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Mitogen-activated Protein Kinase-dependent Activation of the Na^+/H^+ Exchanger Is Mediated through Phosphorylation of Amino Acids Ser^{770} and Ser^{771}*

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We investigated regulation of the type 1 isoform of the Na^+/H^+ exchanger by phosphorylation. Four specific groups of serine and threonine residues in the regulatory carboxyl-terminal tail were mutated to alanine residues: group 1, S693A; group 2, T718A and S723A/S726A/S729A; group 3, S766A/S770A/S771A; and group 4, T779A and S785A. The proteins were expressed in Na^+/H^+ exchanger-deficient cells, and the activity was characterized. All of the mutants had proper expression, localization, and normal basal activity relative to wild type NHE1. Sustained intracellular acidosis was used to activate NHE1 via an ERK-dependent pathway that could be blocked with the MEK inhibitor U0126. Immunoprecipitation of ^{32}P-labeled Na^+/H^+ exchanger from intact cells showed that sustained intracellular acidosis increased Na^+/H^+ exchanger phosphorylation *in vivo*. This was blocked by U0126. The Na^+/H^+ exchanger activity of mutants 1 and 2 was stimulated similar to wild type Na^+/H^+ exchanger. Mutant 4 showed a partially reduced level of activation. However, mutant 3 was not stimulated by sustained intracellular acidosis, and loss of stimulation of activity correlated to a loss of sustained acidosis-mediated phosphorylation *in vivo*. Mutation of the individual amino acids within mutant 3, Ser^{766}, Ser^{770}, and Ser^{771}, showed that Ser^{770} and Ser^{771} are responsible for mediating increases in NHE1 activity through sustained acidosis. Both intact Ser^{770} and Ser^{771} were required for sustained acidosis-mediated activation of NHE1. Our results suggest that amino acids Ser^{770} and Ser^{771} mediate ERK-dependent activation of the Na^+/H^+ exchanger *in vivo*.

The Na^+/H^+ exchanger isoform 1 (NHE1)[3] is a ubiquitously expressed plasma membrane glycoprotein that extrudes a single intracellular proton in exchange for one extracellular sodium. It thereby functions to protect cells from intracellular acidification while facilitating extracellular Na^+ entry into the cytosol (1). Nine isoforms of Na^+/H^+ exchanger have been discovered and are designated NHE1–NHE9. NHE1 was the first isoform discovered (2). Other isoforms have more restricted tissue distributions, and some have predominantly intracellular localization. In mammals, NHE1 plays a key role in regulation of cell pH, cell volume, and cell proliferation and in the metastasis of some types of tumor cells (1, 3). In the myocardium the Na^+/H^+ exchanger plays a critical role in mediating the damage that occurs with ischemia/reperfusion of the heart (4–6) and also is an important mediator of myocardial hypertrophy (7). Clinical trials are attempting to develop NHE1 inhibitors for treatment of various forms of heart disease (8).

The Na^+/H^+ exchanger consists of two domains: a membrane domain of ~500 amino acids and a 315-amino acid hydrophilic, carboxyl-terminal cytosolic domain. The cytosolic domain regulates the membrane domain with phosphorylation having been shown to occur in the distal region of the cytosolic domain, within the last 178 amino acids (9, 10). We have demonstrated that the MAP kinases extracellular signal-regulated kinases 1 and 2 (ERK1/2) are implicated in growth factor activation of NHE1. This was shown in skeletal muscle tissues (11), in smooth muscle (11), in the intact myocardium (10), in isolated cardiomyocytes (10), and in Chinese hamster ovary cells (11). The ERK-dependent pathway was activated in several models of ischemic heart disease, and this resulted in further activation of the Na^+/H^+ exchanger in the myocardium (12). We recently demonstrated that ERK phosphorylates the NHE1 cytosolic domain at one or more of the following amino acids, Ser^{693}, Ser^{766}, Ser^{770}, Thr^{779}, and Ser^{785} (13) *in vitro*. In another report we also showed that amino acids Thr^{717}, Ser^{722}, Ser^{725}, and Ser^{728} are phosphorylated *in vitro* by a p38 MAP kinase-dependent pathway (14). In this report, we characterized the role of these amino acids in the ERK-dependent pathway of phosphorylation and regulation of the Na^+/H^+ exchanger. Our results are the first to define and elucidate this mechanism of regulation of the Na^+/H^+ exchanger *in vivo*.

EXPERIMENTAL PROCEDURES

Materials—The MEK inhibitor U0126 was from Sigma. Anti-HA antibody (Y-11), anti-MAP kinase ERK1, ERK2, anti-phospho-ERK1/2, and anti-$p90^{RSK}$ were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-$p90^{RSK}$ was from Cell Signaling Technology. Protein A-Sepharose beads were from Pierce. [^{32}P]Orthophosphate was from PerkinElmer Life Sciences. 2′,7-bis (2-carboxyethyl)-5(6) carboxyfluorescein-acetoxymethyl ester was from Molecular Probes, Inc. (Eugene, OR).

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[3] The abbreviations used are: NHE1, Na^+/H^+ exchanger isoform 1; HA, hemagglutinin; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; MEK, MAP kinase/ERK kinase.

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All of the other chemicals were of analytical grade and were purchased from Fisher, Sigma, or BDH (Toronto, Canada).

