with CaM inhibitors (50 M). CaM inhibitors blocked phosphorylation of ERK1/2, whereas a PKC inhibitor did not affect NHE-1 activation prior to the
measurement of 5-HT- and Ang II-stimulated phosphorylation
of ERK1/2. CaM inhibitors blocked phosphorylation of ERK1/2, which occurred within 3 min for both Ang II and
5-HT. Thus, the fact that the peak activation of NHE precedes
that of ERK was obtained between 5–10 min of stimulation by either
ligand, and this peak activation lagged behind the peak activation
of NHE, which occurred within 3 min for both Ang II and
5-HT. Thus, the fact that the peak activation of NHE precedes
of ERK activation, we wanted to use a receptor-indepen-
dent method to stimulate NHE. Thus, we used the same
acid loading protocol as was described in the experiments using
BCECF fluorescence to examine the effects on ERK phosphorylation
during an imposed acid load and during the pH recovery
phase (Fig. 5, A and B). Those experiments showed that
intracellular acidification itself did not significantly alter the
level of ERK phosphorylation when measured after 2 or 5 min
of recovery. ERK phosphorylation did not change when “pH
recovery” did not occur (with Na+-depleted buffer or in the
presence of 5 M EIPA). Thus, merely inducing acidification of
the cell interior did not alter ERK phosphorylation. However, when the cells were allowed to recover from the acid load in the
presence of extracellular Na+, a rapid increase in ERK phospho-

FIG. 3. Relationship between 5-HT- and Ang II-activated ERK
and NHE. A, lack of effects of MEK inhibitors on Ang II- and 5-HT-
activated NHE as measured by microphysiometry. B, effects of MEK
inhibitors on Ang II- and 5-HT-activated ERK as measured by using
phosphorylation state-specific antibodies. Data are presented as
means ± S.E. for at least three different assays performed in duplicate
for each condition. * indicates p < 0.01 when compared with vehicle; ‡ indicates p < 0.05 when compared with either Ang II or 5-HT; ‡‡ indicates p < 0.01 when compared with either Ang II or 5-HT. C, effects of
eosmotic replacement of sodium with tetramethylammonium in the
media on Ang II- and 5-HT-induced ERK phosphorylation. Autoradio-
grams are representative of three separate experiments.

regulator of ERK1/2 in rat aortic smooth muscle cells.

Receptor-independent Activation of Na+/H+ in RASM
Results in Activation of ERK—To confirm that activation of NHE
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acid loading protocol as was described in the experiments using
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the cell interior did not alter ERK phosphorylation. However, when the cells were allowed to recover from the acid load in the
presence of extracellular Na+, a rapid increase in ERK phospho-

FIG. 4. Kinetics of 5-HT- and Ang II-activated ERK and NHE. A, cells were treated with 100 nM Ang II with (black diamonds) or without
:white circles) 5 μM EIPA for various periods of time, after which
ERK phosphorylation was measured as described under “Experimental Procedures.” ECAR was also measured by microphysiometry (thick line) and presented as arbitrary units normalized to the peak effect induced
by Ang II. B, cells were treated with 1 μM 5-HT with (black circles) or
without (white circles) 5 μM EIPA for various periods of time, after
which ERK phosphorylation was measured as described under “Exper-
imental Procedures.” ECAR was also measured by microphysiometry
(thick line) and presented as arbitrary units normalized to peak effect
induced by Ang II. All studies were performed at least three times in
duplicate. Data are presented as means ± S.E. for at least three differ-
ent assays performed in duplicate for each condition. C, effects of
inhibitors of PKC and CaM on 5-HT- and Ang II-induced ERK activa-
tion. Cells were pretreated for 30 min with inhibitors (50 μM W7, 1 μM ophobolin, or 1 μM GF109203X) prior to stimulation for 5 min with 100
nM Ang II, 1 μM 5-HT, 1 μM PMA, or vehicle. ERK phosphorylation was
measured by using phosphorylation state-specific antibodies as de-
scribed under “Experimental Procedures.” Experiments were per-
formed at least three times in duplicate. Data are presented as mean ±
S.E. * indicates p < 0.01 when compared with vehicle; ‡ or ‡‡ indicate p < 0.05 or < 0.01 when compared with either Ang II, 5-HT, or PMA
alone.

