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Phenylephrine and sustained acidosis activate the neonatal rat cardiomyocyte Na^+/H^+ exchanger through phosphorylation of amino acids Ser^{770} and Ser^{771}

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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. First published June 19, 2009; doi:10.1152/ajpheart.01231.2008.—The mammalian Na^+/H^+ exchanger isoform 1 (NHE1) is a ubiquitously expressed membrane protein that regulates intracellular pH in the myocardium. NHE1 is also important in mediating myocardial hypertrophy, and the blockage of NHE1 activity prevents hypertrophy and reduces ischemia-reperfusion injury in animal models. We recently demonstrated that extracellular-regulated kinase (ERK)-mediated activation of NHE1 occurs during ischemia-reperfusion of the myocardium. To understand the regulation of NHE1 in the myocardium by phosphorylation, we expressed a series of adenoviruses that express wild-type and mutant cDNA for NHE1. All exogenous cDNA for NHE1 had additional mutations [$Leu^{163}Phe/Gly^{174}Ser$], which increases NHE1 resistance to EMD-87580 (a specific blocker of NHE1) 100-fold, and allowed the measurement of exogenous NHE1 while inhibiting endogenous NHE1. By examining the effects of a series of mutations of the NHE1 cytosolic region, we determined that the amino acids Ser^{770} and Ser^{771} were essential for the acute activation of NHE1 activity in rat cardiomyocytes. The specific mutation of either residue prevented the rapid activation of exchanger activity by a sustained intracellular acidosis through ERK-dependent pathways. The same amino acids were critical to phenylephrine-mediated, ERK-dependent activation of NHE1 activity and increased the phosphorylation in intact rat cardiomyocytes. The results demonstrate that both sustained intracellular acidosis and phenylephrine rapidly activate the NHE1 protein in intact cardiac cells through ERK-dependent pathways that act on a common pathway mediated by amino acids Ser^{770} and Ser^{771} of the cytosolic tail of the protein.

extracellular-regulated kinases-1/2; ischemia-reperfusion; sodium-hydrogen exchanger

THE SODIUM-HYDROGEN EXCHANGER isoform-1 (NHE1) is a ubiquitously expressed plasma membrane glycoprotein. It functions to protect cells from intracellular acidification by extruding a single intracellular proton in exchange for one extracellular sodium (11). Ten isoforms of the Na^+/H^+ exchanger have been discovered (NHE1–NHE10). NHE1 was the first isoform discovered (41) and is ubiquitously distributed. Other isoforms have more restricted tissue distributions, and some have predominantly intracellular localization. In mammals, aside from its role in regulation of intracellular pH (pH_i), NHE1 is also important in the regulation of cell volume and cell proliferation and in the metastasis of some types of tumor cells (3, 11). In the myocardium, the Na^+/H^+ exchanger has several key roles in heart disease. It plays a critical role in mediating the damage that occurs with ischemia-reperfusion of the heart (1, 25) and is an important mediator of myocardial hypertrophy (23). Clinical trials are attempting to develop NHE1 inhibitors for the treatment of various forms of heart disease (2).

There are two major domains in Na^+/H^+ exchanger proteins. An NH_2-terminal membrane domain is ~500 amino acids, and this is followed by a 315 amino acid, hydrophilic, carboxyl-terminal cytosolic domain. The cytosolic domain regulates the membrane domain. Phosphorylation occurs in the distal region of the cytosolic domain within the last 178 amino acids (33, 49). We demonstrated that the mitogen-activated protein kinases (MAPKs) extracellular-regulated kinases 1 and 2 (ERK1/2) are implicated in growth factor activation of NHE1. This was shown in the intact myocardium (33), in isolated cardiomyocytes (33), and in Chinese hamster ovary (CHO) cells (51). The ERK-dependent pathway was activated in several models of ischemic heart disease, and this resulted in the further activation of the Na^+/H^+ exchanger in the myocardium (32). 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}, Ser^{771}, Thr^{779}, and Ser^{785} (28) in vitro. In CHO cells, we showed that amino acids Ser^{770} and Ser^{771} are critical to NHE1 activation. In other reports, we have shown that amino acids Thr^{717}, Ser^{722}, Ser^{725}, and Ser^{728} are phosphorylated in vitro by a p38 MAPK-dependent pathway (24) and that Ser^{726} and Ser^{729} mediate the activation of NHE1 by apoptosis in CHO cells (14).

Several aspects of NHE1 regulation are of special interest in the myocardium. The regulation of NHE1 varies from one tissue to another, even when the same NHE isoform is expressed in different cells (12, 48). The myocardial Na^+/H^+ exchanger has different kinetic characteristics than in other tissues (50). It is activated acutely by both α-adrenergic stimulation (58) and moderate periods of sustained intracellular acidosis (SIA) (17). α-Adrenergic stimulation of the myocardium has been shown to activate ERK-dependent pathways (7), and SIA acts through ERK-dependent pathways (17). Both mechanisms of regulation may be important in activating the protein in heart failure (30), and we have shown that ischemia and reperfusion activate the NHE1 protein and NHE1-directed protein kinases in the intact myocardium (32).

While increases in NHE1 levels have been shown to occur in heart disease (8, 13), the elevation of levels of the NHE1 protein alone in the myocardium does not enhance the susceptibility to ischemia-reperfusion injury and the regulation of NHE1 has recently been suggested to be critical in this regard (22). In this report, we characterized the mechanism of ERK-dependent phosphorylation and the regulation of the Na^+/H^+ exchanger in the myocardium. We demonstrated that phenylephrine-acidosis-ERK-dependent activation of NHE1 in cardiomyocytes is through the phosphorylation of amino acids Ser^{770} and Ser^{771}. Our results are the first to define and elucidate this

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mechanism of regulation of the Na^+/H^+ exchanger in intact neonatal cardiomyocytes.

MATERIALS AND METHODS

Materials. Routine chemicals were of analytical grade and were purchased from Fisher Scientific (Ottawa, ON, Canada), Sigma (St. Louis, MO), or BDH (Toronto, ON). Special chemicals were from the following sources: collagenase type 2 was obtained from Worthington Biochemical (Lakewood, NJ), and 2′,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein AM (BCECF-AM) was from Molecular Probes (Eugene, OR). Platinum R Taq Polymerase was from Invitrogen (Burlington, ON, Canada). EMD-87580 was a generous gift of Dr. N. Beier of Merck (Frankfurter, Germany). PWO DNA polymerase was from Roche Applied Science, and Lipofectamine 2000 reagent was from Invitrogen. The MEK inhibitor U-0126 and phorbol 12-myristate 13-acetate (PMA) was from Sigma. Anti-MAPK ERK1, ERK2, and anti-phospho-ERK1/2 and anti-$p90^{RSK}$ (anti-Ser^{380}) were from Cell Signaling Technology (Beverly, MA). Anti-hemagglutinin (HA) antibody (Y-11) anti-$p90^{rsk}$ was from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-NHE1 antibody (MAB-340) was from Chemicon (Billerica, MA). Protein-A Sepharose beads were from Pierce. [^{32}P]orthophosphate was from Perkin Elmer Life and Analytical Sciences (Boston, MA). *N*-acetyl-Leu-Leu-Nle-CHO (ALLN) was from Calbiochem (La Jolla, CA).