Site-directed Mutagenesis—Mutations in the cytosolic tail of the NHE1 isoform of the Na^+/H^+ exchanger were made to an expression plasmid containing a HA-tagged human NHE1. The plasmid pYN4+ contains the cDNA of the entire coding region of NHE1 (15). Site-directed mutagenesis was performed using amplification with PWO DNA polymerase followed by use of the Stratagene (La Jolla, CA) QuikChange™ site-directed mutagenesis kit as recommended by the manufacturer. Mutations were designed to create a new restriction enzyme site for use in screening transformants. Mutations were made to four regions of the cytosolic regulatory domain of the Na^+/H^+ exchanger (see Fig. 1). The mutants were as follows (only one of each primer pair is shown): Mutant 1, S693A, 5′-GCCCACAAGCTTGACGCACCCACCATG-3′; Mutant 2, T718A/S723/726/729A, 5′-ATCGACCCGGCTGCCCCGCAGGCACCCGAGGCTGTAGATCTGGTGAAT-3′ and 5′-AAGGAGGACCTACCGGTCATCGCCATCGACCCGGCT-3′; Mutant 3, S766/770/771A, 5′-GCATCATGATGCGGGCCAAGGAGACTGCAGCCCCAGGAACCGAC-3′; Mutant 4, T779A/S785A, 5′-CCGACGATGTCTTCGCCCCCGCCCCGAGTGACGCCCCCAGCTCCCAG-3′, (underlined residues indicate newly introduced restriction sites). Site-specific mutagenesis was also done on individual amino acids Ser^{766}, Ser^{770}, and Ser^{771} using the following primers: CATCATGATGCGGGCCAAGGAGACTAGTTCCCCAGGAACCG, GGAGCAAGGAGACTGCTAGCCCAGGAACCGACG, and GCAAGGAGACTTCGGCCCCGGGAACCGACGATG, respectively. DNA sequencing was by the DNA core services laboratory of the Department of Biochemistry to confirm the accuracy of the mutations and the fidelity of the product.

Cell Culture and Stable Transfection—AP-1 cells that lack an endogenous Na^+/H^+ exchanger were used to examine NHE1 activity. Stable cell lines were made of all mutants by transfection with Lipofectamine™ 2000 reagent as described earlier (15). The cells were selected using 800 μg/ml geneticin (G418), and stable cell lines were regularly re-established from frozen stocks at passage numbers between 5 and 15 whenever necessary. For some experiments the MEK inhibitor U0126 was included (10 μM in dimethyl sulfoxide). The inhibitor was included in normal Na^+-containing medium for 10 min prior to NHE1 assay and in all subsequent steps.

To examine *in vivo* phosphorylation of NHE1 proteins AP-1 cells were plated on 100-mm dishes. One day prior to experimentation, the cells were incubated in complete α-minimum essential medium supplemented with 0.5% bovine growth serum overnight at 37 °C in 5% CO_2. The day of experimentation the media was replaced with phosphate-free Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 0.5% bovine growth serum and incubated at 37 °C in 5% CO_2 for 30 min. The medium was removed and replaced with 2 ml of the same medium, to which $H_3{}^{32}PO_4$ was added to a final concentration of 100 μCi/ml medium. The cells were then incubated at 37 °C in 5% CO_2 for 3 h.

SDS-PAGE and Immunoblotting—Immunoblot analysis using anti-HA antibody was used to confirm NHE1 expression in samples from total cell lysates of AP-1 cells. The cell lysates were made as described earlier (12, 16). For Western blot analysis equal amounts of each sample (50 μg of total protein) were resolved on 10% SDS-PAGE. The gel was transferred onto a nitrocellulose membrane and immunostained with peroxidase-conjugated goat anti-mouse antibody (Bio/Can, Mississauga, Canada). The Amersham Biosciences enhanced chemiluminescence Western blotting and detection system was used to detect immunoreactive proteins on x-ray film. Densitometric analysis of x-ray films was carried out using ImageJ 1.35s software (National Institutes of Health, Bethesda, MD).

Cell Surface Expression—Cell surface expression was measured as described earlier (15). Briefly described, the cells were labeled with Sulfo-NHS-SS-Biotin (Pierce), and immobilized streptavidin resin was used to remove surface-labeled Na^+/H^+ exchanger. Equal amounts of the total and unbound proteins were analyzed by SDS-PAGE and Western blotting against the HA tag. Relative amounts of NHE1 on the cell surface were calculated by comparing both the 110- and 95-kDa forms of NHE1.

Na^+/H^+ Exchange Activity—Na^+/H^+ exchange activity was measured using a PTI Deltascan spectrofluorometer. The initial rate of Na^+-induced recovery of cytosolic pH (pH_i) was measured after acute acid load using 2′,7-bis (2-carboxyethyl)-5(6) carboxyfluorescein acetoxymethyl ester (Molecular Probes Inc.) as described earlier (15). Ammonium chloride (50 mM × 3 min.) was used to transiently induce an acid load. The coverslip was then removed from the NH_4Cl containing buffer and placed in a Na^+-free buffer (135 mM *N*-methyl glucamine, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 5.5 mM glucose, 10 mM HEPES, pH 7.3) for ~30 s or until the pH_i had reached a minimum, at which point the coverslip was removed and placed in a normal Na^+ buffer (135 mM NaCl) and allowed to recover for 3 min. There were no differences in buffering capacities of stable cell lines as indicated by the degree of acidification induced by ammonium chloride applications (not shown). Following the pH_i recovery, a three-point pH calibration using Na^+-free calibration buffers (135 mM *N*-methyl glucamine, 135 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 5.5 mM glucose, 10 mM HEPES, at pH 6, 7, and 8) was completed. For some experiments we used a two-pulse assay to induce prolonged intracellular acidosis (17) and to stimulate ERK-dependent pathways and the activity of NHE1. For these experiments the cells were initially acidified with ammonium chloride as described above and then immediately allowed to recover in normal Na^+-containing medium. The cells were then acidified with a second ammonium chloride pulse either with or without a 3-min period in Na^+-free medium. Following pH_i recovery in Na^+ normal buffer, a three-point calibration was completed.

The NHE1 protein activity was determined by measuring the slope of the first linear 20 s of the recovery period and was expressed as ΔpH/s. To calculate the effect of the stimulatory treatment, the rate of recovery for the first pulse was set at 100%, and the rate of recovery of the second pulse was expressed as a percentage relative to the first pulse. Using the percentage values of the second pulse, we compared the effect of sustained acidosis-treated cells to control treated cells (cells that had no sustained acidosis and immediate recovery after the second ammonium chloride induced acidification). The results

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are shown as the means ± S.E., and statistical significance was determined using a Mann-Whitney *U* test.