NHE-1 Regulates ERK in Vascular Smooth Muscle Cells

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Fig. 5. Effects of receptor-independent activation of NHE on ERK phosphorylation. A, effects of imposed intracellular acid load and recovery (after 2 and 5 min) on ERK phosphorylation in RASM cells. Cells were acid loaded by the ammonium chloride pre-pulse method as described under “Experimental Procedures.” Recovery was monitored in the presence of vehicle or 100 nM Ang II, 1 μM 5-HT, or 5 μM EPA. The plots shown are representative of at least four similar experiments for each condition. B, cells were acid loaded for 2 min by the ammonium chloride pre-pulse method as described under “Experimental Procedures” and rapidly challenged again with 20 mM NH₄Cl for 2 min to elicit a rapid alkaline shift. Experiments were performed in Na⁺-replete buffer to block NHE activity. NHE activity was unblocked by addition of isotonic Na⁺-replete buffer for 2 or 5 min. ERK phosphorylation was measured by using phosphorylation state-specific antibodies. * indicates p < 0.01 when compared with pre-acid load basal levels; † indicates p < 0.05 when compared with controls in Na⁺-replete buffer.

Fig. 6. Inhibition of NHE attenuates signals generated by Ang II and 5-HT. Cells were treated with 100 nM Ang II, 1 μM 5-HT, or vehicle in the presence or absence of the NHE-1 inhibitor MIA (10 μM) in isotonic sodium-replete buffer. Cellular extracts were analyzed for activation of ERK (A), MEK (B), or Ras (C). These experiments were performed at least three separate times. * indicates p < 0.01 when compared with vehicle; † indicates p < 0.05; ‡ indicates p < 0.01 when compared with either Ang II or 5-HT alone.

Dissociation can be induced when NHE-1 is activated through a non-receptor-dependent pathway (recovery from an imposed acid load). This either means that NHE-1 is required to activate ERK in these cells or that a rapid ΔpH from acidic (pH 6.6) to near normal (7.10) is required for ERK phosphorylation (and NHE is merely the pathway that mediates the pH recovery in these cells). Next we tried to distinguish between a requirement for NHE-1-mediated proton translocation and ΔpH from acidic toward resting intracellular pH. We acid loaded the cells in Na⁺-depleted buffer to make NHE inactive, challenged them again with 20 mM NH₄Cl to evoke a rapid alkaline shift, and measured the level of ERK phosphorylation. The results presented in Fig. 5C show that alkalinization per se (independent of NHE) caused significant activation of ERK1/2 (∼200%). However, the increase in ERK phosphorylation was much more pronounced (5 times over basal) after NHE activity was stimulated by applying Na⁺-replete isosmotic buffer to the cells, suggesting that enhanced NHE activity is required for ERK phosphorylation.

Position of NHE Relative to MEK and Ras in the ERK Activation Pathway—If NHE is a true component of the signaling pathway through which ERK becomes activated, it should be interposed between known components of the ERK activation pathway. Fig. 6A shows that 10 μM MIA nearly completely prevented increases in ERK phosphorylation induced by 1 μM 5-HT (hatched bar) or 100 nM Ang II (gray bar), confirming the results shown in Figs. 3C and 4A. Moreover, pretreatment with MIA nearly completely attenuated MEK phosphorylation induced by either 5-HT or Ang II (Fig. 6B), suggesting that NHE is located upstream of MEK. When Ras activity was assessed under the same conditions (Fig. 6C), both 5-HT and Ang II induced a doubling of Ras activity in the absence of MIA. In the presence of MIA, neither agonist induced a significant activation of Ras activity. However, MIA itself slightly activated Ras (∼25%), potentially confounding the definitive localization of NHE upstream of Ras. What we can safely conclude from these results is that NHE is at or above the level of Ras in this pathway.

