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Acidosis-mediated regulation of the NHE1 isoform of the Na^+/H^+ exchanger in renal cells

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Odunewu A, Fliegel L. Acidosis-mediated regulation of the NHE1 isoform of the Na^+/H^+ exchanger in renal cells. *Am J Physiol Renal Physiol* 305: F370–F381, 2013. First published May 15, 2013; doi:10.1152/ajprenal.00598.2012.—The mammalian Na^+/H^+ exchanger isoform 1 (NHE1) is a ubiquitous plasma membrane protein that regulates intracellular pH by removing a proton in exchange for extracellular sodium. Renal tissues are subject to metabolic and respiratory acidosis, and acidosis has been shown to acutely activate NHE1 activity in other cell types. We examined if NHE1 is activated by acute acidosis in HEK293 and Madin-Darby canine kidney (MDCK) cells. Acute sustained intracellular acidosis (SIA) activated NHE1 in both cell types. We expressed wild-type and mutant NHE1 cDNAs in MDCK cells. All the cDNAs had a L163F/G174S mutation, which conferred a 100-fold resistance to EMD87580, an NHE1-specific inhibitor. We assayed exogenous NHE1 activity while inhibiting endogenous activity with EMD87580 and while inhibiting the NHE3 isoform of the Na^+/H^+ exchanger using the isoform-specific inhibitor S3226. We examined the activation and phosphorylation of the wild-type and mutant NHE1 proteins in response to SIA. In MDCK cells we demonstrated that the amino acids Ser^{771}, Ser^{776}, Thr^{779}, and Ser^{785} are important for NHE1 phosphorylation and activation after acute SIA. SIA activated ERK-dependent pathways in MDCK cells, and this was blocked by treatment with the MEK inhibitor U0126. Treatment with U0126 also blocked activation of NHE1 by SIA. These results suggest that acute acidosis activates NHE1 in mammalian kidney cells and that in MDCK cells this activation occurs through an ERK-dependent pathway affecting phosphorylation of a distinct set of amino acids in the cytosolic regulatory tail of NHE1.

acidosis; ERK; Na^+/H^+ exchanger; phosphorylation

THE Na^+/H^+ EXCHANGER ISOFORM-1 (NHE1) is a ubiquitously expressed plasma membrane glycoprotein which functions to protect cells from intracellular acidification by extruding one intracellular proton in exchange for a single extracellular sodium (8). Ten isoforms of Na^+/H^+ exchanger have been discovered (NHE1–NHE10). NHE1 was the first isoform discovered and is ubiquitously distributed (9). Other isoforms have more restricted tissue distributions, and some have predominantly intracellular localization. In mammals, aside from its role in regulation of intracellular pH, NHE1 is also important in regulation of cell volume, cell proliferation, and differentiation and in metastasis of some types of tumor cells (2, 8, 39).

Na^+/H^+ exchanger proteins have two major domains. The N-terminal membrane domain is ~500 amino acids and transports ions. This is followed by a hydrophilic, carboxyl-terminal cytosolic domain that is 315 amino acids in human NHE1. The cytosolic domain regulates the membrane domain, and much of the regulation is by phosphorylation occurring in the distal 178 amino acids of the cytosolic domain (24, 36). We demonstrated that the mitogen-activated protein kinases (MAPK) extracellular regulated kinases 1 and 2 (ERK1/2) are implicated in growth factor activation of NHE1 in the myocardium (24) and in Chinese hamster ovary (CHO) cells (38). The ERK-dependent pathway was activated in several models of ischemic heart disease that cause acidosis, and this resulted in further activation of the Na^+/H^+ exchanger in the myocardium (23). We also demonstrated that ERK phosphorylates the NHE1 cytosolic domain at one or more of the following amino acids, Ser^{693}, Ser^{766}, Ser^{770}, Ser^{771}, Thr^{779}, and Ser^{785} (17) in vitro. In CHO cells (19) and in heart cells (4), we showed that the amino acids Ser^{770} and Ser^{771} are critical to NHE1 activation by acidosis.

Isoform one of the Na^+/H^+ exchanger is ubiquitous in mammalian tissues. One tissue in which it is present is the kidney. NHE1 has been identified in renal tissues by Western blotting, characterization of activity, or identification of its mRNA in various renal cell lines and tissues including Madin-Darby canine kidney (MDCK) cells (16), rat proximal tubule cells (3), in M-1 cortical collecting duct cells (20), human embryonic kidney cells (40), renal tissues including renal tubules (27), inner medullary collecting duct (35), and whole renal cortex (1). It has been localized on both the apical and on the basolateral membrane of polarized renal cells MDCK cells (26), although more frequently reported to be on the basolateral membrane only (15, 21). In renal cells the Na^+/H^+ exchanger is involved in a variety of functions including $NaHCO_3$ absorption, NaCl absorption, pH regulation, and maintenance of cell volume (reviewed in Ref. 21). The NHE3 isoform of the Na^+/H^+ exchanger is also present in renal cells and is found on the apical membrane of polarized renal cells (21). Several studies have shown earlier that acidosis activates NHE1 in the myocardium via ERK-mediated phosphorylation (4, 12). The kidney is subject to chronic acidosis, which results in a host of adaptive changes including elevated NHE1 message (22). Long-term metabolic or respiratory acidosis has been reported to elevate messenger RNA levels of NHE1 (14, 22). However, the effects of short-term acidosis on the activity of NHE1 in renal cells have not been well examined. In addition, although ERK 1/2 have been suggested to regulate NHE in kidney cells (10), the molecular mechanisms of ERK-dependent regulation of NHE1 have not been elucidated in renal tissues. In this study, we examine if acidosis acutely activates the NHE1 isoform of the Na^+/H^+ exchanger in renal cells. We determine the mechanism of activation and amino acids involved in regulation of the protein by phosphorylation. Our results are the first demonstration of the amino acids and mechanisms involved in regulation of this protein in the mammalian kidney.

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MATERIALS AND METHODS

Materials. Protein A-Sepharose beads were from Sigma-Aldrich (St. Louis, MO). 2′,7-Bis (2-carboxyethyl)-5(6) carboxyfluorescein acetoxymethyl ester (BCECF-AM) was from Molecular Probes (Eugene, OR). Anti-MAPK (ERK1/2), anti-phospho ERK1/2, anti-$p90^{RSK}$ (recognizing RSK1/RSK2/RSK3), and anti-phospho $p90^{RSK}$ (Ser 380) were from Cell Signaling Technology (Beverly, MA). Anti-hemagglutinin (HA) antibody (Y-11) was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-NHE1 antibody was a gift from BD Biosciences. Anti-NHE3 antibody was a generous gift of Dr. Todd Alexander of the University of Alberta. S3226 was a generous gift from Dr. Sergio Grinstein of University of Toronto Hospital for Sick Kids, (Toronto, Canada). EMD87580 was a generous gift from Dr. N. Beier of Merck (Frankfurter, Germany). Lipofectamine 2000 reagent was from Invitrogen (Carlsbad, CA). [^{32}P]orthophosphate was from Perkin Elmer Life and Analytical Sciences (Boston, MA). All other chemicals used were of analytical grade and purchased from Fisher Scientific (Ottawa, ON), Sigma, or BDH (Toronto, Canada).

Site-directed mutagenesis. Site directed mutagenesis was performed as previously described (19). Mutations in the regulatory region of the cytosolic tail of NHE1 were made to the expression plasmid $pYN4^+$, which contains the cDNA coding for HA-tagged human NHE1 (31).

The mutants used were as follows: *mutant 3*: S776/770/771A (IRM3); *mutant 4*: T779A/S785A (IRM4); and individual mutations to S770 and S771 to alanine (S770A and S771A) and were described earlier (4). All mutants had additional mutations L163F/G174S which increased NHE1 resistance to inhibition ~100-fold (5).

Cell culture and stable transfection. MDCK cells were passaged in Ham's F-12/DMEM supplemented with 5% FBS and 50 μg/ml gentamicin. HEK293 cells were passaged in DMEM/high glucose supplemented with 10% FBS, 100 μg/ml dentamicin, and 25 mM HEPES. All cells were maintained at 37°C in 5% CO_2.

The cells were stably transfected with the mutant plasmid constructs using Lipfectamine 2000 reagent in accordance with the manufacturer's instructions. Transfected cells were selected with 800 μg/ml geneticin (G418).

Cell surface processing was measured in MDCK cells as described earlier (19, 32). Cell surface proteins were labeled with sulpho-NHS-SS-Biotin (Pierce Chemical, Rockford, IL). After solubilization a total fraction was examined and an unbound fraction that was incubated with immobilized streptavidin as described earlier. We have earlier noted that bound labeled NHE1 does not reliably elute from streptavidin resin, so we examined total and unbound fractions (32). Total fraction and unbound fraction were loaded on 10% acrylamide gels for SDS-PAGE. NHE1 (upper and lower bands) were detected by Western blotting, and quantified using ImageJ software.

Immunoprecipitation of phosphorylated NHE1. For some experiments, cells expressing NHE1 were labeled with [^{32}P]inorganic phosphate. The media were removed, and the plates were washed two times with phosphate-free DMEM. Then, 8 ml of phosphate-free DMEM were added for 30 min at 37°C, and the plates were washed 2× with more media. Then, 2 ml of phosphate-free DMEM were added with $H_3{}^{32}PO_4$ added to a final concentration of 100 μCi/ml media. Following incubation with [^{32}P] for 3 h, stably transfected MDCK cells were subjected to sustained intracellular acidosis (SIA) for 3 min. Cell lysates were then prepared using RIPA buffer containing 150 mM NaCl, 80 mM NaF, 50 mM Tris·HCl (pH 8.0), 5 mM EDTA, 1 mM EGTA, 5 mM Na pyrophosphate, 1 mM Na orthovanadate and protease inhibitors, 0.1 mM PMSF, 0.1 mM benzamidine, and 40 μM ALLN. Where indicated, detergents were added as follows, 1.0% (vol/vol) NP-40, 0.5% (wt/vol) deoxycholate, and 0.1% (wt/vol) SDS. Ice-cold RIPA buffer without detergents was added to the cells, which were then placed on dry ice for 5 min. After thawing on ice, cells were scraped and put into Eppendorf tubes and sonicated on ice for ~15 s. The cells were centrifuged at 14,000 rpm for 30 min at 4°C. The supernatant was removed, and the pellets were resuspended in RIPA buffer with detergents and sonicated for ~15 s. The cells were further centrifuged at 10,000 rpm for 30 min at 4°C.