Immunoprecipitation of NHE1 Protein—Cell lysates were used for the immunoprecipitation of exogenously expressed NHE1 from AP-1 cells. The lysates were precleared by incubation with 20 μl of 100 mg/ml protein A-Sepharose beads at 4 °C for 30 min in detergent-containing radioimmune precipitation assay buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% SDS, 0.1% Triton X-100, 1 mM EGTA, and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, and 0.1% (v/v) protease inhibitor mixture (12)) and 1% (w/v) bovine serum albumin. After removal of beads by centrifugation, the supernatant was incubated with 1.4 μg/ml of an anti-HA antibody for 2 h at 4 °C. Protein A-Sepharose beads were blocked in detergent-containing radioimmune precipitation assay buffer with 1% (w/v) bovine serum albumin for 30 min at 4 °C. The blocking solution was removed by centrifugation, and a sample with HA antibody was added and rotated end-over-end overnight at 4 °C. The samples were centrifuged to remove the supernatant, and after washing the bound protein was eluted from the washed beads by incubating with 45 μl of 1× SDS-PAGE sample loading buffer. After SDS-PAGE, samples were transferred to nitrocellulose membranes and radioactivity was detected by exposure of x-ray film. Immunoblotting with anti-HA antibody was used to check the level of NHE1 in the samples and correct for any variation in immunoprecipitation efficiency. The results are the means ± S.E. of at least three experiments, and statistical significance was determined using a Mann-Whitney *U* test.

RESULTS

We initially produced a number of mutations to the NHE1 cytosolic regulatory domain that could be used to provide *in vivo* analysis of effects on the regulation and activity of the Na^+/H^+ exchanger. Our mutants were in four locations in the cytosolic domain (Fig. 1). Mutants 1, 3, and 4 were chosen because our recent results have shown that ERK can phosphorylate one or more of the specific amino acids chosen in these regions *in vitro* (13). Mutant 2 was made because of our recent results showing that phosphorylation within these amino acids is involved in p38 mitogen-activated phosphorylation of NHE1 (14).

To study the effect of these mutations, we made stable cell lines of control and mutant NHE1 proteins in AP-1 cells that lack their own endogenous NHE1 protein. At least two stable cell lines were made of each protein type. Fig. 2 illustrates some of the characteristics of each type of protein. The mutants displayed varying levels of expression and cell surface targeting, but all were functionally active at levels that were similar to that of controls (Fig. 2*C*). The expression levels varied from 40 to 118% of that of the wild type Na^+/H^+ exchanger (Fig. 2*A*). AP-1 cells that were either mock or untransfected showed no HA tag immunoreactivity and no NHE1 activity. Our laboratory has earlier observed that with protein mistargeting, the NHE1 protein may be found predominantly as an 85-kDa protein that is bereft of glycosylation (15). This was not the case with these mutant proteins because the majority of the immunoreactive species were present as the larger size of about 105 kDa. We also

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634  MILRNNLQKT  RQRLRSYNRH  TLVADPYEEA  WNQMLLRRQK  672

673  ARQLEQKINN  YLTYPAHLKD  SPTMSRARIG  SDPLAYEPKE  712
                             #1
713  DPPVITIDPA  SPOSPESVDL  VNEELKGKVL  GLSRDPAKVA  752
              #2
753  EEDEDDDGGI  MMRSKETSSP  GTDDVFTPAP  SDSPSSQRIQ  792
                       #3                  #4
796  RCLSDPGPHP  EPGEGEPFFP  KGQ  815
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FIGURE 1. **Carboxyl-terminal 181 amino acids of the NHE1 isoform of the Na^+/H^+ exchanger.** Bold residues indicate Ser/Thr residues that were mutated to alanine. The groups of residues that were mutated are *underlined* and *numbered*.

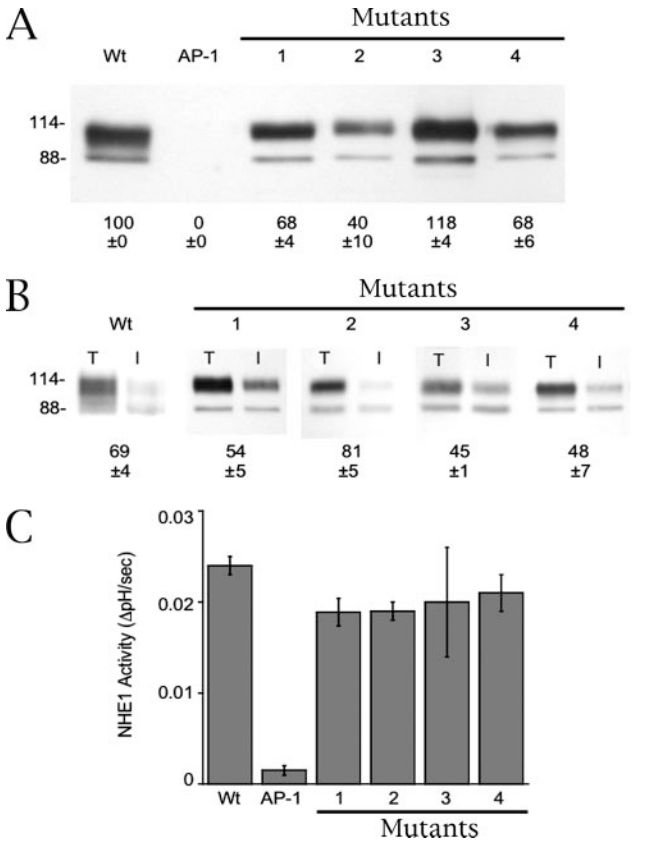

FIGURE 2. **Characterization of wild type and mutant NHE1 proteins in AP-1 cells.** *A*, Western blot analysis of cell extracts from control and stably transfected AP-1 cells. Cell extracts were prepared from control (AP-1) cells and from cells stably transfected with cDNA coding for HA-tagged cells: wild type NHE1 (*Wt*), mutants 1–4 contained mutations as described for Fig. 1. The *numbers* indicate values relative to the wild type and are the means ± S.E. of at least three determinations. *B*, subcellular trafficking of NHE1 proteins. The cells were treated with Sulfo-NHS-SS-Biotin, solubilized, and biotin-labeled proteins were bound to streptavidin-agarose beads as described under "Experimental Procedures." A sample of the total cell lysate (*T*) and an equivalent amount of unbound lysate (*I*, intracellular) were run on SDS-PAGE. Western blotting was with an anti-HA antibody to identify NHE1 protein. The *numbers* indicate the percentage of surface protein and are the means ± S.E. of at least three determinations. *C*, NHE activity was measured after transient induction of acute acid load as described under "Experimental Procedures." The results are the means ± S.E. of at least four determinations.

examined the surface targeting of the various mutants. We found (Fig. 2*B*) that all targeted to the plasma membrane in amounts similar to that of the wild type. 69% of the wild type NHE1 protein was at the cell surface, whereas 45–81% of the expressed mutant protein targeted to the plasma membrane.