Construction of adenoviral NHE1 genes. The NHE1 gene and various mutants of NHE1 were used to make adenoviruses for the transfection of primary cultures of neonatal rat cardiomyocytes. Initially, the HA-tagged Na^+/H^+ exchanger (35) was mutated to make it resistant to inhibition by amiloride analogs. The NHE1 isoform of the exchanger (i.e., present in cardiomyocytes) is sensitive to inhibition by amiloride analogs. Other isoforms of the NHE (i.e., NHE3) are much more resistant to inhibition (37). The double mutation $Leu^{163}Phe/Gly^{174}Ser$ in NHE1 increases the resistance to inhibition, similar to NHE3 resistance. The plasmid $pYN4^+$ contains cDNA coding for the HA-tagged NHE1 protein and has been described earlier (44). The mutation $Leu^{163}Phe/Gly^{174}Ser$ (Table 1) was made in the NHE1 in the plasmid $pYN4^+$ as described earlier and conferred an ~100-fold increase in resistance to inhibition (5, 35).

To localize the amino acids that are important in the regulation of the NHE1 protein in the myocardium, several phosphorylation-defective mutant NHE1 cDNAs were used. They were chosen based on our earlier study in which we demonstrated that four regions of the NHE1 COOH-terminus were susceptible to ERK-dependent phosphorylation (28). The mutants were as follows: *mutant 1*, S693A; *mutant 2*, T718A/S723/726/729A; *mutant 3*, S766/770/771A; and *mutant 4*, T779A/S785A. The mutants were also made of the individual amino acids Ser^{770} and Ser^{771}, respectively (Table 1). The mutation of $pYN4^+$ plasmids was as described earlier (31). To make the phosphorylation-defective mutant NHE1 cDNAs resistant to inhibition, an *Age*I-*Spe*I fragment was replaced from the resistant mutant. This contained the $Leu^{163}Phe/Gly^{174}Ser$ mutation of the membrane domain while excluding the phosphorylation domain.

Table 1. *Summary of mutations in NHE1 protein*

Description	Label
L163F/G174S	IRM
S693A	1
T718A, S723/726/729A	2
S766/770/771A	3
T779A, S785A	4
S770A	S770A
S771A	S771A

IRM, inhibitor-resistant mutant. All mutations were made in the full-length Na^+/H^+ exchanger isoform 1 (NHE1). All mutants expressed contained the IRM mutation in addition to other mutations indicated. 1–4, *mutants 1–4*.

Adenoviral vectors were made for the expression of the NHE1 isoform of the Na^+/H^+ exchanger. Control and mutated inhibitor-resistant Na^+/H^+ exchangers were amplified using a high-fidelity polymerase (Platinum R Taq Polymerase, Invitrogen) from the plasmid $pYN4^+$ using the primers MackpYNF CCGGGGTACCGCCACCATGGTTCTGCGGTCTGG and MackPynR GGAAGCTTAAGCTTCTACTGAGCAGCGTAATCTGGAAC that flanked the Na^+/H^+ exchanger cDNA with *Kpn*I and *Hind*III sites, respectively. The product was cloned into a modified adenovirus (19) using *Kpn*I and *Hind*III sites of the amplified insert and of the pAdTrack-CMV vector. After directional ligation, the product was sequenced to confirm identity and fidelity. After linearization with *Pac*I the pADTrack-CMV-NHE1 construct was and used to transform *Escherichia coli* (BJ5183) that contain the vector pAdEasy-1. Recombinant pAd-NHE1 mutants were screened by restriction enzyme mapping, amplified, purified, and used to transfect E1-transformed human embryonic kidney cells (293A). The transfection efficiency was monitored by green fluorescent protein (GFP) expression, which was on the same construct as the NHE1 cDNA. For the large-scale production of adenovirus, viral-containing supernatants were used to reinfect the cells. High-titer viral stocks were purified by CsCl centrifugation, followed by dialysis and titering using plaque assays (5).

Preparation and characterization of isolated cardiomyocytes. Primary cultures of cardiomyocytes were prepared from 5- to 6-day-old neonatal Sprague-Dawley rat heart ventricles as described previously (33). Isolated primary cardiomyocytes were plated onto glass coverslips for pH_i measurements or onto Corning culture dishes or flasks when harvesting cell extracts. The myocytes were maintained for 3 days before infection in medium containing Dulbecco's modified Eagle's medium (DMEM/F12) supplemented with 10% bovine growth serum (FBS), 10 μg/ml transferrin, 10 μg/ml insulin, 10 ng/ml selenium, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mg/ml bovine serum albumin (BSA), 5 μg/ml linoleic acid, 3 mM pyruvic acid, 0.1 mM minimum essential medium (MEM) nonessential amino acids, 10% MEM vitamin, 0.1 mM bromodeoxyuridine, and 30 mM HEPES (pH 7.4). For some experiments, the cells were infected with NHE1-expressing adenovirus, and after 24 h, the cells were labeled with [^{32}P]inorganic phosphate. The media was removed, and the plates were washed two times with phosphate-free DMEM. Phosphate-free DMEM (8 ml) was then added for 30 min at 37°C, and the plates were washed 2× with more media. Phosphate-free DMEM (2 ml) was added with $[H_3]^{32}PO_4$, added to a final concentration of 100 μCi/ml media. The cells were then incubated at 37°C in 5% CO_2-95% room air for 3 h. In some experiments, the cells were treated with 50 ng/ml phorbol ester (PMA) for 20 min. All animals in this study were used in accordance with Canadian Council on Animal Care regulations with a protocol approved by that agency for their use for this purpose.

pH_i measurement. To examine NHE1 activity in isolated cardiomyocytes, the cells were infected with wild-type or various mutants of pAd-NHE1. For infection, the cells were simultaneously infected and placed in serum-reduced medium (0.5% FBS) overnight with 10 μg/ml gentamicin. Standard infections were at a multiplicity of infection of 20. The cells were routinely infected for 24 h before pH_i measurement. The pH_i of an entire population of cells grown on coverslips was measured using a PTI Deltascan spectrofluorometer. The initial rate of the Na^+-induced recovery of pH_i was measured after ammonium chloride (50 mM × 3 min) induced acute acid load using BCECF-AM. The recovery was in the presence of 135 mM NaCl and was measured during the first 20 s after the return of NaCl at 37°C as described previously (44). A calibration curve was done with nigericin at the end of every experiment to calibrate pH_i to fluorescence as described earlier (44). To test the effects of the SIA experiments, the cells were incubated in low-serum media (0.5% FBS) overnight and then treated with a two-pulse acidification assay. The SIA treatment was as described earlier and has been previously

described by others (17) and ourselves (31). The cells were treated with ammonium chloride two times and allowed to recover in NaCl containing medium. For the first treatment with ammonium chloride, the cells were rinsed with Na^+-free medium for 10–20 s, and when the decline in pH_i had stabilized, NaCl was added immediately to allow for pH_i recovery. For the second pulse, the cells were maintained in Na^+-free medium for 3 min before the reintroduction of 135 mM NaCl to cause SIA (31). For one group of experiments ($n = 5$), the SIA was induced in the presence of normal 135 mM NaCl but was maintained by the addition of 20 μM EMD-87580. A brief washout period of 10–15 s removed EMD-87580 before recovery. The values of the Na^+/H^+ exchanger activity obtained after sustained acidosis were compared with the values obtained in two-pulse assays without sustained acidosis in the second pulse. The assays were done in either the presence or absence of 10 μM EMD-87580. EMD-87580 was dissolved in PBS and added during ammonium chloride treatment in Na^+-free medium and during recovery in NaCl. Our laboratory (5) previously determined that 10 μM EMD-87580 was sufficient to inhibit endogenous NHE1 in isolated cardiomyocytes but does not inhibit exogenous NHE1 with the $Leu^{163}Phe/Gly^{174}Ser$ mutation. To test the effect of phenylephrine on NHE1 activity, the cells were maintained in normal serum media overnight and were treated for 6 min with 100 μM freshly made phenylephrine, which was maintained throughout the assay. For these experiments, we did not use a two-pulse protocol but rather a series of experiments with a single pulse of cells either untreated or treated with phenylephrine, which gave a better response in the cells. All of these experiments were done with simultaneously prepared cells and were compared on the same day. All results are shown as means ± SE, and statistical significance was determined using the Wilcoxon-Mann-Whitney rank sum test.