To immunoprecipitate NHE1 via the HA-tag, the supernatant was incubated with 1.4 μg/ml of rabbit HA-probe (Y11) sc-805 (Santa Cruz) antibody and rocked overnight at 4°C. Then, 100 μl of prepared protein-A-Sepharose beads were added to the supernatant with antibody, and the mixture was rocked for 1 h at 4°C. The beads were

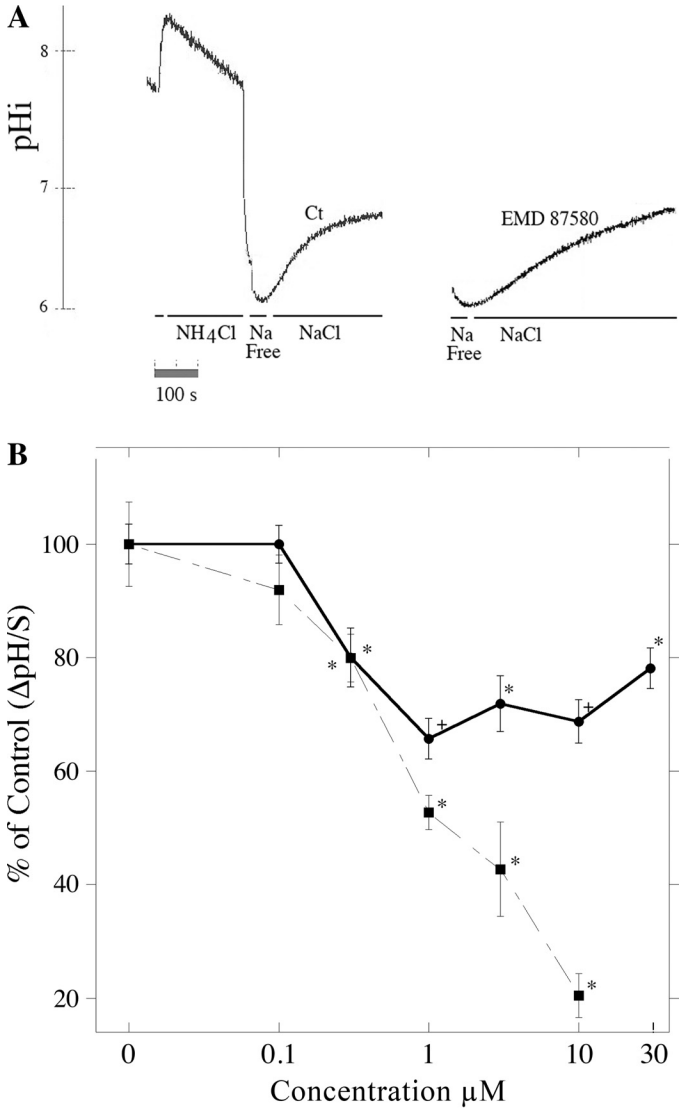

Fig. 1. Characterization of the Na^+/H^+ exchanger in Madin-Darby canine kidney (MDCK) cells. *A*: examples of traces illustrating the effect of EMD87580 (10 μM) on recovery from an acute acid load. Intracellular pH was examined in cells that were transiently acidified using ammonium chloride as described in MATERIALS AND METHODS. Periods of NH_4Cl, NaCl, and Na-free solution are indicated. An entire example of the recovery in the absence of EMD87580 is indicated. For treatment with EMD87580, only the recovery after acidification is shown. Ctl, control. *B*: summary of endogenous NHE1 activity in the presence of varying concentrations of EMD87580 (●) or S3226 (■). Intracellular pH was measured as described in *A*, and recovery from acidosis was in the presence of varying concentrations of inhibitor present in the entire recovery stage. Values are means ± SE of at least 6 experiments. $^*P < 0.05$, $^+P < 0.01$, significantly different from the control. Absolute value of control activity of NHE1 in MDCK cells was 0.21 ± 0.023 ΔpH/min.

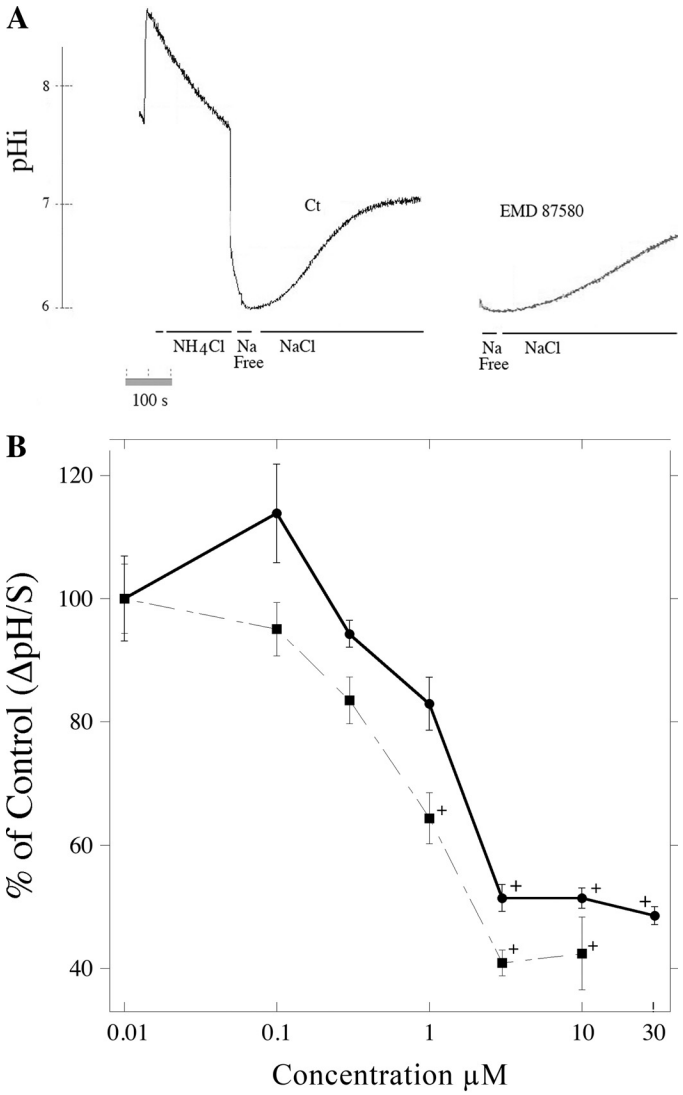

Fig. 2. Characterization of the Na^+/H^+ exchanger in HEK cells. *A*: examples of traces illustrating the effect of EMD87580 (10 μM) on recovery from an acute acid load. Intracellular pH was examined as described in Fig 1*A*. *B*: summary of endogenous NHE1 activity in the presence of varying concentrations of EMD87580 (●) or S3226 (■). Intracellular pH was measured as described in Fig. 1*A*, and recovery from acidosis examined was in the presence of varying concentrations of EMD87580, which was present in the entire recovery stage. Values are means ± SE of at least 6 experiments. $^+P < 0.01$, significantly different from the control. Absolute value of control activity of NHE1 in HEK cells was 0.25 ± 0.012 ΔpH/min.

collected by centrifugation at 4,000 rpm for 30 s and washed twice with RIPA buffer. The bound proteins were eluted from the beads by solubilizing the proteins in 50 μl of 1× SDS-PAGE sample-loading buffer at 37°C for 15 min. The beads were spun down, and the supernatant containing immunoprecipitated NHE1 was run on SDS-PAGE gel. After transfer to nitrocellulose membrane, radioactivity was detected using a Typhoon 9400, variable mode imager (GE Healthcare, Piscataway, NJ).

SDS-PAGE and immunoblotting. Expression of NHE1 mutants was confirmed by immunoblotting using anti-HA antibody (Boehringer Mannheim, Laval, Quebec). Equal amounts of each sample were resolved on 10% SDS-PAGE gels. The gels were transferred to nitrocellulose membrane and immunostained with anti-HA monoclonal antibody and peroxidase-conjugated goat anti-mouse antibody (Bio/Can, Mississauga, Canada). Immunoreactive proteins were detected on X-ray film using the Amersham enhanced chemiluminescence Western blotting and detection system.

For the detection of ERK1/2, phospho-ERK1/2, $p90^{RSK}$, and phospho $p90^{RSK}$, antibodies to these proteins were used according to the manufacturer's instruction and visualized with the appropriate secondary antibodies. Gels were transferred to a nitrocellulose membrane, and immunoreactive proteins were viewed on the membrane using the Li-COR fluorescence labeling and detection systems (LI-COR Biosciences, Lincoln, Nebraska). For some experiments, immunoreactive proteins were detected on X-ray film by using s antibodies conjugated to horse radish peroxidase.

Densitometric analysis was carried out using ImageJ 1.35s software (National Institute of Health, Bethesda, MD).

Intracellular pH measurement. To determine the effect of SIA on NHE1 in kidney cells, MDCK and HEK293 cells, grown to ≥90% confluency on coverslips, were incubated overnight in reduced (0.5%) serum media. BCECF was used to measure the rate of intracellular pH (pH_i) recovery after acute acid load by ammonium chloride (50 mM × 3 min). NHE1 activity was measured as the slope of the first 20 s of recovery from acidification and was expressed as ΔpH per second as described earlier (32). Where indicated, cells were subjected to a two-pulse acidification assay using a PTI Deltascan spectrofluorometer as previously described (4). NH_4Cl prepulse was used to acidify the cells both times. In the first pulse, acidification of cells by NH_4Cl removal was followed by ~20-s incubation in a Na^+-free buffer after which the cells were allowed to recover in a normal Na^+ buffer. The second pulse was the same except that acidification induced by NH_4Cl withdrawal was allowed to occur for 3 min in Na^+-free buffer before recovery in normal Na^+ buffer. SIA was not introduced in the second pulse for control cells where indicated. For one series of experiments, SIA was induced in the presence of normal external Na^+, by use of a high concentration of the NHE inhibitor 1 mM 5-(*N*,*N*-hexamethylene)-amiloride to inhibit any NHE1- or NHE3-dependent Na^+/H^+ exchanger activity.