We initially characterized the effect of sustained acidosis on NHE1 activity and on ERK1/2 and $p90^{RSK}$ (Fig. 3). AP-1 cells

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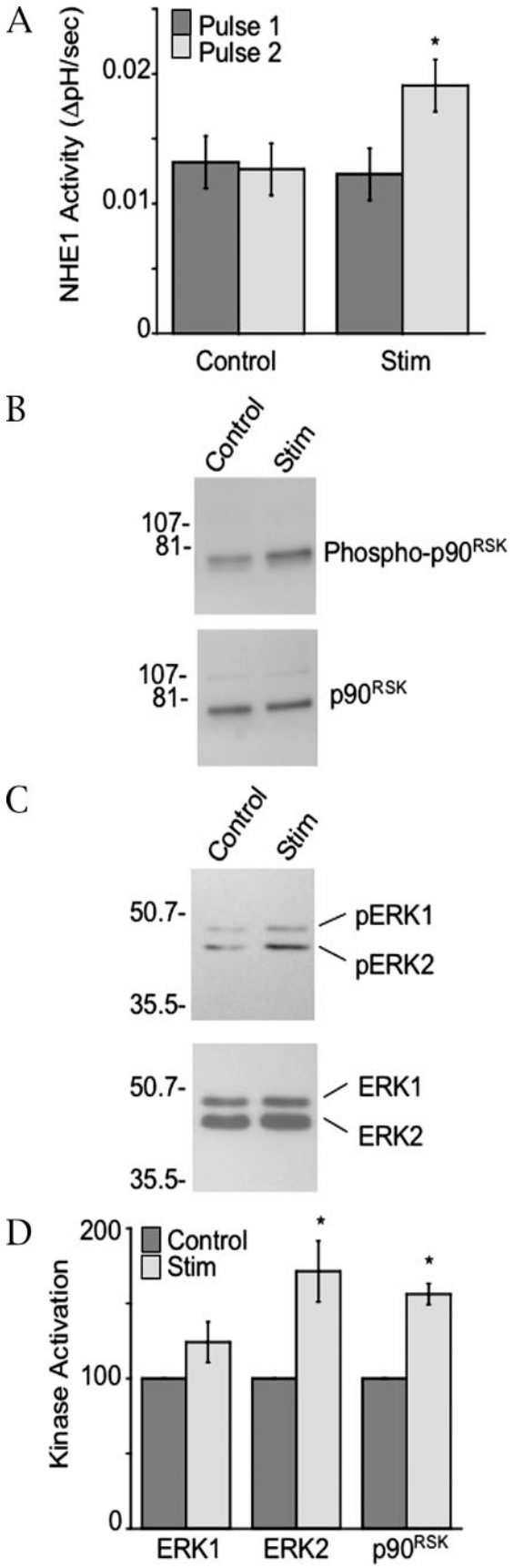

FIGURE 3. **Characterization of sustained acidosis activation of NHE1 activity and ERK-dependent pathways.** *A*, AP-1 cells expressing wild type NHE1 were subjected to dual ammonium chloride prepulse with or without sustained intracellular acidosis as described under "Experimental Procedures." The rate of recovery for each acid pulse was determined, and the values of the second pulse were compared with those of the first. *Control* indicates the second pulse was in the absence of sustained intracellular acidosis. *Stim* indicates the second pulse administered sustained acidosis. The results are the means ± S.E. of at least 10 separate assays. * indicates that the value of the second pulse is significantly higher than that of the control at $p > 0.05$. *B*, representative Western blots of p90RSK from control or sustained acidosis-treated cells. *Upper panel*, cells blotted with anti-phospho-P90RSK antibody. *Lower panel*, corresponding Western blot with anti-p90RSK antibody. *C*, representative Western blots of ERK1/2 from control or sustained acidosis-treated cells. *Upper panel*, cells blotted with anti-phospho-ERK antibody. *Lower panel*, corresponding Western blot with anti-p90RSK antibody. *D*, summary of kinase activation by sustained intracellular acidosis. The levels of activated kinase are expressed relative to the levels of controls. The results are the means ± S.E. of at least three experiments. * indicates significantly higher than control at $p > 0.05$.

expressing wild type NHE1 were subjected to dual ammonium chloride prepulse, with or without sustained intracellular acidosis. The rate of recovery of the two pulses is indicated. In cells that were not subjected to sustained intracellular acidosis, the second recovery was equivalent to the first. In cells that were subjected to sustained acidosis, the second rate of recovery was significantly elevated relative to the first (Fig. 3*A*). Because ERK-dependent pathways were reported to be activated by sustained intracellular acidosis (17), we confirmed that this pathway was activated (Fig. 3, *B–D*). Sustained acidosis resulted in significant activation of both ERK2 and $p90^{RSK}$. ERK1 was not significantly activated. The reason for the lack of activation was not clear but could be due to difficulty in quantification of the smaller amount of immunoreactive protein of this type.

We examined the effects on NHE1 activity and phosphorylation level following an acute acid load in the presence of U0126 (Fig. 4). To assess the effect on NHE1 activity, four two-pulse activity assay treatments were completed: a control two-pulse assay plus Me_2SO, a stimulatory two-pulse assay plus Me_2SO, a control two-pulse assay plus 10 μM U0126 in Me_2SO, and a stimulatory two-pulse assay plus 10 μM U0126 in Me_2SO. The rate of pH_i recovery for the first and second pulse of each set of treatments was compared, with the first pulse of each set of treatments set at 100% and the second pulse rate of pH_i recovery expressed as a percentage relative to it. The results are shown in Fig. 4*A*, which illustrates the NHE1 activity of the second pulse relative to the first. In the absence of U0126 there is a statistically significant increase of 67% in NHE1 activity following sustained intracellular acidosis. Control cells did not show a difference in NHE1 activity between the first and second pulses in the presence of Me_2SO. In contrast, in the presence of 10 μM U0126, there was no longer an increase in exchanger activity following sustained intracellular acidosis. Both the control and stimulatory treatment in the presence of U0126 resulted in no significant increase in NHE1 activity during the second recovery from acidosis.