Buffering capacity (B; in $mmol \cdot l^{-1} \cdot pH\ unit^{-1}$) was determined essentially as described earlier (22) by varying the amount of NH_4Cl and by the observing the change of cell pH produced by this load. Isolated cardiomyocytes that were infected with various adenovirus were BCECF loaded and were incubated with NH_4-containing buffer as described earlier (22).

The buffering capacity was determined as follows: $B = \Delta NH_{4i}^+ \Delta pH_i[NH_4Cl_o \times 10(pK_a - pH_i)]/[1 + 10(pK_a - pH_o)]$, where NH_4Cl_o is extracellular NH_4Cl and pH_o is extracellular pH. The equilibrium among NH_4^+, NH_3, and pH in the extracellular medium was determined by the Henderson-Hasselbach relation using a pK_a for NH_4^+ of 9.21.

SDS-PAGE and immunoblotting. To confirm NHE1 expression, the cell lysates were made from isolated cardiomyocytes as described earlier (43). For Western blot analysis, equal amounts of up to 100 μg of each sample were resolved on 10% SDS-polyacrylamide gels. Nitrocellulose transfers were immunostained using anti-HA monoclonal antibody for NHE1 detection (Boehringer Mannheim, Laval, QC, Canada) and peroxidase-conjugated goat anti-mouse antibody (Bio/Can, Mississauga, ON, Canada). MF-20 anti-myosin antibody was from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). To visualize and quantify immunoreactive proteins, we used Li-COR fluorescence labeling and detection systems (LI-COR Biosciences, Lincoln, NE). To detect ERK1/2 and phospho-ERK1/2, the two antibodies were used together at 1:1,000 dilution and then visualized with the appropriate secondary antibodies. To detect $p90^{rsk1/2}$ and phosphorylated $p90^{rsk1/2}$, antibodies for each were both used at a dilution of 1:1,000. For the detection and quantification of phosphorylated proteins, we used a Typhoon 9400 Variable Mode Imager (GE Healthcare, Piscataway, NJ).

Immunoprecipitation of phospho-NHE1 protein. Cell lysates were used for the immunoprecipitation of exogenously expressed NHE1 from isolated cardiomyocytes incubated with $[^{32}P]$. Radioimmunoprecipitation assay (RIPA) buffer contained (in mM) 50 Tris (pH 8.0), 150 NaCl, 80 NaF, 5 EDTA, 1 EGTA, 25 sodium pyrophosphate, and 1 sodium orthovandate and protease inhibitors containing 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, and 40 μM ALLN. Detergents were added where indicated at concentrations of 1% Nonidet P-40, 0.1% SDS, and 0.05% deoxycholate. After an incubation in $[^{32}P]$-containing medium, the cells were washed in phosphate-free buffer and treated with ammonium chloride to induce SIA where appropriate. Ice-cold RIPA buffer (3 ml) without detergents was added, and the cells were placed on dry ice to freeze them. After thawing on ice, the cells were scraped into centrifuge tubes and sonicated for 10 s. The cells were centrifuged at 35,000 rpm × 1 h, and 1 ml of RIPA buffer with detergents was added to the pellet. After 15 s of further sonication, the sample was centrifuged at 10,000 rpm × 30 min, and the supernatant was used for immunoprecipitation of NHE1 via the HA tag.

For immunoprecipitation of NHE1, 1 ml of lysates was precleared by an incubation with 100 μl of 100 mg/ml protein-A Sepharose beads at 4°C for 30 min in detergent-containing RIPA buffer and 1% (wt/vol) BSA. After the removal of the beads by centrifugation, the supernatant was incubated with 7.5 μl (1.4 μg/ml) of rabbit anti-HA antibody for 2 h at 4°C. Protein-A Sepharose beads were blocked in detergent-containing RIPA buffer with 1% (wt/vol) BSA for 1 to 2 h at 4°C. The supernatant with antibody was added to 100 μl of prepared protein-A Sepharose beads and agitated overnight at 4°C. The beads were collected by centrifugation at 7,000 rpm × 30 s and washed 4× with RIPA buffer before the final collection. After being washed, the bound protein was eluted from the washed beads by incubation with 45 μl of 1× SDS-PAGE sample-loading buffer. After SDS-PAGE, the samples were transferred to nitrocellulose membranes, and radioactivity was detected by using a Typhoon 9400, Variable Mode Imager (GE Healthcare, Piscataway, NJ). Immunoblotting with anti-HA antibody was used to check the level of NHE1 in the samples and correct for any variation in immunoprecipitation efficiency. Quantification was done with the Odyssey-scanning system (LI-COR Biosciences). The results are means ± SE of at least three experiments, and statistical significance was determined using a Wilcoxon signed-rank test. To compare the levels of one infected set of cells with another, experiments were always done simultaneously with equal amounts of cells, equal radioactivity, and in parallel at the same time. Exposures were made simultaneously of samples run in the same SDS-PAGE. The amount of immunoprecipitated NHE1 was quantified using the Odyssey scanning system (LI-COR Biosciences) for each sample immunoprecipitated, and the quantification of phosphorylated proteins was done with the Typhoon 9400, Variable Mode Imager (GE Healthcare).

RESULTS

To study the regulation of the NHE1 protein in isolated cardiomyocytes, we developed a series of adenoviral vectors that express the mammalian NHE1 protein. A series of mutations were made in the cytoplasmic regulatory region that contained putative phosphorylation sites for regulatory protein kinases. All of the NHE1 proteins expressed contained a $Leu^{163}Phe/Gly^{174}Ser$ mutation that made them resistant to drug inhibition. This allowed us to inhibit the activity of the endogenous NHE1 protein present in cardiomyocytes while the exogenous NHE1 protein remained active. Mutant NHE1 proteins were initially cloned into the pAdTrack-CMV vector to begin adenoviral construction. For all such constructs, we confirmed that the clone was being expressed in this vector by transfecting AP-1 cells that lack their own NHE and checked for NHE activity. In addition, Western blot analysis was used to confirm that the protein was expressed (not shown). After completion of the recombinant adenovirus construction, we initially checked for the expression and activity of the exogenous NHE1 protein in isolated cardiomyocytes. Figure 1 shows typical results. Wild-type NHE1 protein was expressed in