Where noted, assays were carried out in the presence of 10 μM EMD87580 (a NHE1-specific inhibitor) and 3 μM S3226 (a specific NHE3 inhibitor). We have determined that these concentrations suf-

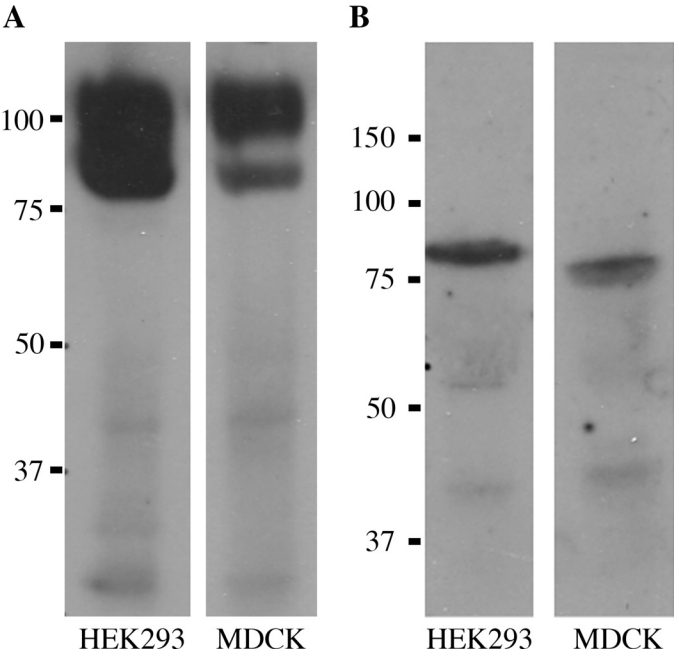

Fig. 3. Western blot analysis of cell extracts from MDCK and HEK293 cells. Cell extracts were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies against anti-NHE1 (*A*) and anti-NHE3 (*B*). Results are typical of 3 experiments.

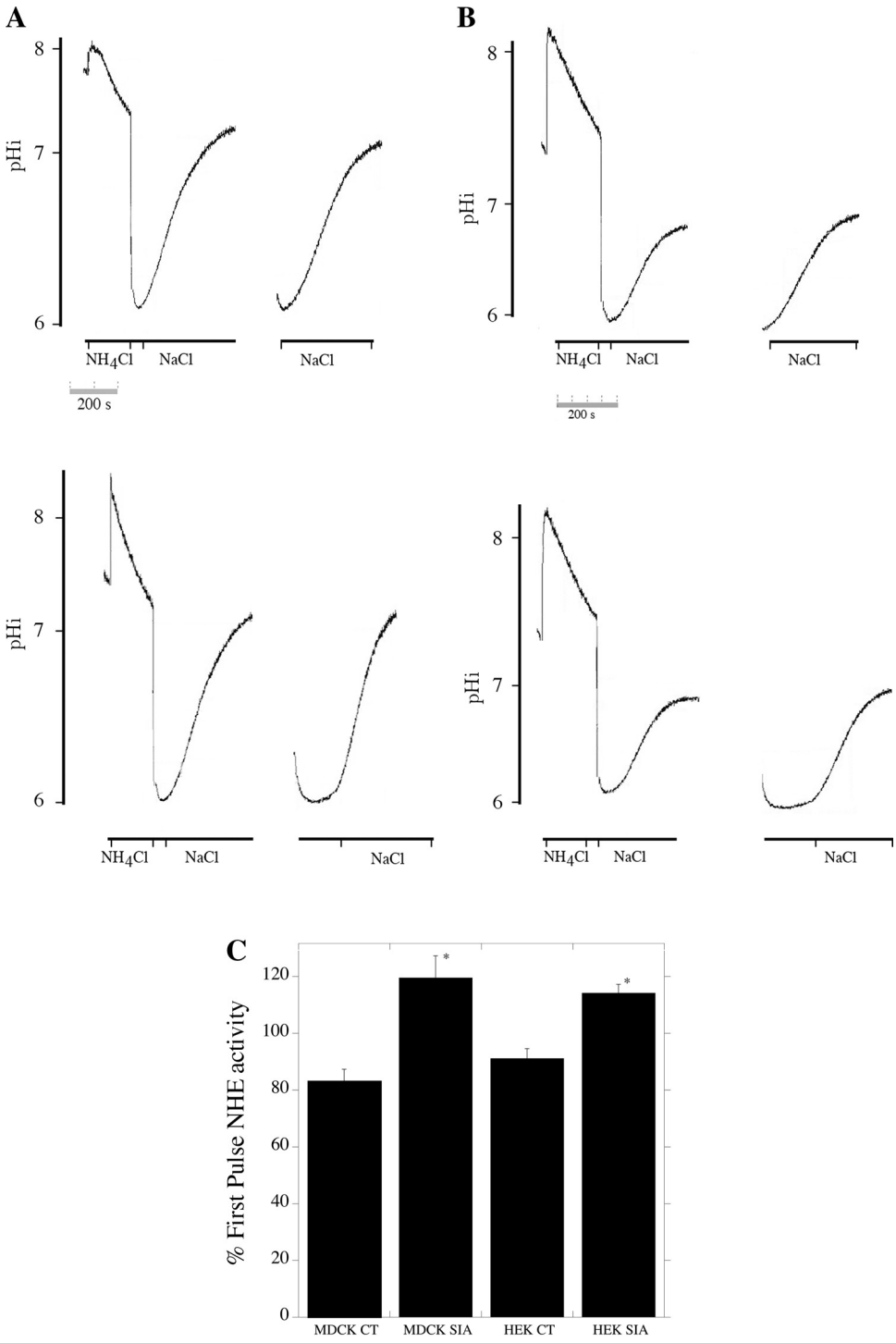

Fig. 4. Effect of sustained intracellular acidosis on NHE1 activity of MDCK and HEK293 cells. Cells were subjected to a 2-pulse Na^+/H^+ exchanger activity assays, and the activity of the exchanger in the 2nd pulse was compared with that of the 1st pulse. The 2nd pulse was done either with or without a 3-min period of sustained intracellular acidosis immediately prior to recovery. *A* and *B*: examples of traces of dual pulse assay on MDCK (*A*) and HEK293 (*B*) cells. *Top*: example of a dual pulse assay illustrating pHi with ammonium chloride treatment and recovery after transient acidosis. For brevity, only the recovery after the 2nd ammonium chloride pulse is shown. *Bottom*: as in the *top* but for the 2nd recovery cells were maintained in sustained acidosis for 3 min before reintroduction of NaCl. After ammonium chloride treatment and before each recovery from acidosis, cells were maintained either a brief or extended period in Na^+-free medium. *C*: summary of NHE1 activity in dual pulse assays of MDCK and HEK293 cells. Two pulse assays were used, and in experimentals, the 2nd pulse contained an extended 3-min period of sustained intracellular acidosis (SIA). Controls (CT) did not have a sustained acidosis in the 2nd pulse. Values summarized are means ± SE of 6–10 experiments. $*P < 0.05$, significantly different from the control. Absolute value of control activity of NHE1 in MDCK and HEK cells was 0.25 ± 0.023 and 0.23 ± 0.01 ΔpH/min, respectively.

ficiently inhibited endogenous NHE1 and NHE3, respectively, in kidney cells, but 10 μM EMD87580 do not inhibit NHE1, which has the L163F/G174S mutation (5). EMD87580 was dissolved in PBS while S3226 was dissolved in DMSO. In some cases, the MEK inhibitor U0126 was used before evaluation of phospho-ERK, phospho-RSK, or NHE1 activity. U0126 was dissolved in DMSO and added to a final concentration of 10 μM. U0126 or control (DMSO) was added for 10 min before assay.

Results are shown as the means ± SE and statistical significance was determined using the Wilcoxon signed-rank test.

RESULTS

We have previously (4, 13, 19) demonstrated that sustained intracellular acidosis activates the NHE1 isoform of the Na^+/H^+ exchanger. Initial experiments characterized the Na^+/H^+ exchanger in two cell lines derived from renal tissues, MDCK and HEK293 cells. They also examined whether sustained intracellular acidosis could stimulate Na^+/H^+ exchanger activity. EMD87580 is a specific inhibitor of the NHE1 isoform

of the Na^+/H^+ exchanger. At a concentration of 10 μM, the NHE1 isoform is largely inhibited while the NHE3 isoform of the Na^+/H^+ exchanger is resistant to inhibition until much higher concentrations are used (5, 6). Figure 1, *A* and *B*, illustrates the effect of addition of the inhibitor EMD87580 on the Na^+/H^+ exchanger activity of MDCK cells. Concentrations of EMD87580 of 1 μM or higher inhibited ~30% of the NHE activity, consistent with a significant, though minority presence of the NHE1 isoform of the Na^+/H^+ exchanger.

Figure 2, *A* and *B*, illustrates similar experiments with HEK cells. At concentrations >1 μM EMD87580 inhibited ~50% of the Na^+/H^+ exchanger activity of these cells consistent with the presence of the NHE1 isoform of the Na^+/H^+ exchanger.

S3226 is a Na^+/H^+ exchanger inhibitor reported to have specificity for the NHE3 isoform of the Na^+/H^+ exchanger (28). To determine if this isoform was present in MDCK and HEK cells, we tested the effect of this inhibitor on Na^+/H^+ exchanger activity in these cell types. Figures 1*B* and 2*B* show that the presence of S3226 potently inhibited up to 80% and of Na^+/H^+ exchanger activity in MDCK cells while in HEK cells the effect plateaued at 60% inhibition.