To assess the effect of MEK inhibition on NHE1 phosphorylation, four sets of experiments were completed: a control treatment, a stimulatory treatment, a control treatment plus 10 μM U0126 in Me_2SO, a stimulatory treatment plus 10 μM U0126 in Me_2SO. The phosphorylation levels of each treatment were corrected for loading, and the level of phospho-NHE1 after positive stimulation was plotted (Fig. 4, *b* and *c*). In the absence of the MEK inhibitor, U0126, the stimulated/control ratio of

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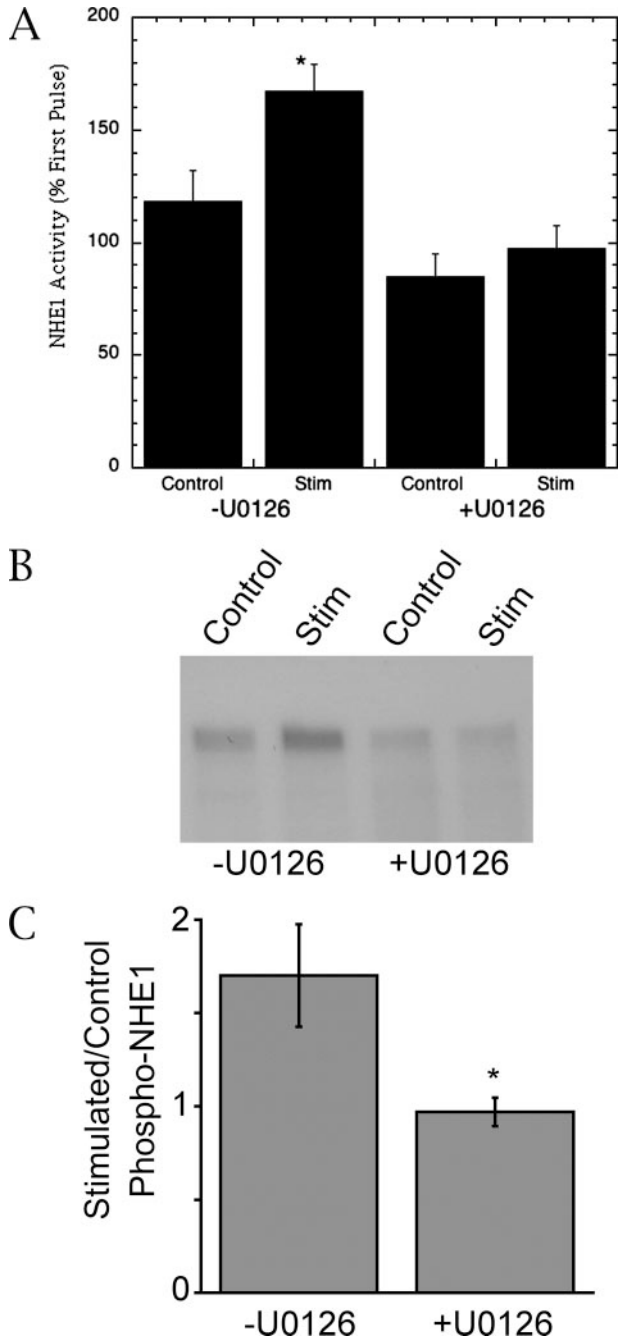

FIGURE 4. **Effect of U0126 on sustained intracellular acidosis activation of the Na^+/H^+ exchanger.** *A*, AP-1 cells expressing wild type NHE1 were subjected to dual ammonium chloride prepulse with or without sustained intracellular acidosis as described under "Experimental Procedures." The rate of recovery for each acid pulse was determined, and the values of the second pulse are shown relative to those of the first. *Control* indicates that the second pulse was in the absence of sustained intracellular acidosis. *Stim* indicates the second pulse administered sustained acidosis. − or + indicates the presence or absence of 10 μM U0126. The results are the means ± S.E. of at least six separate assays. * indicates the value of the second pulse is significantly higher than that of the control at $p > 0.01$. *B* and *C*, effect of sustained acidosis on NHE1 phosphorylation. Cells containing HA-tagged NHE1 were subjected to either control or sustained acidosis (*Stim*) in the presence or absence of U0126 after incubation in [^{32}P]orthophosphate-containing medium. NHE1 was immunoprecipitated and samples were examined by autoradiography. *B*, example of autoradiogram of 110-kDa NHE1 protein. *C*, analysis of stimulated/control ratio of Phospho-NHE1 levels in immunoprecipitates. The samples were corrected for immunoprecipitate protein levels as described under "Experimental Procedures." The results are the means of at least five independent experiments. * indicates significantly different at $p > 0.05$, Wilcoxon Mann-Whitney rank sum test or Mann-Whitney *U* test.

phospho-NHE1 was 1.7 ± 0.3, whereas in the presence of 10 μM U0126, the ratio was 0.97 ± 0.08. The phospho-NHE1 ratio in the presence of the inhibitor was significantly lower than that observed for phospho-NHE1 in the absence of the inhibitor.

To assess the effect of mutations of phosphorylation sites of the NHE1 cytoplasmic domain, we examined NHE1 stimulatory activity and NHE1 phosphorylation levels following sustained intracellular acidosis. Four sets of mutant NHE1 proteins were examined with mutations at phosphorylation sites in the cytosolic domain of the NHE1 protein (Fig. 1). Each mutant was subjected to either control or sustained acidosis treatment, and the rate of recovery following an acid load was compared. The results (Fig. 5*A*) demonstrate that mutants 1 and 2 have a level of stimulation that is the same as the wild type NHE1 protein. Mutant 4 has lower level of stimulation; however, it still is not significantly different from wild type. Mutant 3 does not have an increased level of activity, and this is significantly different from that observed for the wild type protein.