ERK Regulation by NHE Occurs Downstream of EGF Receptor Phosphorylation—It is generally accepted that Gq-coupled receptors activate ERK in vascular smooth muscle cells through transactivation and phosphorylation of the EGF receptor (56–60). Therefore, we next sought to establish whether NHE regulates ERK activation in RASM cells upstream of the EGF receptor. Fig. 7A shows that isotonic substitution of sodium with tetramethylammonium does not attenuate EGF-induced ERK activation. Similarly, pretreatment of RASM cells

Experimental Procedures.
with 5 μM EIPA does not affect EGF-mediated increases in ERK phosphorylation. These findings suggest that EGF-mediated activation of ERK occurs either upstream of or in parallel with NHE activation. We next sought to study the pathway by which EGF activates NHE in RASM cells (Fig. 7B). Pretreatment of RASM cells with 50 μM PD98059 (MEK inhibitor) did not change the ability of EGF to activate NHE, showing lack of a role for MEK/ERK pathway in EGF-induced NHE activation. To test the possible involvement of PKC in NHE activation by EGF, we pretreated cells with a PKC inhibitor (1 μM GF109203X) for 30 min prior to stimulation with 10 ng/ml EGF. This treatment did not affect NHE activation by EGF but was able to inhibit PMA (1 μM)-elicited proton efflux, showing that this maneuver blocked PKC in our conditions. Finally, we pretreated cells with two different inhibitors of CaM (50 μM W7 and 1 μM ophiobolin), both of which almost completely inhibited EGF-mediated phosphorylation of the EGFR and that inhibition of NHE with 10 μM MIA has no effect on Ang II- or 5-HT-induced transphosphorylation of the EGFR. This suggests that NHE is not upstream of EGFR phosphorylation when it is induced through transactivation by Ang II or 5-HT.

**DISCUSSION**

The regulatory relationships between NHE and ERK have been the subject of a number of studies over the last decade. Because both proteins can serve mitogenic functions and because both are activated by similar stimuli, it has been hypothesized that one may be a regulator for the other. Indeed, in some cell types ERK plays a clear role in either the short- or long-term activation of NHE. However, in other cell types ERK has not been demonstrated to play a regulatory role in the activation of NHE. The current study investigated the roles of NHE and ERK (as stimulated by either 5-HT or Ang II) in the activation of each other in RASM cells. What is new about this work is that we have found evidence to support a novel role for NHE in the activation of ERK in RASM cells. This evidence includes 1) dual stimulation of NHE and ERK by Ang II and 5-HT, with the activation of NHE preceding that of ERK; 2) similar concentration-response relationships for the stimulation of NHE and the phosphorylation of ERK by 5-HT and Ang-II; 3) blockade of the activation of ERK induced by 5-HT and Ang II by chemical inhibition of NHE; 4) blockade of the activation of ERK induced by 5-HT and Ang II by removal of sodium from incubation buffers; and 5) phosphorylation of ERK during recovery from an imposed acid load, a maneuver that induces receptor-independent activation of NHE. Moreover, in the case of receptor-induced activation of ERK, NHE appears to be located upstream of MEK and ERK and parallel to Ang II- and 5-HT-mediated transactivation of the EGFR receptor. NHE intersects the classical pathway of activation of ERK at or above the level of Ras. The specificity of the blockade of ERK phosphorylation by maneuvers that inhibit NHE suggests that this effect is not an artifact. Fig. 8 depicts one possible scheme that can account for our findings.

Although the regulation of ERK by NHE-1 has not previously been thoroughly documented, it certainly is not farfetched, especially when one considers that specific inhibitors of NHE have been shown to reduce neointimal proliferation in a rat model of carotid artery injury (61). Ang II and 5-HT are both potent vasoconstrictors, and Ang II has been shown to play major roles in various cardiovascular diseases, including left ventricular hypertrophy and hypertension (62). Thus, our findings have potential implications for the regulation of vascular tone as well as for vascular pathobiology.