To confirm the presence of the NHE1 and NHE3 isoforms of the Na^+/H^+ exchanger Western blotting was used. Figure 3*A* demonstrates that the NHE1 isoform of the protein was present. There was a characteristic larger molecular mass isoform and a smaller size protein corresponding to the fully and partially or deglycosylated forms of the NHE1 protein, respectively, that we have described earlier (32). Figure 3*B* demonstrates the presence of the NHE3 isoform of the Na^+/H^+ exchanger. A characteristic ≈80- to 85-kDa immunoreactive band was present. There was a slight difference in the apparent molecular mass of NHE3 between the two cell types, which could be due to differences in glycosylation.

We next examined whether a sustained intracellular acid load stimulated activity of the Na^+/H^+ exchanger in MDCK and HEK293 cells. We (4, 19) and others (12) have demonstrated this phenomenon earlier in other cell types. The results are shown in Fig. 4. Cells were subjected to a two pulse assay with acidosis induced by ammonium chloride withdrawal. In the second pulse, acidosis was maintained for 3 min before reintroduction of NaCl and recovery of pH_i by the Na^+/H^+ exchanger. Figure 4*C* illustrates a summary of the results and Fig. 4, *A* and *B*, examples of activity. In MDCK cells, the recovery from the second pulse was slightly reduced, compared with the first pulse, in the absence of a sustained acidosis. In contrast, after 3 min of sustained acidosis, the second recovery was elevated compared with the first pulse and was significantly greater than the value obtained in the absence of acidosis. Similar results were obtained with HEK293 cells though to a lesser extent. SIA caused a smaller, but significant, increase in NHE activity (Fig. 4*C*). In one series of experiments we used 5-(*N*,*N*-hexamethylene)-amiloride to cause SIA in MDCK cells in the presence of normal external Na^+. Under these conditions, SIA induced a significant increase in the rate of recovery from acidosis, which was 43% greater than recovery in the absence of SIA (not shown).

For our experiments, we included S3226 as an inhibitor of the NHE3 isoform of the Na^+/H^+ exchanger. To confirm that the dose of S3226 that we used does not inhibit the NHE1 isoform of the exchanger, we tested the effect of 10 μM S3226 on activator protein-1 (AP-1) cells that were transfected with the inhibitor-resistant isoform of the Na^+/H^+ exchanger that we used earlier in our experiments (5). AP-1 cells have their endogenous Na^+/H^+ exchanger deleted and only possess that Na^+/H^+ exchanger that we add to the cells (25). Figure 5 demonstrates that 10 μM S3226 did not inhibit activity of the exogenous NHE1 protein.

Since we demonstrated (Fig. 4) that SIA produced an enhancement of NHE1 activity, we examined which amino acids of the regulatory cytosolic domain of NHE1 might be involved. We had previously demonstrated that two regions containing phosphorylatable amino acids are potentially important in this regard. Ser^{766}, Ser^{770}, and Ser^{771} were in one region (called region 3) and amino acids Thr^{779} and Ser^{785} of region 4 (4). Ser^{703} (13) and the phosphorylatable amino acids Ser^{693} and Thr^{718}, Ser^{723}, Ser^{726}, and Ser^{729} (4, 19) were not involved in the SIA response in several cell types. We therefore made stable cell lines of cells that contained plasmids that express NHE1 with mutations in region 3 and region 4. The NHE1 isoform that was expressed contained mutations in transmembrane segment IV that made the protein resistant to inhibitors. EMD87580 inhibitor (and S3226) were added to the assay so that only exogenous NHE1 activity was measured. The results are shown in Fig. 6. Figure 6*A* demonstrates that the various stable cell lines in MDCK cells expressed the HA-tagged NHE1 protein. Similar results were obtained in HEK cells (not shown). In MDCK cells, cell lines with NHE1 with a mutation in Ser^{771} or with the mutations in IRM3 and IRM4 were not stimulated by sustained intracellular acidosis (Fig. 6*B*). However, the wild-type NHE1 protein and mutation of Ser^{770} to Ala did not prevent stimulation of activity by SIA. In contrast in HEK cells (Fig. 6*C*), none of the mutations prevented SIA from stimulating NHE1 activity, suggesting that the mechanism of stimulation of NHE1 activity was different in these cell types.

We examined if the mutations we introduced altered surface targeting of the wild-type and mutant NHE1 protein in MDCK cells. The results are shown in Fig. 6*D*. Wild-type, IRM3, IRM4, and the S770A and S771A mutants had similar, although not identical, levels of targeting to the plasma membrane, which was consistent with results shown earlier (19).

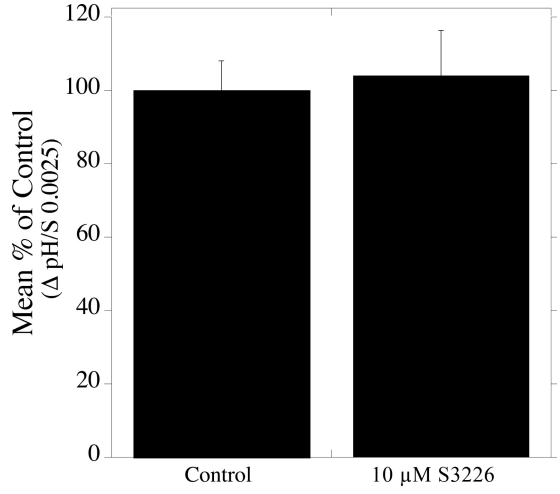

Fig. 5. Effect of 10 uM S3226 on activity of the NHE1 protein in AP-1 cells. An inhibitor-resistant (5) mutant form of the NHE1 protein was introduced into AP-1 cells as described earlier. Activity of the NHE1 isoform of the protein was determined in the presence or absence of S3226 as described in the MATERIALS AND METHODS. S3226 was present during recovery from SIA.