We then compared the phosphorylation levels of the wild type NHE1 to the mutants. Preliminary experiments demonstrated that mutant 1 and 2 were comparable with wild type in their phosphorylation levels and did not differ in response to sustained intracellular acidosis (not shown). We examined the phospho-NHE1 levels of the wild type and mutants 3 and 4 in more detail, and they were 1.74 ± 0.32, 0.75 ± 0.07, and 1.28 ± 0.29, respectively. Fig. 5*B* illustrates a representative autoradiograph of the results, whereas Fig. 5*C* summarizes the results of at least six independent experiments. With mutation 3, the stimulated/control phospho-NHE1 ratio is significantly lower than that that observed for wild type NHE1. Mutation 4 did not significantly reduce the level of phospho-NHE1 after sustained acidosis treatment.

To determine which amino acids of mutant 3 were responsible for mediating the effects of sustained intracellular acidosis, individual mutations to alanine were made to amino acids Ser^{766}, Ser^{770}, and Ser^{771}. Stable cell lines expressing these mutant proteins were then established. Fig. 6*A* is a Western blot demonstrating the expression of the three mutant cell lines. Expression levels were similar to that of the wild type NHE1. We also determined that surface targeting of the NHE1 mutants was not changed in comparison with the wild type protein (not shown). The three mutant cells lines and the mutant NHE1 protein with all three amino acids mutated to alanine were examined in the two-pulse assay. We determined the ability of sustained intracellular acidosis to stimulate the NHE1 protein during the second recovery from acidosis. The results are shown in Fig. 6*B*. The triple mutant, with all three amino acids mutated to alanine, was not activated by sustained intracellular acidosis. In addition, mutation of either Ser^{770} or Ser^{771} eliminated activation of these mutants by sustained acidosis. In contrast, mutation of Ser^{766} to alanine did not eliminate activation of the protein by prolonged acidosis.

DISCUSSION

Phosphorylation of the Na^+/H^+ exchanger isoform I mediates ~50% of the stimulatory effect of growth factors (9). Although kinase consensus and phosphorylation sites have been identified in the cytoplasmic domain *in vitro* (10, 18, 19),

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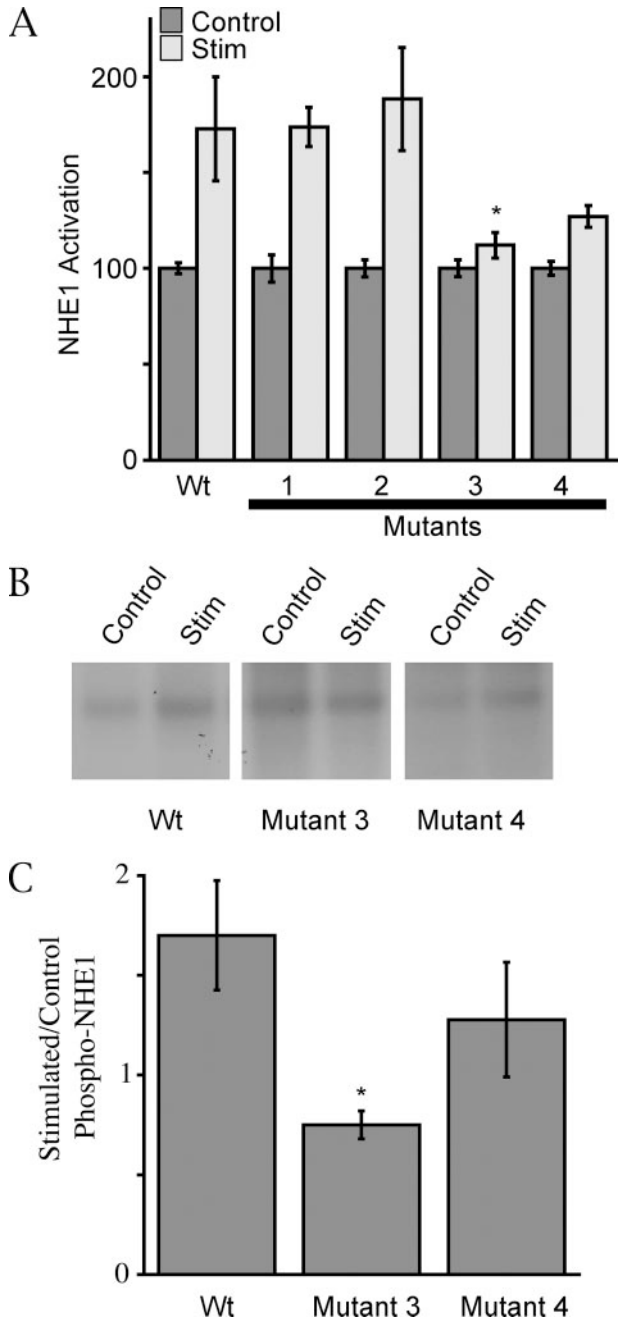

FIGURE 5. **Effect of mutations on wild type and mutant NHE1 activity and phosphorylation levels.** *A*, NHE1 activity was measured using dual ammonium chloride prepulse with or without sustained intracellular acidosis. The rate of recovery for each acid pulse was determined, and the values of the second pulse were compared with those of the first. *Control* indicates that the second pulse was in the absence of sustained intracellular acidosis. *Stim* indicates the second pulse administered sustained acidosis. The results are the means ± S.E. of at least 10 independent assays. * indicates that the mutant NHE1 activity after acidosis treatment is significantly different from that observed in the wild type NHE1. *B* and *C*, effect of sustained acidosis on wild type and mutant NHE1 phosphorylation. Cells containing HA-tagged NHE1 were subjected to either control of sustained acidosis (*Stim*). NHE1 was immunoprecipitated, and the samples were examined by autoradiography. *B*, example of autoradiogram of 110-kDa NHE1 protein. *C*, analysis of stimulated/control ratio of phospho-NHE1 levels in NHE1 wild type and mutant immunoprecipitates. The samples were corrected for immunoprecipitated protein levels as described under "Experimental Procedures." The results are the means of at least six independent experiments. * indicates significantly different at $p > 0.05$, Wilcoxon Mann-Whitney rank sum test or Mann-Whitney *U* test.