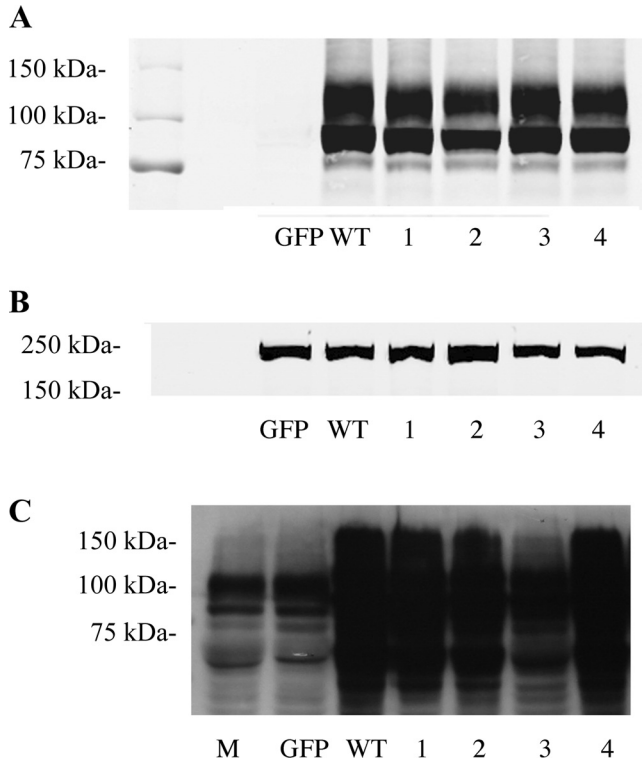
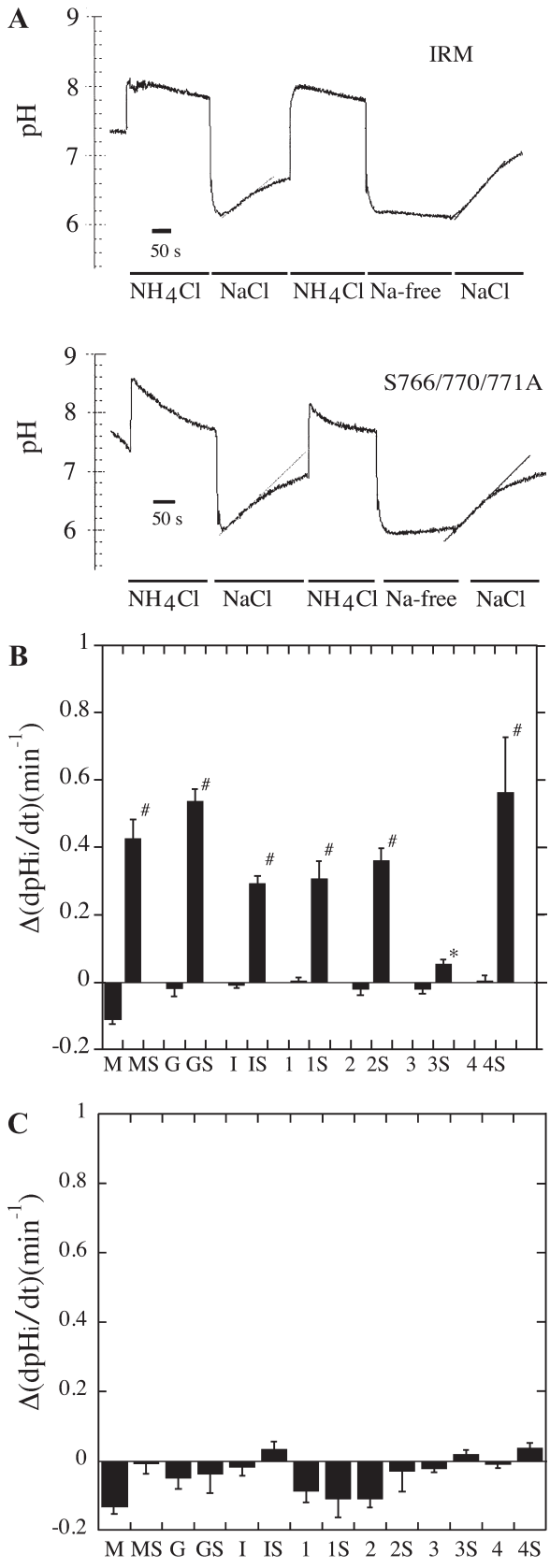


Fig. 1. Western blot analysis of Na^+/H^+ exchanger isoform 1 (NHE1) expression in isolated cardiomyocytes transfected with adenovirus-containing NHE1 protein (pAdNHE1) or mutant NHE1 protein. Isolated cardiomyocytes were made from neonatal rat hearts as described in the MATERIALS AND METHODS. Equal numbers of cells were then infected with pAdNHE1 containing the hemagglutinin (HA)-tagged inhibitor-resistant NHE1 protein. WT and 1–4, cell extracts from isolated cardiomyocytes infected with adenovirus containing wild-type NHE1 (with inhibitor resistant mutation) and with phosphorylation *mutations 1–4*, respectively; GFP, cell extract of isolated cardiomyocytes transfected with adenovirus expressing green fluorescent protein alone; M, mock-infected cells. *A*: Western blot of cell extracts blotted with anti-HA antibody. *B*: samples immunoblotted for myosin heavy chain. *C*: samples were immunoblotted for NHE1 using antibody against the COOH-terminus of the protein.

isolated cardiomyocytes as detected by antibody against the HA tag (Fig. 1*A*). Both a glycosylated and a deglycosylated or partially deglycosylated form of the NHE1 protein are visible as described earlier (44). The cells transfected with adenovirus expressing GFP alone showed no detectable HA-tagged NHE1 protein, whereas the cells transfected with adenovirus containing the NHE1 *mutants 1–4* all expressed the HA-NHE1 protein. An antibody against myosin heavy chain (Fig. 1B) confirmed that isolated cardiomyocyte proteins were present and transferred for immunoblotting. Figure 1C illustrates a Western blot against the endogenous NHE1 protein using a commercial antibody against the NHE1 cytoplasmic tail. The full-length NHE1 protein was detected in the uninfected cells and in cells infected with NHE1 containing adenovirus. Exogenous NHE1 protein was slightly larger in apparent molecular weight, likely due to the added HA tag. Degradation products of NHE1 were also present. The level of NHE1 protein was roughly estimated as increased threefold, though the quantification was compromised by the presence of endogenous NHE1 in the virally infected samples.

Isolated cardiomyocytes were infected with adenovirus with or without NHE1 cDNA insert. Over 99% of the cells appeared to be infected as shown by fluorescence of the GFP marker protein that is contained in adenovirus GFP (not shown). Fluorescence from GFP was only a small fraction of that of BCECF and did not interfere with pH_i measurement. A calibration curve was done for every experiment between pH_i and fluorescence. The infection of the cardiomyocytes with NHE1 containing adenovirus did not alter the resting intracellular pH of cells. In addition, there were no differences in the initial degree of acidification induced by ammonium chloride between NHE1-infected cells and controls (i.e., GFP-infected cells) in either of the two pulses. For two-pulse assays, we compared the values obtained in the second pulse with SIA to those obtained in a matched two-pulse assay without SIA. There were no differences in the initial pH of recovery of the second pulse compared with the first pulse or when comparing a two-pulse assay with SIA with a two-pulse assay without SIA. The increased period of incubation in Na^+-free medium changed the duration and not the extent of intracellular acidosis. For example, the minimum pH obtained with the first pulse in the GFP-infected cells was 6.05 ± 0.07, whereas that of the second pulse was 6.03 ± 0.06. Similar results were obtained with other groups of infected cardiomyocytes. To confirm that we had expressed various functional inhibitor-resistant mutants (IRMs) in isolated cardiomyocytes, we measured the NHE1 activity in isolated cardiomyocytes infected with NHE1 containing adenovirus for 24 h at a multiplicity of infection of 20. The activity was measured in cells using a two-pulse assay with the second pulse in the presence or absence of 10 μM EMD-87580. We previously determined that this concentration inhibited the endogenous NHE1 protein, whereas the NHE1 mutant with the $Leu^{163}Phe/Gly^{174}Ser$ mutations was unaffected (5). GFP-infected cells had a first pulse rate of recovery of 0.0111 ± 0.0016 ΔpH/s. This was not different in the second pulse when there was no sustained acidosis. When we compared the second pulse in the presence of 10 μM EMD-87580 to the first pulse in the absence of EMD-87580, we found an ~85–90% decrease in Na^+/H^+ exchanger activity in uninfected or GFP-infected cells. However, the same comparison of cells infected with the NHE1 inhibitor-resistant mutation showed an ~40–45% decrease in activity. The decline in activity due to 10 μM EMD-87580 in infected cells is likely due to the decreased activity of the endogenous Na^+/H^+ exchanger. Most of the 55% of the remaining activity was likely due to the activity of the inhibitor resistant NHE1 protein. There was no significant change in the absolute rate of recovery of *mutants 1–4* or of the single-mutant NHE1 proteins. In the absence of SIA or phenylephrine, the NHE1 activity after the second ammonium chloride pulse was equivalent to the first in all mutants and controls. There was also no difference in the buffering capacity of cells infected with various types of adenovirus relative to uninfected cells (supplemental Fig. 1; note: supplemental material is posted with the online version of this article).