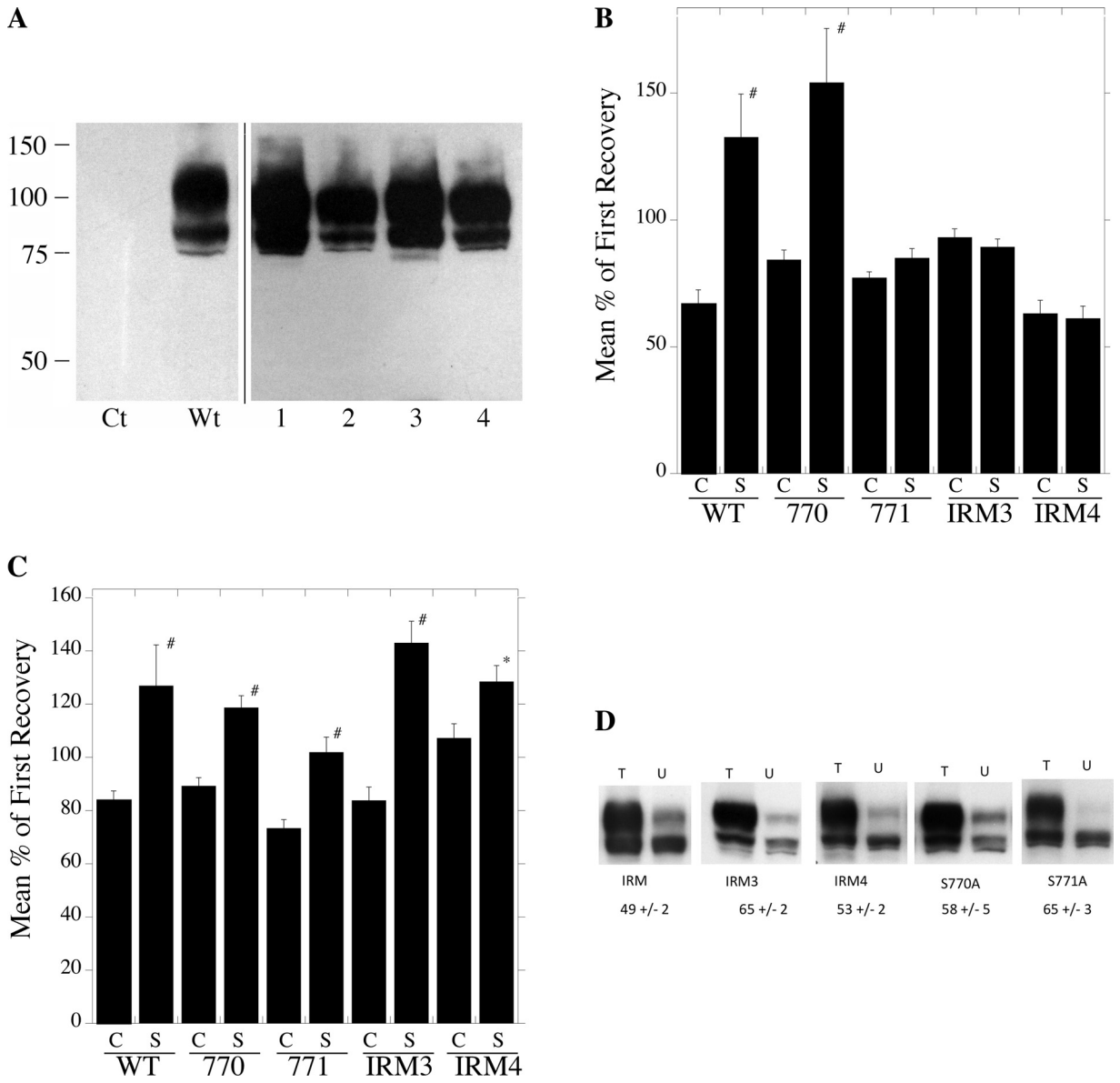

Fig. 6. Analysis of mutated and wild-type NHE1 protein in MDCK and HEK cells. *A*: Western blot analysis of whole cell lysates of stable MDCK cell lines expressing HA-tagged NHE1 protein. Ct, control, whole cell lysate of MDCK cells mock transfected; Wt, lysate of MDCK cells stably transfected with wild-type NHE1 protein. *Lanes 1–4*: stable cell lines of NHE1 protein containing the mutants, S770A, S771A, IRM3, and IRM4, respectively. (Cells were not normalized for protein concentration.) *B* and *C*: effect of mutations of phosphorylation sites on induction of activity of NHE1 by SIA. *B*: stable cell lines of wild-type (WT) or indicated mutant NHE1 proteins in MDCK cells. Cells lines were subjected to dual pulse NHE activity assay essentially as described in Fig. 4. NHE1 activity was assayed in the presence of 10 μM EMD87580 and 3 μM S3226 as indicated. Value of the 2nd recovery from acidosis was compared with the 1st recovery, and results are expressed as percent of the 1st pulse. "C" indicates a dual pulse assay control in the absence of sustained intracellular acidosis. "S" indicates the 2nd pulse was after a period of sustained intracellular acidosis. *C*: as in *B* except in HEK 293 cells. Results are means ± SE of at least 6 experiments. $^*P < 0.05$, $^\#P < 0.01$, significantly different from the control. Absolute values of ΔpH change/min for all MDCK cell types uncorrected for levels of protein expression were from 0.23 to 0.43. For HEK cells, the values were between 0.34 to 1.2 ΔpH/min. *D*: surface localization of NHE1 of wild-type and mutant proteins. Sulfo-NHS-SS-biotin-treated MDCK cells were lysed proteins were solubilized and subsequently treated as described in the MATERIALS AND METHODS. Equal samples of total lysates (T) and unbound (representing intracellular) lysates (U) were resolved on SDS-PAGE and probed with anti-HA antibody to identify NHE1 protein. Amount of surface localized NHE1 was calculated from densitometric analysis by taking the (total protein) − (unbound protein) = (membrane surface localized protein). Percentage of the total NHE1 protein localized to the plasma membrane is indicated. Results are means ± SE; n = at least 3 experiments.

To examine the pathways that were involved in mediating the SIA in MDCK cells, we examined a time course of activation of ERK and $p90^{rsk}$ that had previously been suggested to be involved in activation of NHE1 in other tissues (4, 19). Figure 7*A* illustrates the results that are summarized in Fig. 7, *B* and *C*. Treatment of cells with SIA resulted in an increased amount of P-ERK and P-RSK that peaked at 3 min in MDCK cells for P-ERK. The levels were increased significantly for pERK at 3 min and were significantly elevated for P-RSK at 3 and 5 min. P-ERK levels in HEK 293 cells had a tendency to increase and peaked ~50% over the levels of control, but the changes were more variable and not statistically significant.

To directly determine which amino acids may be involved in regulation of NHE1 by phosphorylation, we examined the

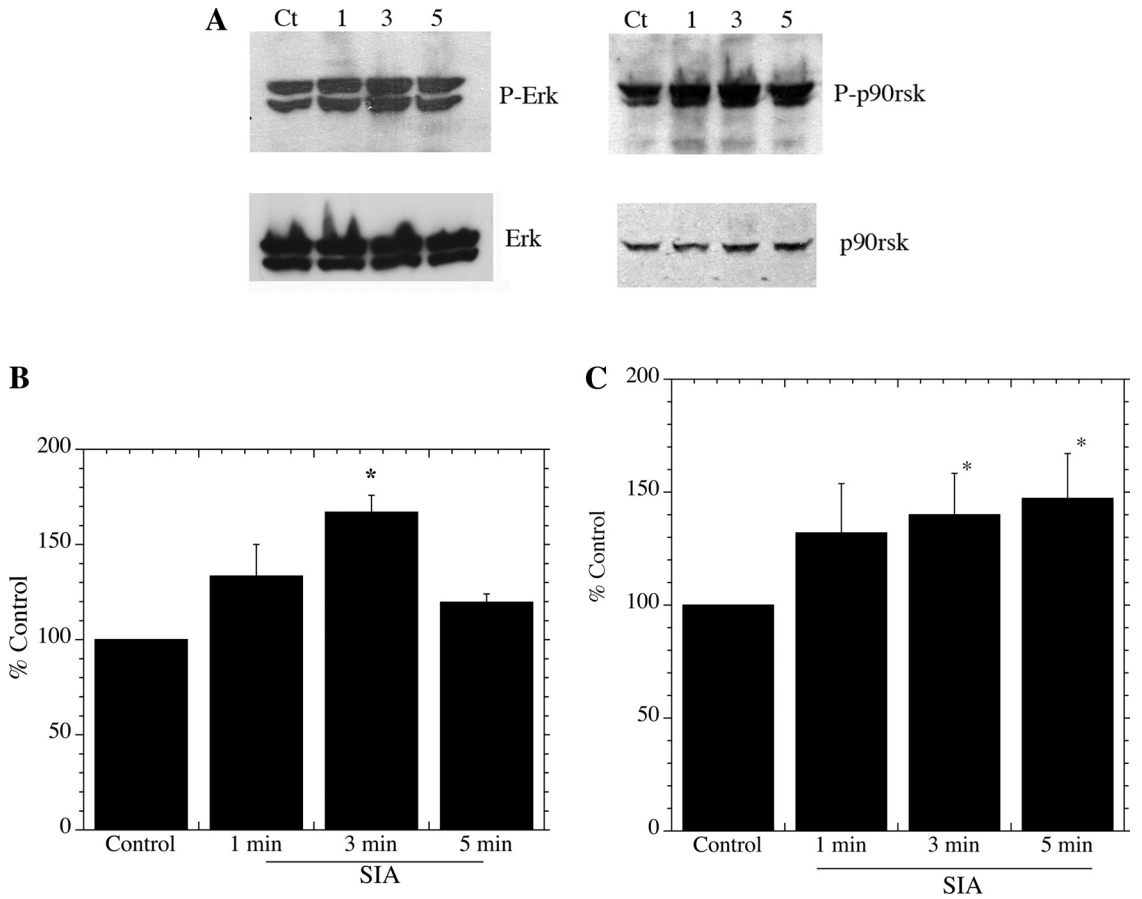

Fig. 7. Time course of activation of ERK and $p90^{rsk}$ by sustained intracellular acidosis in MDCK cells. Cells were subjected to SIA for the times indicated and the level of phospho-ERK and phosphor-$p90^{rsk}$ was examined by Western blotting as described in MATERIALS AND METHODS. *A*: examples of Western blotting for phospho-ERK and phosphor-$p90^{rsk}$. ERK and $p90^{rsk}$ were immunoblotted to control for the absolute level of the protein. *B* and *C*: summary of experiments examining phospho-ERK (*B*) and phosphor-$p90^{rsk}$ (*C*) levels relative to the levels of ERK and $p90^{rsk}$. Results are means ± SE of 3–5 experiments. $^*P < 0.05$, significantly different from the control.

effect of mutation of several putative phosphorylation sites on the level of phosphorylated protein in the presence or absence of SIA. Initially, we compared the basal level of phosphorylation of the wild-type NHE1 protein and of several mutants in MDCK cells. The results (Fig. 8, *A* and *B*) showed that mutation of amino acids Ser^{770} and Ser^{771} or of Thr^{779} and Ser^{785} in the IRM4 mutant caused large decreases in the basal level of phosphorylation of the NHE1 protein.

To determine which amino acids may be involved in activation of NHE1 by SIA, we examined the relative levels of phosphorylation of the NHE1 protein in wild-type and mutant NHE1 proteins in the presence or absence of SIA. The results are shown in Fig. 8, *C* and *D*. SIA induced increased phosphorylation in the wild-type NHE1 protein and in the protein with a mutation at Ser^{770}. In contrast, the other mutants, S771A, IRM3, and IRM4 all showed no increase in phosphorylation in response to SIA and in fact showed a slight decrease in phosphorylation levels. It was necessary to add more of some experimental protein to obtain a detectable signal, which may have caused inaccuracy in these measures, so these values should be considered as only estimates of the radioactivity level.

To further confirm the role of the ERK pathway in acidosis-mediated activation of NHE1 in MDCK cells, we tested the effect of the inhibitor U0126 on the activation of the ERK pathway and on activation of the NHE1 protein. Figure 9, *A* and *C*, demonstrates that treatment with U0126 dramatically reduces the level of phosphor-ERK protein and prevented activation of the ERK by SIA. The levels of phopho-$p90^{rsk}$ were less affected, declining only slightly (Fig. 9, *B* and *D*). Treatment of MDCK cells with U0126 also prevented activation of the cells by sustained intracellular acidosis (Fig. 10).

DISCUSSION

Renal tissues are subject to both chronic metabolic or respiratory acidosiss which result in a host of adaptive changes. While it has been demonstrated that chronic acidosis elevates renal NHE1 message levels (14, 22), the acute effects on activity are not well characterized. We recently demonstrated that acute acidosis activates NHE1 via an ERK-dependent pathway in CHO cells (13, 19) and in isolated cardiomyocytes (4). In these cell types amino acids, Ser^{770} and Ser^{771} were shown to be phosphorylated in response to sustained intracellular acidosis and were important in stimulation of NHE1 activity. While the acute effects on the NHE3 isoform of the Na^+/H^+ exchanger have been well characterized in renal cell types (21), the NHE1 isoform has not been well studied.