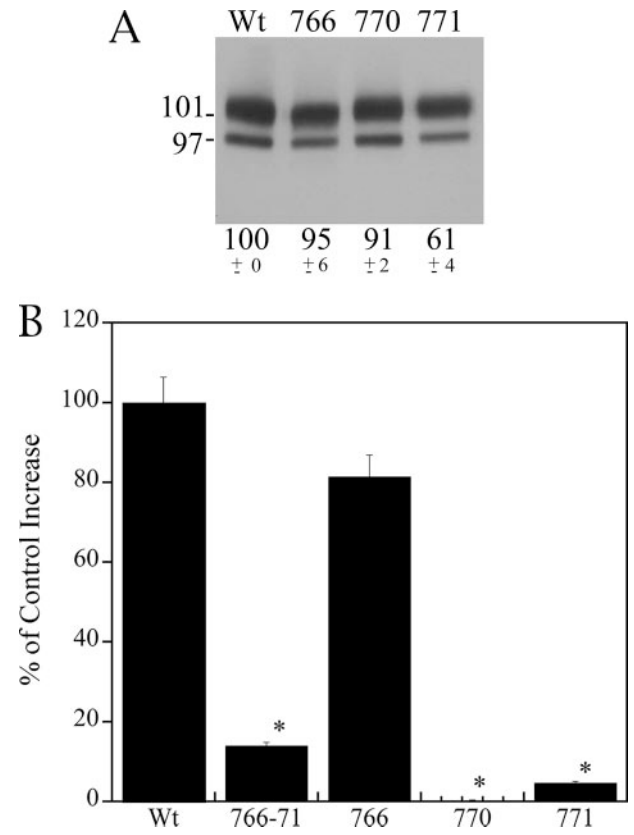

FIGURE 6. **Characterization of wild type and NHE1 proteins with mutations in amino acids Ser^{766}, Ser^{770}, and Ser^{771}.** *A*, Western blot analysis of cell extracts from control and stably transfected AP-1 cells. Cell extracts were prepared from control (AP-1) cells and from cells stably transfected with cDNA coding for HA-tagged Na^+/H^+ exchanger proteins: wild type NHE1 (*Wt*), mutants S766A, S770A, and S771A contained mutations as described under "Experimental Procedures." The *numbers* indicate values relative to the wild type and are the means ± S.E. of at least three determinations. *B*, NHE activity was measured after dual transient induction of acute acid load as described under "Experimental Procedures." The second pulse was after a 3-min sustained acidosis. The results are expressed relative to the control (wild type) and are the percentages of the increased rate of recovery of the second acid pulse, in comparison with the initial pulse. The values are the means ± S.E. of at least six determinations. *Wt* refers to the wild type NHE1 protein, and *766*, *770*, and *771* refer to the NHE1 proteins with the S766A, S770A, and S771A mutations, respectively. *766-71* refers to the NHE1 mutant protein with all three mutations, S766A, S770A, and S771A.

only Ser^{703} has been identified as a residue phosphorylated *in vivo* (20). The present study identifies a novel set of residues that play a critical role *in vivo* in NHE1 stimulation via sustained intracellular acidosis. We examined ERK-dependent phosphorylation because in several earlier studies we have shown both ERK-dependent phosphorylation of the NHE1 protein and ERK-dependent stimulation of activity (10, 11). Sustained intracellular acidosis was earlier shown to be a reliable method of stimulation of Na^+/H^+ exchanger activity by ERK-dependent pathways (17). In preliminary experiments we found that it was a more reliable method of activation of NHE1 in this cell type in comparison with receptor-mediated activation.

We chose four sets of mutations to examine regulation of NHE1 phosphorylation. Three of these were identified earlier in *in vitro* phosphorylation experiments with ERK (13), and one region was previously identified as phosphorylated by p38 (14). Three of the sites of mutation contained more than one potential phosphorylation site. The residues selected for mutation

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were chosen as groups rather than single residue mutations, with the exception of Ser^{693}. First, this approach allowed us to examine a manageable amount of mutant proteins. Second, it has been suggested that groups of phosphorylatable residues sometimes act together in concerted manner, and mutation of single residues can sometimes not be sufficient to alter physiological events. For example, in the cystic fibrosis transmembrane regulator protein, it has been demonstrated that some phosphorylatable residues are dependent on the simultaneous phosphorylation of other residues as groups to exert their physiological effect (21).

Mutation of the selected residues did not have large effects on the normal functioning of the NHE1 protein. Although the expression and targeting levels varied somewhat, in all cases the mutant protein was expressed and properly targeted. Evaluation of the functional state of the mutated proteins proved that the mutations did not abolish basal Na^+/H^+ exchanger ability. We concluded that the mutations do not cause severe detrimental effects to the functional state of the protein and that the mutant proteins can be used to further study the role of specific residues in NHE1 regulation.

We have shown earlier that stimulation of NHE1 activity results in phosphorylation of the protein and that MEK inhibition results in decreased phosphorylation of the protein *in vivo* (10). Previously, we used hormonal stimulation of phosphorylation in isolated cardiomyocytes, whereas in this study, we used sustained acidosis to stimulate NHE1 activity in this cell type. We found that this method of stimulation of these cells resulted in increased activity and phosphorylation of the protein that could be blocked by MEK inhibition. Our results on stimulation of activity of NHE1 were similar to those of Haworth *et al.* (17). It should be noted that our results differ from those Hayashi *et al.* (22), who did not find that sustained intracellular acidosis stimulates NHE1 activity. However, in their study they did not serum-deprive NHE1 prior to treatment. We found that this is necessary for the effects of sustained intracellular acidosis. The cells that were not serum-starved were not stimulated by sustained intracellular acidosis (not shown). This suggests that NHE1 needs to be in a resting or basal state for acidosis-mediated stimulation to be effective.

When we stimulated the ERK pathway by sustained intracellular acidosis, we found that of the four sets of amino acids that we mutated, only mutant 3 (S766A/S70A/S71A) was significantly affected in its stimulation of NHE1 activity. In addition, with mutation of these residues, NHE1 is not phosphorylated in response to sustained intracellular acidosis treatment. This implicated one or all of these residues as being specifically phosphorylated by the ERK-dependent pathway, resulting in the activation of NHE1. Mutant 3 contained three phosphorylatable amino acids, Ser^{766}, Ser^{770}, and Ser^{771}. Individual mutations to alanine of each of these amino acids demonstrated that either amino acid Ser^{770} or Ser^{771} is required for sustained acidosis to activate the NHE1 protein. Mutation of Ser^{766} had little to no effect on this activation of the NHE1 protein. These results suggest that these two amino acids are necessary to mediate phosphorylation-dependent activation of NHE1 via the ERK2/p90RSK pathway.