We characterized the effect of SIA on the activity of various NHE1 proteins in isolated cardiomyocytes (Fig. 2). The uninfected and GFP-infected isolated cardiomyocytes were assayed in the absence of EMD-87580. The second rate of recovery increased ~50–75% over the initial rate of recovery (Fig. 2, *A* and *B*). A similar effect occurred in isolated cardiomyocytes when SIA was induced by using EMD-87580 in the presence of normal sodium (not shown). When cardiomyocytes were



infected with adenovirus-expressing inhibitor-resistant NHE1 protein, in the presence of EMD-87580 they behaved in a similar fashion to these controls. The second rate of recovery was elevated about 60% greater than that of the controls. This confirmed that exogenous NHE1 in cardiomyocytes was functional and responded similarly to the endogenous NHE1 present in cells. Similar results were found with *mutants 1*, *2*, and *4*. The second pulse was not significantly different from that obtained with the wild-type NHE1 inhibitor-resistant protein. There was a slight decrease in the rate of recovery of *mutant 4*, though this was not significantly different from control values. In contrast, when cells were infected with the NHE1-mutant protein 3, with mutations to Ala at amino acids 766, 770, and 771, there was significantly less activation by SIA compared with the activation achieved with wild-type, inhibitor-resistant NHE1.

Because previous reports have shown that the stimulation of NHE1 by acidosis is ERK dependent (17, 32), we examined the effect of the ERK pathway inhibitor U-0126 on the ability of SIA to activate NHE1 activity. The results (Fig. 2*C*) showed that the blockage of these pathways prevented the activation of the NHE1 protein in isolated cardiomyocytes that were treated with SIA. This occurred in cells that were uninfected and infected with either GFP, the IRM NHE1, or the IRM NHE1 with the *mutants 1–4*. In all cases there was no significant increase in NHE1 activity by treatment with SIA.

To confirm that SIA-activated ERK pathways of isolated cardiomyocytes subjected to SIA, we examined the phosphorylation levels of ERK1/2 and $p90^{rsk}$. In these experiments we examined the phosphorylation state of the kinases after SIA and before recovery from acidosis and after SIA so that we could examine the state of the kinase at the initial stages of NHE1 recovery. The results are shown in Fig. 3. The upper panels in Fig. 3, *A* and *B*, were with anti-phospho-kinase antibodies, whereas the lower panels were with anti-kinase

Fig. 2. Effect of sustained intracellular acidosis (SIA) on NHE1 activity of isolated cardiomyocytes. G, infected with GFP containing virus without NHE1; IRM or I, infected with WT inhibitor-resistant NHE1 adenovirus. In M and GFP-infected cells, activity was in the absence of EMD-87580. For other cells, activity was measured in the presence of 10 μM EMD-87580. Cells were uninfected or infected with various adenovirus types at an multiplicity of infection of 20 for 24 h. The cells were then subjected to two pulse Na^+/H^+ exchanger activity assays, and the activity of the exchanger in the second pulse was compared with that of the first pulse. The second pulse was done after a 3-min period of SIA immediately before recovery. *A*: examples of traces of dual-pulse assay. *A*, *top*: WT (IRM) inhibitor-resistant NHE1. *A*, *bottom*: example of dual-pulse assay of *mutant 3* with mutation S766/770/771A. Lines drawn at tangent to the rate of recovery are for illustrative purposes only. Periods of NH_4Cl, NaCl, and Na-free solution are indicated. A brief period of Na-free incubation after the initial NH_4Cl pulse is not illustrated. *B*: summary of NHE1 activity in dual-pulse assays of WT and NHE1 mutants. Two pulse assays were done, and in experimentals, the second pulse contained an extended period of sustained acidosis as illustrated in *A*. Controls did not have a sustained acidosis in the second pulse. A summary of the difference between the second pulse minus the first pulse is shown. #$P < 0.05$, significantly different from the first pulse; *$P < 0.01$, significantly different relative to IRM NHE1. Values are means ± SE of at least 10 experiments. The suffix S indicates that in the second pulse, the group was treated with sustained acidosis. Where S was absent, the second pulse did not contain an extended period of acidosis. dpH_i/dt, change of intracellular pH over time. *C*: effect of SIA on NHE1 activity in the presence of U-0126. Cells were treated and measured as described in *B*, except in the presence of 3 μM U-0126 treatment. U-0126 treatment was 10 min of preincubation before assay, and U-0126 was then present for the entire assay period.