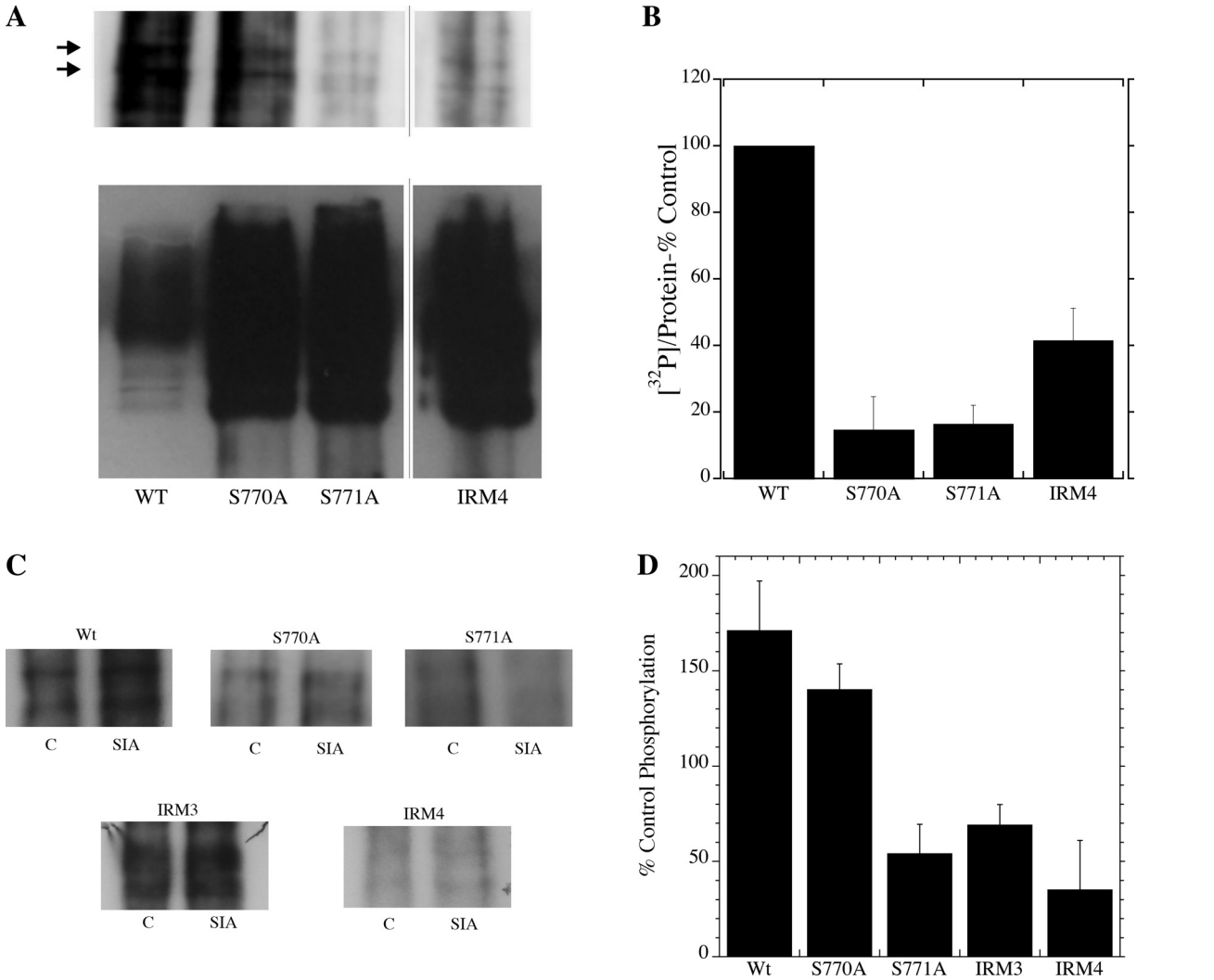

Fig. 8. Effect of mutation of phosphorylation sites on phosphate incorporation in the NHE1 protein. *A*: stable cell lines of wild-type or NHE1 mutant-containing MDCK cells. Cells were incubated in [^{32}P]-containing media and immunoprecipitated as described in MATERIALS AND METHODS. Cells were not stimulated by SIA. *Top*: [^{32}P] profile of immunoprecipitate. *Bottom*: immunoblot of immunoprecipitate using anti-HA tag antibody. *B*: summary of experiments in *A*. Results are means ± SE of at least 3 experiments. *C*: effect of SIA on phosphorylation levels of wild-type or NHE1 mutant-containing MDCK stable cell lines. Cells were incubated in [^{32}P]-containing media, and NHE1 was immunoprecipitated as described in MATERIALS AND METHODS after SIA treatment. Examples of [^{32}P] levels of immunoprecipitates are shown. *D*: summary of experiments in *C*. Phosphorylation levels were corrected for the levels of immunoprecipitated protein which was determined using anti-HA tag antibody. Results are means ± SE of at least 3 experiments.

Whether it is activated by short-term acidosis and any putative molecular mechanism is unknown. We initially characterized two renal-derived cell lines HEK293 cells and MDCK cells. HEK293 cells were derived by transformation of primary cultures of human embryonic kidney cells with adenovirus DNA (11). MDCK cells are a well-characterized epithelial cell line derived from the kidney of a female adult cocker spaniel. MDCK are derived from the distal tubule/collecting duct and have retained morphological traits and enzymatic properties characteristic of their tissue of origin. They have been reported to express NHE1 at both apical and basolateral sides of these cells (16).

Initial experiments determined that both NHE1 and NHE3 protein and activity were present in HEK and MDCK cells, confirming previous observations. While it has been reported that endogenous NHE3 is not expressed in MDCK cells (26), other reports have shown that it can be induced in this cell type and a basal level of expression was shown (33, 34). NHE1-dependent activity was shown by inhibition with EMD87580. Additionally, we found that we were able to inhibit endogenous NHE3 with the compound S3226. S3226 is a specific inhibitor of NHE3 with very low efficacy of inhibition of NHE1 (28). We confirmed that S3226 was ineffective in inhibition of NHE1 by examining its effectiveness in AP-1 cells that expressed only NHE1 isoform (Fig. 5). This experiment also confirmed that NHE1 with the IRM mutation was not sensitive to inhibition by S3226. We therefore included S3226 in assays of NHE1 activity so that we could examine effects on NHE1, free of any residual activity of NHE3.

When we treated either MDCK or HEK cells with sustained intracellular acidosis for a period of 3 min, both cell types had increased NHE1 activity upon recovery. EMD87580 attenuated NHE1 activity with or without SIA treatment (not shown). ERK activation occurred within 3 min in both cell types. We

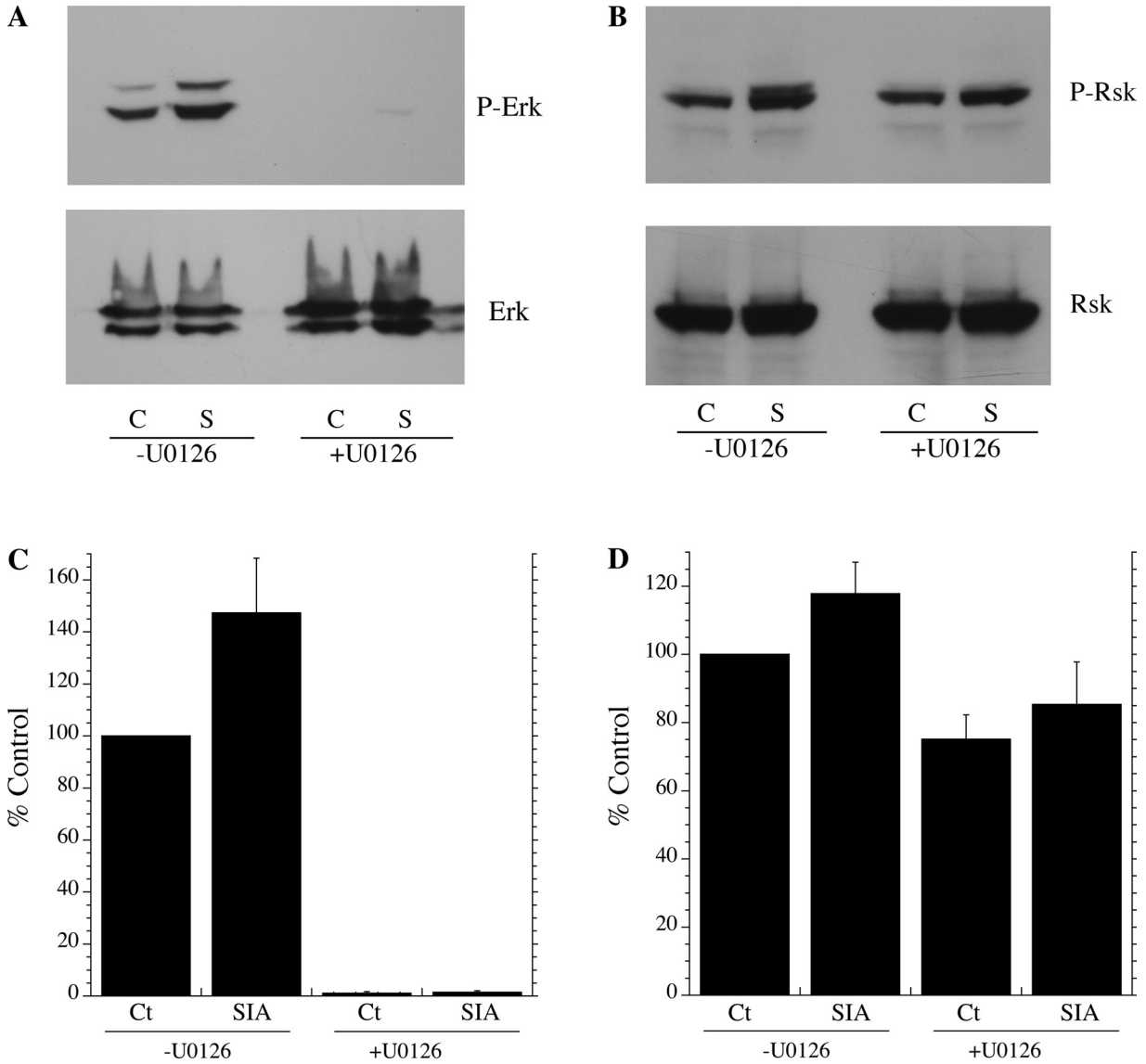

Fig. 9. Effect of U0126 on phosphorylation levels and activation of ERK and $p90^{rsk}$ by sustained intracellular acidosis. *A*: example of Western blotting for ERK and phospho-ERK. *B*: example of Western blotting for RSK and phospho-RSK. *C* and *D*: summary of experiments in *A* and *B*, respectively. Results are means ± SE of at least 3 experiments.

found more consistent activation of ERK in MDCK cells as opposed to HEK cells. Depletion of intracellular Na^+ could theoretically alter the cellular Na^+ gradient and augment NHE1 activity directly. We therefore induced SIA by use of NHE inhibitors, and this SIA induced in the presence of normal Na^+ still caused an increase in NHE1 activity. Other studies have also demonstrated that SIA stimulates NHE1 activity in the presence of normal external Na^+ (12, 13). Prolonged depletion of extracellular sodium can deplete intracellular sodium; however, our assays only used 3 min of sodium depletion making it unlikely that intracellular sodium was depleted (7, 41).