Our results differ from that of a previous report, where Ser^{703} (20) was found to be important in NHE1 regulation. However, in that study a different cell type was used, and cells were stimulated with serum, which could act to enhance NHE1 activity through a large number of pathways. In our hands, we have found that the level of *in vitro* phosphorylation by ERK or $p90^{rsk}$ of a fusion protein of the NHE1 cytosolic domain did not vary with a S703A mutation (unpublished observations). Similarly, we demonstrated that p38-dependent phosphorylation does not vary with the S703A mutation (14). These results suggest that Ser^{703} is not critical, at least for MAP kinase-dependent pathways in these cell types. We have also earlier demonstrated that amino acids Thr^{717} and $Ser^{722/725/728}$ of rabbit NHE1 are involved in p38-mediated activation of NHE1 in some cell types. We therefore examined the effects of mutation of these amino acids in the present study. However, mutation of these amino acids did not affect activation of NHE1 or phosphorylation levels. We have previously examined the effects of p38 inhibition on NHE1 activity and phosphorylation in the myocardium and were unable to demonstrate any significant effects of p38 inhibition in the myocardium (12). The present results are in keeping with the idea that the Thr^{717} and $Ser^{722/725/728}$ region is not significant in ERK-mediated activation of NHE1 but is involved in p38 activation of NHE1 in some specific cell types (12, 14).

To draw a connection between the activation of ERK and $p90^{RSK}$ kinases and the stimulation of NHE1 activity, the MEK1 specific inhibitor U0126 was used to block the activation of ERK1/2 and subsequently $p90^{RSK}$ activation. U0126 is a selective inhibitor of MEK1 activation by the Raf protein kinase (23). It inhibited sustained acidosis enhancement of the activity of the Na^+/H^+ exchanger plus its phosphorylation. Based on these results we suggest a pathway whereby sustained acidosis activates ERK2 and subsequently $p90^{RSK}$ via the Raf→MEK1 cascade, leading to NHE1 phosphorylation at residues Ser^{770} and Ser^{771} (Fig. 7). It seems likely also that hormonal activation of the ERK-dependent pathway, which leads to activation of NHE1, likely proceeds through this same pathway. Further experiments are necessary to demonstrate this. We have earlier shown that serum activation of isolated cardiomyocytes leads to increased phosphorylation of NHE1 *in vivo*. In addition, MEK inhibition blocked this activation (10).

The canonical consensus sequence of ERK is generalized as Pro-Xaa-Ser/Thr-Pro where Xaa is a neutral or basic amino acid and $n = 1$ or 2 (24). Nevertheless, ERK-dependent phosphorylation and regulation of the other membrane proteins such as the tumor necrosis factor-α receptor has been reported at nonconsensus ERK phosphorylation sites such as a minimal S/TP. This was demonstrated by others both *in vivo* and *in vitro* with purified ERK2 (25). Within the mutant 3 region (Fig. 1), Ser^{771} is followed by a proline residue, which makes it most likely that this is the residue that is directly recognized by ERK2. ERK-dependent phosphorylation can precede and be a prerequisite for phosphorylation of other nearby residues by other protein kinases such as glycogen-synthase kinase 3 (26). The glycogen-synthase kinase consensus sequence ((S/T)*XXX*(p(S)/p(T))) would be optimal for amino acid Ser^{766} being phosphorylated if Ser^{770} was phosphorylated (27). However, because we

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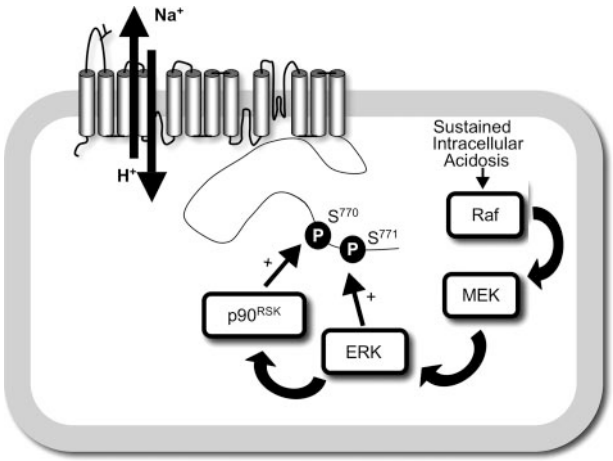

FIGURE 7. **Mechanism of activation of NHE1 by sustained intracellular acidosis.** Cellular events occurring during the treatment of cells during sustained intracellular acidosis are shown. Sustained acidosis results in activation of Raf and subsequent activation of MEK. MEK activates ERK, which subsequently activates $p90^{RSK}$. The kinases ERK and $p90^{RSK}$ directly phosphorylate NHE1 at amino acids Ser^{770} and Ser^{771}.

found that this amino acid is not critical to sustained acidosis activation of NHE1, it seems unlikely that this pathway is involved or is critical for this activation of the NHE1 protein. In the case of $p90^{RSK}$, a consensus site of R*X*R*XX*S (28) is similar to amino acids 765–770, with Ser^{770} being a possible site of phosphorylation. Because we found that Ser^{770} is necessary for activation of NHE1 by sustained acidosis, it is possible that $p90^{rsk}$ activation of NHE1 is necessary for some of the response we demonstrated. It is interesting that both amino acids, Ser^{770} or Ser^{771}, were necessary for sustained acidosis activation of NHE1. Mutation of either one to alanine was sufficient to eliminate the effect of sustained acidosis. The co-requirement of these amino acids is similar to that observed in some other membrane proteins. For example in the voltage-dependent calcium channel, Ser^{409} and Ser^{447} are substrates for ERK1/2-mediated phosphorylation and are required for activation of the channel. Mutation of either amino acid reduces the ERK-stimulated calcium current (29). Having a requirement for two phosphorylated amino acids adjacent to each other for a physiological response represents an interesting control mechanism that is perhaps tightly regulated. Overall, the clustering and requirement of phosphorylatable residues in this region is intriguing but requires further experimentation to elucidate the interrelationships of these amino acids.

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