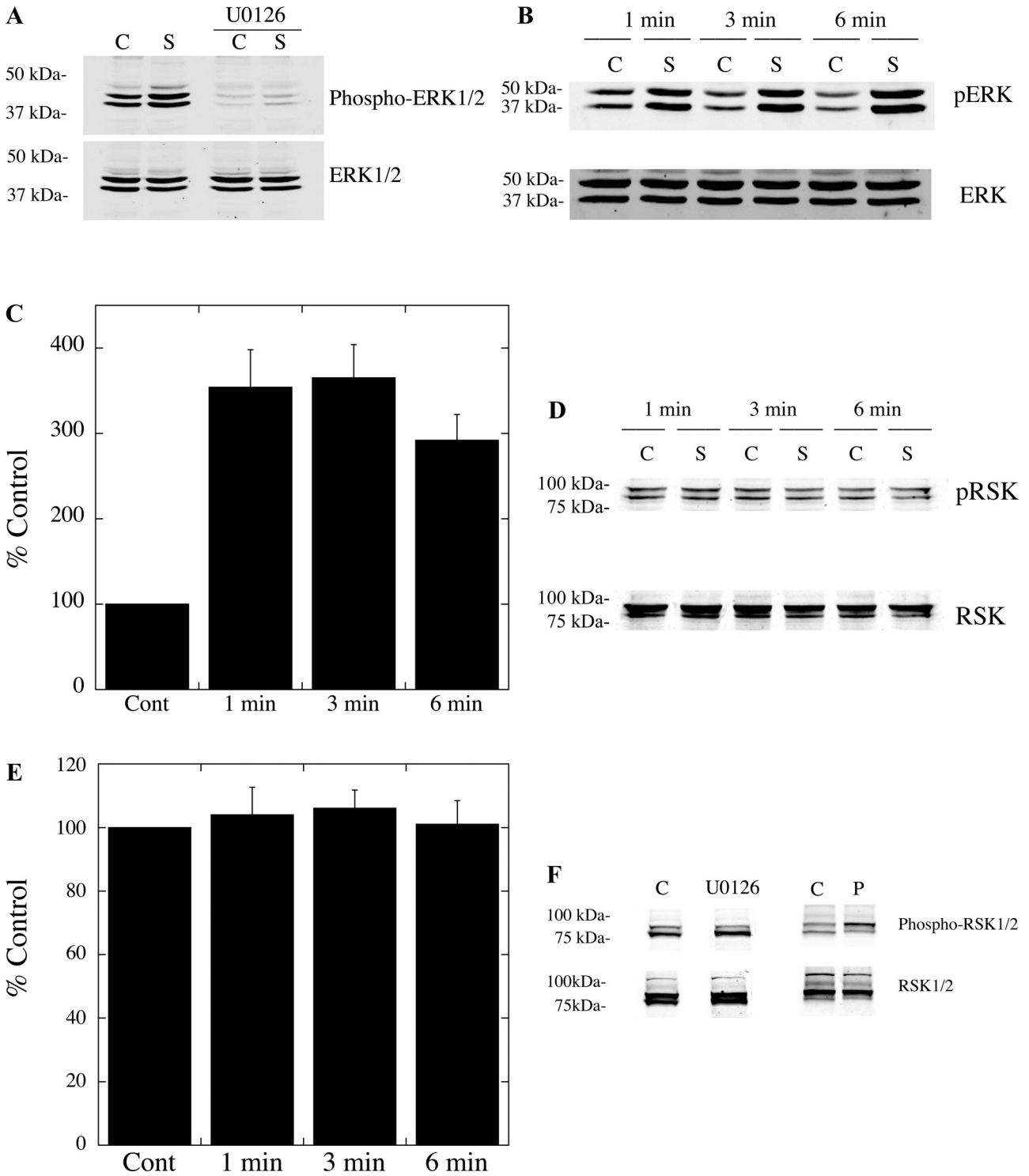


Fig. 3. Effects of SIA on activation of ERK and $p90^{rsk}$ of isolated cardiomyocytes. Isolated cardiomyocytes were subjected to SIA as described under *pH_i measurement*. *A*: Western blot with ERK (*bottom*) and anti-phospho-ERK (pERK; *top*) antibodies. Where indicated, cells were treated in the presence of 10 μM U-0126. C, control (Cont) cells; S, cells subjected to SIA. *B* and *C*: time course of activation of ERK by SIA: Western blot of pERK and ERK protein after 1, 3 and 6 min of SIA stimulation (*B*) and summary of effects of SIA on ERK activation (*C*). *D* and *E*: time course of activation of $p90^{rsk}$ by SIA: Western blot of phospho-$p90^{rsk}$ and $p90^{rsk}$ protein after 1, 3, and 6 min of SIA stimulation (*D*) and summary of effects of SIA on $p90^{rsk}$ activation (*E*). RSK1/2, $p90^{rsk1/2}$. Values are means ± SE of at least 3 experiments. *F*: Western blot of $p90^{rsk}$ with anti-$p90^{rsk}$ (*bottom*) and anti-phospho-$p90^{rsk}$ (*top*) antibodies. Where indicated, cells were untreated (C) or treated with 10 μM U-0126 or 50 ng/ml PMA (P). Results are typical of 3 experiments.

antibodies that were used to normalize the results. Figure 3*A* illustrates that SIA resulted in a large and significant increase in phospho-ERK1/2 levels. This was blocked by U-0126. Figure 3, *B* and *C*, shows a time course of ERK1/2 activation. ERK1/2 was rapidly activated within 1 min of stimulation. Figure 3, *D–F*, shows the same kind of analysis of $p90^{rsk}$. However, there was no significant increase in the phosphorylation levels of $p90^{rsk}$ isoforms 1 and 2 with the SIA stimulation over the 6-min acidosis period examined (Fig. 3, *D* and *E*). We also examined the effect of U-0126 on the basal level of phospho-$p90^{rsk}$ (Fig. 3*F*). U-0126 did not decrease the level of phospho-$p90^{rsk}$, indicating that the protein was not already stimulated. However, a treatment with phorbol esters caused a significant increase in the level of phospho-$p90^{rsk}$, confirming that we could detect the elevation of phospho-$p90^{rsk}$ in our system.

We examined the effect of intracellular acidosis and ERK inhibition on the phosphorylation levels of the NHE1 in cardiomyocytes in vivo. SIA increased the level of phosphorylation of the NHE1 IRM protein in isolated cardiomyocytes. Figure 4*A* shows an example of an autoradiogram that demonstrates increased phosphorylation of the NHE1 protein in response to sustained acidosis. This occurred for the wild-type NHE1 and for *mutants 1*, *2*, and *4*. There was no apparent increase in the level of phosphorylation in *mutant 3* in response to sustained acidosis. In addition, in the presence of the compound U-0126, an increased phosphorylation did not occur for any of the NHE1 proteins. The level of phosphorylation of *mutant 3* was always much less than that of the wild-type and of the other mutant proteins. The exposure times for *mutant 3* protein were increased greatly to facilitate the accurate quantification of the levels of phosphorylation. Figure 4*B* is a summary of the experiments. All experiments were paired and examined the levels of NHE1 phosphorylation of wild-type and mutant proteins comparing the phosphorylation levels of NHE1 protein with and without sustained acidosis. The results indicate that the phosphorylation level increased ~40–45% in