We made stable cell lines of MDCK and HEK cells expressing the NHE1 protein. The exogenous protein was both HA tagged and contained mutations that render it resistant to inhibition by EMD87580 (4, 5). We were therefore able to measure exogenous NHE1 activity by inhibiting endogenous NHE1 activity with EMD87580 and inhibiting any NHE3 activity with S3226. We examined the effect of SIA on the rate of NHE1 activity in both HEK and MDCK cells. For these experiments we examined activity of the wild-type NHE1 protein and of the NHE1 protein with the mutations Ser^{770}, Ser^{771}, S776/770/771A (IRM3), and T779A/S785A (IRM4). HEK cells gave a pattern of activation of NHE1 that was independent of the nature of the mutant protein. In contrast, mutation of Ser^{771} or the IRM3 and IRM4 group of mutations specifically eliminated activation of the NHE1 protein in MDCK cells while the wild-type NHE1 protein and the S770A mutant were activated by SIA. We examined basal phosphorylation levels of the exogenous wild-type NHE1 protein in comparison wild type. Mutation of either Ser^{770}, Ser^{771}, or the IRM4 site caused large decreases in the overall level of basal phosphorylation of the NHE1 protein. This indicated that a significant amount of the rapidly exchangeable phosphate in-

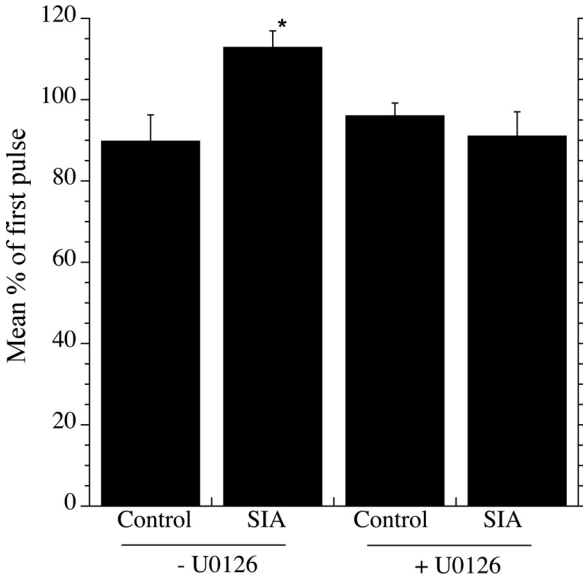

Fig. 10. Effect of U0126 on NHE1 activity after SIA. MDCK cells expressing NHE1 were subjected to a two pulse Na^+/H^+ exchanger activity assays as described in Fig. 4. Rate of recovery after the 2nd pulse was compared with that of the 1st in the presence or absence of sustained intracellular acidosis. S3226 was present in all assays (3 μM). U0126 was present (10 μM) where indicated. Values summarized are means ± SE of at least 10 experiments. *$P < 0.05$, significantly different from the control.

corporation into the NHE1 protein was in these amino acids. There was an indication that mutation of one site might affect phosphorylation levels at other sites, since both the S770A and S771A appeared to cause large reductions in the level of NHE1 basal phosphorylation. This may be through a change in the protein kinase consensus site or by a change in the conformation of the protein in this region, which affects phosphorylation of the adjacent site.

To gain insights into the mechanism by which SIA stimulated NHE1 activity, we examined the effect of SIA on the phosphorylation levels of the wild-type and mutant NHE1 proteins in MDCK cells. We found that our results mirrored the effects on NHE1 activity. Mutation of amino acids S771A or the IRM4 site prevented increases in the phosphorylation level of NHE1 in response to SIA. However, the mutation of S770A did not prevent an increase in NHE1 phosphorylation in response to SIA. These results suggest that amino acid Ser^{771} and the phosphorylation sites T779/S785, in the IRM4 site, are important targets of NHE1 when stimulated by SIA.

These results have both similarities and differences from those that were reported earlier. In the present study we showed that Ser^{771} was critical in activation of NHE1 by SIA and that when it was mutated there was no enhancement of NHE1 phosphorylation levels by SIA. In contrast Ser^{770} was not critical for either activation of NHE1 activity or for elevated phosphorylation in response to SIA. However, previously (4, 19) we demonstrated that in CHO cells and in isolated cardiomyocytes both Ser^{770} and Ser^{771} were important in activation of NHE1 by SIA. Ser^{771} precedes Pro^{772} and forms a consensus sequence for ERK (29). We had earlier suggested that Ser^{770} may form part of the kinase binding site (4). Differences in conformation of this part of the tail region, perhaps induced by binding of tissue-specific regulatory proteins, might account for the differences shown in this study.

The IRM4 region of NHE1 contains Thr^{779} and Ser^{785}. In the present study, we found that this region was important in activation of NHE1 activity by SIA. Mutation of these two amino acids to Ala prevented increased phosphorylation of NHE1 by SIA. Previously, in CHO cells (19), we showed that mutation of this site also prevented increases in NHE1 activity in response to SIA and there was an intermediate effect on phosphorylation levels. However, in isolated cardiomyocytes, mutation of this region did not prevent activation of the NHE1 protein and only had a partial effect on elevation of phosphorylation levels. It appears as though the relative importance of this region varies, depending on the cell type. Tissue-specific regulation of NHE1 has been reported earlier. In the myocardium for example, the Hill coefficient of activation is near 3, steeper than in some other cell types indicating a different regulatory mechanism (37).

An interesting observation of the present study was that the total level of basal NHE1 phosphorylation was greatly reduced by mutation of Ser^{770} and Ser^{771}. Additionally, mutation of Thr^{779} and Ser^{785} also reduced the total level of basal NHE1 phosphorylation. We earlier reported that in cardiomyocytes mutation of Ser^{770} and Ser^{771} reduced the basal level of phosphorylation of NHE1 greatly, while mutation of Thr^{779} and Ser^{785} did not have this effect in these cells. Our results in the present study with MDCK cells followed the same pattern, although we found mutation of Thr^{779} and Ser^{785} had a more significant effect, which correlated with their functional role. Why mutation of either Ser^{770} or Ser^{771} would reduce most of the basal level of phosphorylation may be because mutation of one of the sites affects the others accessibility to regulation.

A critical role of ERK-dependent pathways in SIA was initially described by Haworth et al. (12). We also demonstrated that ERK is critical in SIA activation of NHE1 in CHO cells and in cardiomyocytes (4, 19). The studies demonstrated that ERK and $p90^{rsk}$ can be activated by sustained acidosis in several cell types (4, 12, 19). Our results in MDCK cells confirm the importance of this pathway. U0126, the MEK inhibitor, prevented SIA activation of NHE1. It also blocked activation of the ERK-dependent pathway by SIA. The effect of U0126 was more pronounced on ERK phosphorylation levels than it was with $p90^{rsk}$. We have earlier demonstrated that ERK can directly phosphorylate the NHE1 cytosolic domain in vitro at multiple regions including regions encompassing Ser^{770}, Ser^{771}, Thr^{779}, and Ser^{785} (18). The present results demonstrate that this pathway of activation of ERK and $p90^{rsk}$ is also active in MDCK cells. While phosphorylation of Ser^{703} by $p90^{rsk}$ has been demonstrated to be important in some pathological circumstances (18), we demonstrated that it was not important in activation of the Na^+/H^+ exchanger by SIA in both CHO cells and cardiomyocytes (13). The present results are consistent with these observations and suggest that phosphorylation sites that are further downstream are more critical in activation of NHE1 by SIA.

While our experiments demonstrated that SIA activated NHE1 in HEK cells, we did not demonstrate that mutation of any of the residues Ser^{770}, Ser^{771}, Thr^{779}, and Ser^{785} played a significant role in this activation in this cell type. This contrasted with results in MDCK cells and with our results in CHO cells and isolated cardiomyocytes. In HEK 293 cells, it seems clear that activation of NHE1 by SIA occurs by another mechanism, which has yet to be elucidated. While this result

appears surprising, recently results have suggested that HEK293 cells have an unexpected close relationship to neuronal cells and are not typical kidney cells. They have a pattern of intermediate filament expression similar to that of early differentiating neurons, and despite their wide use as kidney cells, they have properties of neuronal lineage cells and not more typical of kidney cells (30). This may explain their different regulation of NHE1 when compared with MDCK cells.

Overall, our study demonstrates for the first time that SIA acutely activates NHE1 activity in kidney cells. We have defined specific residues that are involved in this process and the pathway of activation involved. Future studies will examine how phosphorylation mediates its stimulatory effects.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: A.O. and L.F. conception and design of research; A.O. performed experiments; A.O. and L.F. analyzed data; A.O. and L.F. interpreted results of experiments; A.O. and L.F. prepared figures; A.O. and L.F. edited and revised manuscript; A.O. and L.F. approved final version of manuscript; L.F. drafted manuscript.