The relationships between ERK and NHE appear to be somewhat complex, although in RASM cells NHE activation is closely associated with activation of ERK. Indeed, inhibition of NHE activity by depriving the extracellular sodium or by blockade with MIA or EIPA prevents activation of ERK by two G protein-coupled receptor ligands, Ang II and 5-HT. Those same maneuvers have no effect on EGF-stimulated ERK, suggesting that there are some differences in the pathways used by Ang II and 5-HT to activate ERK when compared with that used by EGF. Interestingly, the close connection between NHE and ERK activation is further underscored by the observation that receptor-independent activation of NHE also results in ERK phosphorylation only when the exchanger is allowed to mediate recovery from an imposed intracellular acid load. Thus, NHE activation is necessary for Ang II- and 5-HT-induced activation of ERK and is sufficient to activate ERK under conditions of an imposed acid load. In
It is likely that the critical role of NHE in G protein-coupled receptor-mediated activation of ERK will be restricted to specific cell types and receptors. Indeed, we have only been able to detect this relationship in cells of contractile phenotype, although we have not yet performed a broad screen of cells. Much of the previous work in this area has focused on the role of ERK in activating NHE, although that relationship also seems to depend upon cell type and the means used to stimulate NHE. This point was nicely illustrated in work performed by Kanda et al. (66) in which thrombin-induced activation of NHE in A10 cells could be blocked by a MEK inhibitor, whereas platelet-derived growth factor-induced activation of NHE could not. The cellular specificity of the relationship between NHE-1 and ERK could be mediated by alternate accessory components of each signaling pathway or by cell-specific compartmentalization of scaffolded signal transduction platforms (67). In any case, the current work demonstrates that NHE can regulate ERK in RASM cells.

In summary, we have demonstrated that in RASM cells NHE activation plays a necessary role in Ang II- and 5-HT-mediated activation of ERK. Interestingly, this stimulation of ERK through an NHE-dependent pathway could be mimicked by acid loading of RASM cells, a receptor-independent method of activating NHE. Studies with EGF receptors suggested that EGF receptor transactivation was also critical for Ang II- and 5-HT-mediated activation of NHE. In contrast, NHE activation played no role in EGF receptor-mediated stimulation of ERK. Mapping studies demonstrated that the NHE- and EGF receptor-dependent upstream pathways converged distal to the ERK. As stated previously, one must acknowledge that Ang II- and 5-HT-activated EGFR, one must acknowledge that Ang II- and 5-HT-induced transactivation of the EGFR may not perfectly mimic EGF-mediated activation of the EGFR. It is also possible that NHE plays an accessory role in Ang II- and 5-HT-induced activation of ERK by facilitating cytoskeletal reorganization or by altering Na$^+$ or H$^+$ concentrations in cellular microdomains, thereby affecting enzyme activity or protein-protein interactions. The speculation regarding the cytoskeletal effects of NHE is particularly intriguing in light of recent work by Barber and co-workers (1, 63-65) showing important functional links between NHE activity and the cytoskeleton. Moreover, some cytoskeletal regulatory effects of NHE-1 require ion translocation, whereas others do not (65).

In that regard, our data support the need for active ion transport by NHE-1 in the activation of ERK by Ang II and 5-HT. The data in Fig. 5A demonstrate that the simple act of intracellular acidification is not sufficient to activate ERK. Rather, recovery of pH in the presence of sodium is required, suggesting a key role for NHE in this process. However, our data do not allow us to distinguish between a specific requirement for NHE-1-mediated proton translocation and ΔpH, from acidic toward resting intracellular pH.
ERK Is Regulated by Sodium-Proton Exchanger in Rat Aortic Vascular Smooth Muscle Cells
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doi: 10.1074/jbc.M304907200 originally published online November 4, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M304907200

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