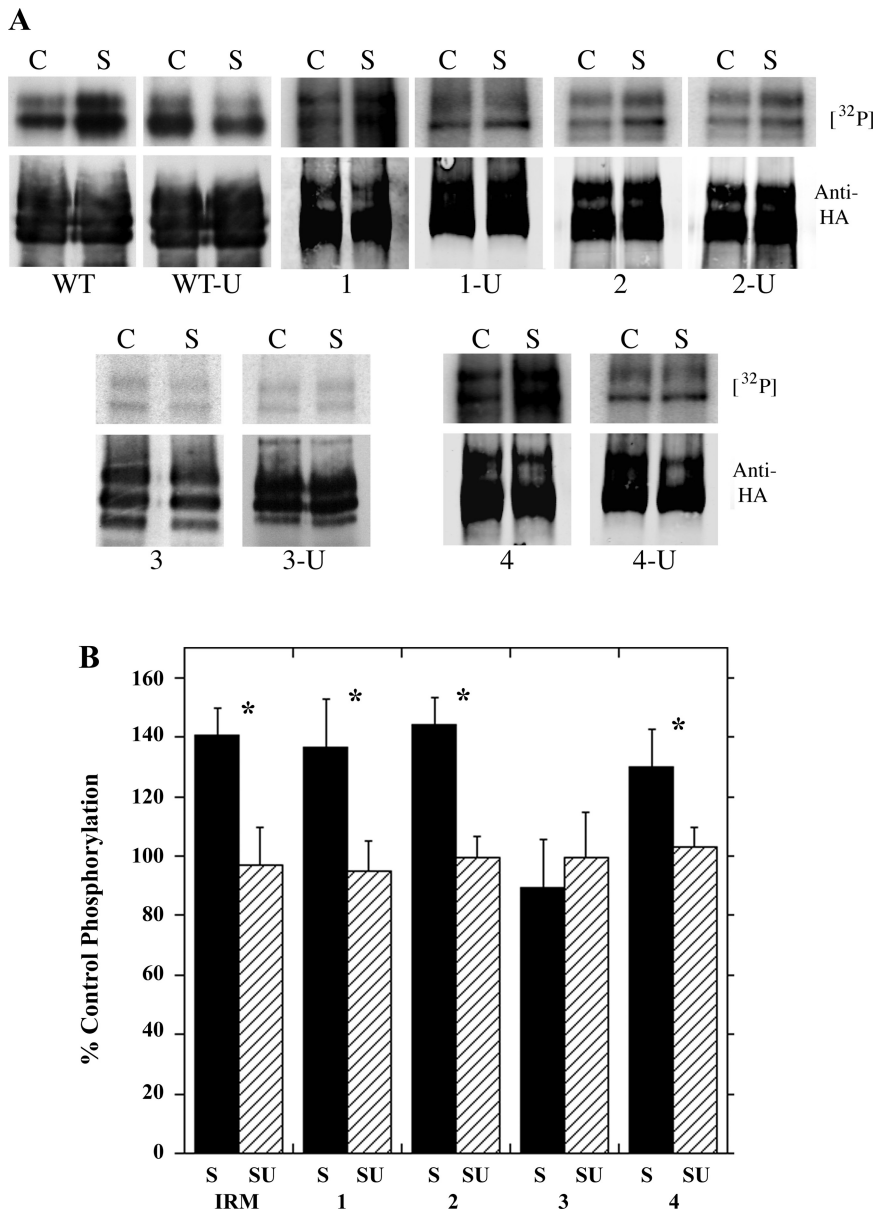


Fig. 4. Effects of SIA on phosphorylation levels of NHE1 protein in isolated cardiomyocytes. *A*: examples of NHE1 protein immunoprecipitated from cardiomyocytes incubated with [^{32}P]. *A*, *top*: phosphorylated protein immunoprecipitated with anti-HA tag to obtain exogenous, introduced NHE1 protein. *A*, *bottom*: anti-HA Western blot used to correct for the amount of immunoprecipitated HA-NHE1 protein. C, immunoprecipitated NHE1 protein from control cardiomyocytes; S, immunoprecipitated NHE1 protein from cardiomyocytes stimulated for 3 min by sustained acidosis; U, appropriate type of NHE1 protein was immunoprecipitated from cardiomyocytes incubated with [^{32}P] in the presence of 3 μM U-0126 throughout the experiment as described in MATERIALS AND METHODS. Exposure times for WT and *mutants 1*, *2*, and *4* were equivalent. The exposure time for examination of [^{32}P] of *mutant 3* was increased severalfold to make the bands visible. *B*: summary of phosphorylation levels of NHE1 protein from isolated cardiomyocytes stimulated by sustained acidosis in the presence or absence of 3 μM U-0126. Black and hatched bars represent stimulated by sustained acidosis (S), and stimulated in the presence of U-0126 (SU). Values are means ± SE of at least 3 experiments. *$P < 0.05$, significantly elevated over the level of control.

response to sustained acidosis in wild-type NHE1 and in *mutants 1* and *2*. There was a smaller increase in *mutant 4*, which was still significantly elevated over control values. *Mutant 3* showed no increase in the level of phosphorylation in response to acidosis. In all cases, we measured the amount of immunoprecipitated protein and normalized the phosphorylated levels using these values (Fig. 4*B*). Similar experiments were done in the presence of U-0126 to determine whether the pathways involved in phosphorylation were ERK dependent. In the presence of this inhibitor (Fig. 4*B*, hatched bar), there was no activation of the NHE1 protein. In a single set of experiments, we compared the absolute level of phosphorylation of all the mutant proteins with that of the wild-type in the same autoradiogram. Only the level of *mutant 3* was reduced compared with the wild-type and was ~5% of the total wild-type NHE1 protein (not shown).

To localize the exact amino acid(s) involved in the regulation of activity of NHE1 in the myocardium, we studied the effects of mutation of amino acids Ser^{770} and Ser^{771}. We had previously shown that the mutation of amino acid 766 was not involved in the regulation of activity in CHO cells (31). Figure 5 summarizes the results. Figure 5, *A* and *B*, shows that the Na^+/H^+ exchanger activity of neither mutant S770A or S771A was activated by sustained acidosis. As demonstrated earlier (Fig. 4), all mutants (except *mutant 3*) and wild-type NHE1 were activated by sustained acidosis. In contrast, the Na^+/H^+ exchanger activity of mutants S770A and S771A were not activated by SIA. We therefore examined the effect of sus-