REFERENCES

1. **Baum M, Biemesderfer D, Gentry D, Aronson PS.** Ontogeny of rabbit renal cortical NHE3 and NHE1: effect of glucocorticoids. *Am J Physiol Renal Fluid Electrolyte Physiol* 268: F815–F820, 1995.
2. **Cardone RA, Casavola V, Reshkin SJ.** The role of disturbed pH dynamics and the Na^+/H^+ exchanger in metastasis. *Nat Rev* 5: 786–795, 2005.
3. **Carraro-Lacroix LR, Ramirez MA, Zorn TM, Reboucas NA, Malnic G.** Increased NHE1 expression is associated with serum deprivation-induced differentiation in immortalized rat proximal tubule cells. *Am J Physiol Renal Physiol* 291: F129–F139, 2006.
4. **Coccaro E, Karki P, Cojocaru C, Fliegel L.** Phenylephrine and sustained acidosis activate the neonatal rat cardiomyocyte Na^+/H^+ exchanger through phosphorylation of amino acids Ser^{770} and Ser^{771}. *Am J Physiol Heart Circ Physiol* 297: H846–H858, 2009.
5. **Coccaro E, Mraiche F, Malo M, Vandertol-Vanier H, Bullis B, Robertson M, Fliegel L.** Expression and characterization of the Na(+)/H (+) exchanger in the mammalian myocardium. *Mol Cell Biochem* 302: 145–155, 2007.
6. **Counillon L, Scholz W, Lang HL, Pouysségur J.** Pharmacological characterization of stably transfected Na^+/H^+ antiporter isoforms using amiloride analogs and a new inhibitor exhibiting anti-ischemic properties. *Mol Pharmacol* 44: 1041–1045, 1993.
7. **Ellis D.** The effects of external cations and ouabain on the intracellular sodium activity of sheep heart Purkinje fibres. *J Physiol* 273: 211–240, 1977.
8. **Fliegel L.** The Na^+/H^+ exchanger isoform 1. *Int J Biochem Cell Biol* 37: 33–37, 2005.
9. **Fliegel L.** Regulation of myocardial Na^+/H^+ exchanger activity. *Basic Res Cardiol* 96: 301–305, 2001.
10. **Gekle M, Freudinger R, Mildenberger S, Silbernagl S.** Aldosterone interaction with epidermal growth factor receptor signaling in MDCK cells. *Am J Physiol Renal Physiol* 282: F669–F679, 2002.
11. **Graham FL, Smiley J, Russell WC, Nairn R.** Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* 36: 59–74, 1977.
12. **Haworth RS, McCann C, Snabaitis AK, Roberts NA, Avkiran M.** Stimulation of the plasma membrane Na^+/H^+ exchanger NHE1 by sustained intracellular acidosis. Evidence for a novel mechanism mediated by the ERK pathway. *J Biol Chem* 278: 31676–31684, 2003.
13. **Karki P, Coccaro E, Fliegel L.** Sustained intracellular acidosis activates the myocardial Na(+)/H(+) exchanger independent of amino acid Ser(703) and p90(rsk). *Biochim Biophys Acta* 1798: 1565–1576, 2010.
14. **Krapf R, Pearce D, Lynch C, Xi XP, Reudelhuber TL, Pouyssegur J, Rector FCJ.** Expression of rat renal Na^+/H^+ antiporter mRNA levels in response to respiratory and metabolic acidosis. *J Clin Invest* 87: 747–751, 1991.
15. **Kuwahara M, Sasaki S, Uchida SC, Cragoe EJ Jr, Marumo F.** Different development of apical and basolateral Na/H exchanger in LLC-PK1 renal epithelial cells: characterization by inhibitors and antisense oligonucleotides. *Biochim Biophys Acta* 1220: 132–138, 1994.
16. **Lagana A, Vadnais J, Le PU, Nguyen TN, Laprade R, Nabi IR, Noel J.** Regulation of the formation of tumor cell pseudopodia by the Na(+)/H(+) exchanger NHE1. *J Cell Sci* 113: 3649–3662, 2000.
17. **Liu H, Stupak J, Zheng J, Keller BO, Brix BJ, Fliegel L, Li L.** Open tubular immobilized metal ion affinity chromatography combined with MALDI MS and MS/MS for identification of protein phosphorylation sites. *Anal Chem* 76: 4223–4232, 2004.
18. **Maekawa N, Abe J, Shishido T, Itoh S, Ding B, Sharma VK, Sheu SS, Blaxall BC, Berk BC.** Inhibiting p90 ribosomal S6 kinase prevents (Na+)-H+ exchanger-mediated cardiac ischemia-reperfusion injury. *Circulation* 113: 2516–2523, 2006.
19. **Malo ME, Li L, Fliegel L.** Mitogen-activated protein kinase-dependent activation of the Na^+/H^+ exchanger is mediated through phosphorylation of amino acids Ser^{770} and Ser^{771}. *J Biol Chem* 282: 6292–6299, 2007.
20. **Markos F, Healy V, Harvey BJ.** Aldosterone rapidly activates Na^+/H^+ exchange in M-1 cortical collecting duct cells via a PKC-MAPK pathway. *Nephron Physiol* 99: p1–9, 2005.
21. **Moe OW, Alpern RJ.** Na^+/H^+ exchange in mammalian kidney. In: *The Na^+/H^+ Exchanger*, edited by Fliegel L. Austin TX: R. G. Landes, 1996, p. 21–46.
22. **Moe OW, Miller RT, Horie S, Cano A, Preisig PA, Alpern RJ.** Differential regulation of Na/H antiporter by acid in renal epithelial cells and fibroblasts. *J Clin Invest* 88: 1703–1708, 1991.
23. **Moor A, Gan XT, Karmazyn M, Fliegel L.** Activation of Na^+/H^+ exchanger-directed protein kinases in the ischemic and ischemic-reperfused rat myocardium. *J Biol Chem* 27: 16113–16122, 2001.
24. **Moor AN, Fliegel L.** Protein kinase mediated regulation of the Na^+/H^+ exchanger in the rat myocardium by MAP-kinase-dependent pathways. *J Biol Chem* 274: 22985–22992, 1999.
25. **Murtazina R, Booth BJ, Bullis BL, Singh DN, Fliegel L.** Functional analysis of polar amino-acid residues in membrane associated regions of the NHE1 isoform of the mammalian Na^+/H^+ exchanger. *Eur J Biochem* 268: 4674–4685, 2001.
26. **Noel J, Roux D, Pouyssegur J.** Differential localization of Na^+/H^+ exchanger isoforms (NHE1 and NHE3) in polarized epithelial cell lines. *J Cell Sci* 109: 929–939, 1996.
27. **Paillard M.** Na^+/H^+ exchanger subtypes in the renal tubule: function and regulation in physiology and disease. *Exp Nephrol* 5: 277–284, 1997.
28. **Schwark JR, Jansen HW, Lang HJ, Krick W, Burckhardt G, Hropot M.** S3226, a novel inhibitor of Na^+/H^+ exchanger subtype 3 in various cell types. *Pflügers Arch* 436: 797–800, 1998.
29. **Sharrocks AD, Yang SH, Galanis A.** Docking domains and substrate-specificity determination for MAP kinases. *Trends Biochem Sci* 25: 448–453, 2000.
30. **Shaw G, Morse S, Ararat M, Graham FL.** Preferential transformation of human neuronal cells by human adenoviruses and the origin of HEK 293 cells. *FASEB J* 16: 869–871, 2002.
31. **Slepkov ER, Chow S, Lemieux MJ, Fliegel L.** Proline residues in transmembrane segment IV are critical for activity, expression and targeting of the Na^+/H^+ exchanger isoform 1. *Biochem J* 379: 31–38, 2004.
32. **Slepkov ER, Rainey JK, Li X, Liu Y, Cheng FJ, Lindhout DA, Sykes BD, Fliegel L.** Structural and functional characterization of transmembrane segment IV of the NHE1 isoform of the Na^+/H^+ exchanger. *J Biol Chem* 280: 17863–17872, 2005.
33. **Su HW, Wang SW, Ghishan FK, Kiela PR, Tang MJ.** Cell confluency-induced Stat3 activation regulates NHE3 expression by recruiting Sp1 and Sp3 to the proximal NHE3 promoter region during epithelial dome formation. *Am J Physiol Cell Physiol* 296: C13–C24, 2009.
34. **Su HW, Yeh HH, Wang SW, Shen MR, Chen TL, Kiela PR, Ghishan FK, Tang MJ.** Cell confluence-induced activation of signal transducer and activator of transcription-3 (Stat3) triggers epithelial dome formation

via augmentation of sodium hydrogen exchanger-3 (NHE3) expression. *J Biol Chem* 282: 9883–9894, 2007.
35. **Sun AM, Liu Y, Centracchio J, Dworkin LD.** Expression of Na^+/H^+ exchanger isoforms in inner segment of inner medullary collecting duct. *J Membr Biol* 164: 293–300, 1998.
36. **Wakabayashi S, Bertrand B, Shigekawa M, Fafournoux P, Pouyssegur J.** Growth factor activation and "H^+-sensing" of the Na^+/H^+ exchanger isoform 1 (NHE1). *J Biol Chem* 269: 5583–5588, 1994.
37. **Wallert MA, Fröhlich O.** Na^+-H^+ exchange in isolated myocytes from rat heart. *Am J Physiol Cell Physiol* 257: C207–C213, 1989.
38. **Wang H, Silva NLCL, Lucchesi PA, Haworth R, Wang K, Michalak M, Pelech S, Fliegel L.** Phosphorylation and regulation of the Na^+/H^+ exchanger through mitogen-activated protein kinase. *Biochemistry* 36: 9151–9158, 1997.
39. **Wang H, Singh D, Fliegel L.** The Na^+/H^+ antiporter potentiates growth and retinoic- acid induced differentiation of P19 embryonal carcinoma cells. *J Biol Chem* 272: 26545–26549, 1997.
40. **Willoughby D, Masada N, Crossthwaite AJ, Ciruela A, Cooper DM.** Localized Na^+/H^+ exchanger 1 expression protects Ca^{2+}-regulated adenylyl cyclases from changes in intracellular pH. *J Biol Chem* 280: 30864–30872, 2005.
41. **Wu ML, Vaughan-Jones RD.** Interaction between Na^+ and H^+ ions on Na-H exchange in sheep cardiac Purkinje fibers. *J Mol Cell Cardiol* 29: 1131–1140, 1997.