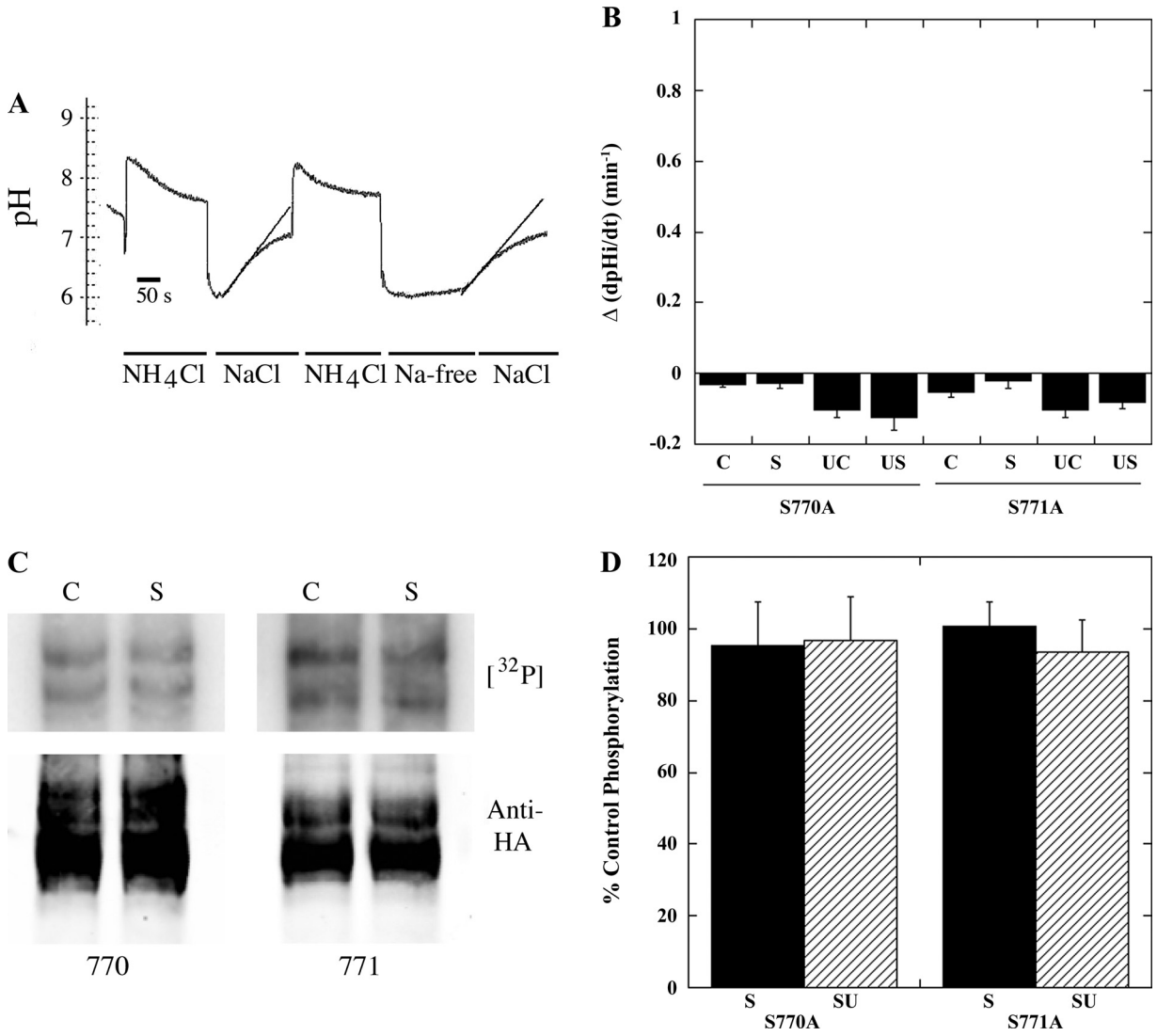


Fig. 5. Effect of mutation of amino acids Ser^{770} and Ser^{771} on NHE1 activity and phosphorylation levels in isolated cardiomyocytes. Isolated cardiomyocytes were prepared and infected with adenovirus containing mutation of either Ser^{770} or Ser^{771}. The activity and phosphorylation levels of the mutant NHE1 protein were determined as described in MATERIALS AND METHODS. *A*: examples of dual-pulse assay for S770A mutant as described in Fig. 2*A*. *B*: effect of sustained acidosis on activity of NHE1 mutants. Bars represent C stimulated by S and in the presence of U. Values are means ± SE of at least 10 experiments. *C*: example of effect of sustained acidosis on phosphorylation levels of mutant NHE1 proteins. *C*, *top*: phosphorylated protein immunoprecipitated with anti-HA tag to obtain exogenous, introduced NHE1 protein. *C*, *bottom*: anti-HA Western blot used to correct for the amount of immunoprecipitated HA-NHE1 protein. *D*: summary of effects of SIA on phosphorylation levels of S770A and S771A mutant NHE1 proteins in isolated cardiomyocytes. Values are means ± SE of at least 3 experiments.

tained acidosis on the phosphorylation levels of these mutant proteins (Fig. 5, *C* and *D*). There was also no increased phosphorylation of these mutant proteins in response to sustained acidosis.

While sustained acidosis has been shown to activate the NHE1 protein in the myocardium, we also wanted to examine whether the hormonal-induced activation of the NHE1 protein in the myocardium was mediated by the same mechanism. We therefore tested the effect of phenylephrine on the activity and phosphorylation of wild-type and mutant NHE1 proteins. Figure 6*A* illustrates the results on the stimulation of NHE1 activity with phenylephrine. Phenylephrine caused between a 35–55% increase in the rate of recovery from an acid load. In the absence of EMD-87580 and without exogenous NHE1 protein, both the uninfected cells and the cells infected with GFP protein had the Na^+/H^+ exchanger activity stimulated by phenylephrine. In the presence of the inhibitor EMD-87580, the mutant exogenous Na^+/H^+ exchanger activity was activated by phenylephrine in all cases, except in *mutant 3* and in the single NHE1 mutants S770A and S771A.

Further experiments were done to examine the effects of the mutations of amino acids in *region 3* on the phosphorylation levels of the NHE1 protein in response to hormonal stimulation. The results are shown in Fig. 6, *B* and *C*. In response to stimulation with phenylephrine, the phosphorylation levels of wild-type NHE1 protein were significantly increased. The inhibitor U-0126 prevented this increase. In contrast to the wild-type protein, the phosphorylation levels of *mutant 3* were not increased in response to phenylephrine. The absolute level of phosphorylation of the *mutant 3* protein was again reduced, relative to that of the wild-type protein.

To confirm that phenylephrine was acting through the ERK pathways in our isolated cardiomyocytes, we examined the phosphorylation levels of ERK1/2 and $p90^{rsk}$. The results are shown in Fig. 7. Figure 7, *A* and *B*, illustrates the results of immunoblotting with anti-phospho-ERK1/2 and $p90^{rsk}$ antibodies, and Fig. 7, *C* and *D*, summarizes the results. The stimulation of cells with 100 μM of phenylephrine for 6 min resulted in a large increase in the level of phospho-ERK1/2. The inhibitor U-0126 eliminated this increase. Phenylephrine did not cause a significant increase in the level of phospho-RSK1/2. The results shown are a summary of both ERK (type 1 and 2) and RSK 1 and RSK 2. In both cases, the same results were shown for the individual analysis of the proteins (not shown).

DISCUSSION

NHE1 plays a critical role in a number of cardiovascular disorders including ischemia reperfusion damage to the myocardium and heart hypertrophy. Lately, the literature suggests that the regulation of NHE1, rather than just the amount of protein, is critical in its role in the etiology of heart disease (22). NHE1 has a proton sensor that prevents activity at higher pH_i values (6); thus the absolute level of the protein may not be as critical as its regulation, which changes the activity profile across varying pH_i. We have shown earlier that NHE1-directed kinase activity and ERK1/2 are activated in the intact myocardium in response to ischemia-reperfusion (32) and reactive oxygen species (40), suggesting that the ERK1/2 pathway could be involved in myocardial pathologies. Therefore, we investigated the precise mechanism of the regulation of NHE1 in the myocardium. We used two methods of stimulation of the Na^+/H^+ exchanger in cardiomyocytes, SIA and α-adrenergic stimulation, and examined whether they both act through a common set of phosphorylation sites. SIA resulted in a significant elevation of activity of both endogenous and exogenous Na^+/H^+ exchanger in isolated cardiomyocytes. In addition, ERK was strongly activated by SIA and both NHE1 activity and ERK activation were blocked by the inhibition of this pathway with U-0126. It should be noted that SIA did not cause an increase in the degree of acidification but only an increase in the length of acidosis of cardiomyocytes, so that differences in the rate of recovery were not due to differences in initial pH_i.

Our experimental model used SIA to stimulate NHE1 activity. It is possible that SIA decreases intracellular Na^+ concentrations that could contribute to the stimulation of NHE1 activity. A prolonged depletion of extracellular sodium can deplete intracellular sodium (9), and extreme decreases in intracellular sodium stimulate NHE1 activity (56). However, under the conditions used in our assay with only 3 min of sodium depletion, the changes in intracellular sodium were likely not enough to cause this effect (9, 56). Also, the effects of the ERK inhibitor suggest that sustained acidosis activated NHE1, specifically dependent on the protein kinase activation of the protein as reported earlier (17, 31). In addition, Haworth et al. (17) demonstrated that sustained acidosis in the presence of normal external sodium still activated NHE1, and we replicated this result.

Our model system also used the adenoviral system to express exogenous NHE1. As it has a strong promoter, NHE1 was expressed to higher levels than the endogenous protein. An overexpression of the protein likely causes some mistargeting of NHE1. Nevertheless, the effects we saw were clearly due to the plasma membrane activity of the introduced protein, as we had inhibited endogenous NHE1 with EMD-87580, and our assay measures only the activity of plasma membrane protein. In addition, the functional effects that exogenous NHE1 demonstrated correlated with the mutations and effects on phosphorylation. We used the HA tag to immunoprecipitate exogenous NHE1 protein so that endogenous NHE1 was not a complicating factor in immunoprecipitation of NHE1.

Our choice of potential amino acids-mediating NHE1 activity was determined by our earlier results. We previously found that ERK plays a critical role in NHE1 regulation in the myocardium (32, 33). We showed that there were four potential regions of the ERK-dependent phosphorylation of NHE1 (21, 28), and within one of these, Ser^{770} and Ser^{771} played a critical role in the sustained acidosis-mediated activation of NHE1 in CHO cells (31). For this reason, we examined which amino acids within these four regions are involved in the regulation of NHE1 in the myocardium. We found that Ser^{770} and Ser^{771} play a critical role in the NHE1 regulation in the myocardium. This occurred for the regulation of NHE1 through both sustained acidosis or through adrenergic regulation of NHE1. In both cases, the mutation of these two amino acids abrogated the ability of these two stimuli to increase the activity of the protein. This did not occur with the mutation of any of the other amino acids in *regions 1*, *2*, or *4*. This clearly indicated that these amino acids were involved in these stimulatory pathways of NHE1 in the myocardium and that both

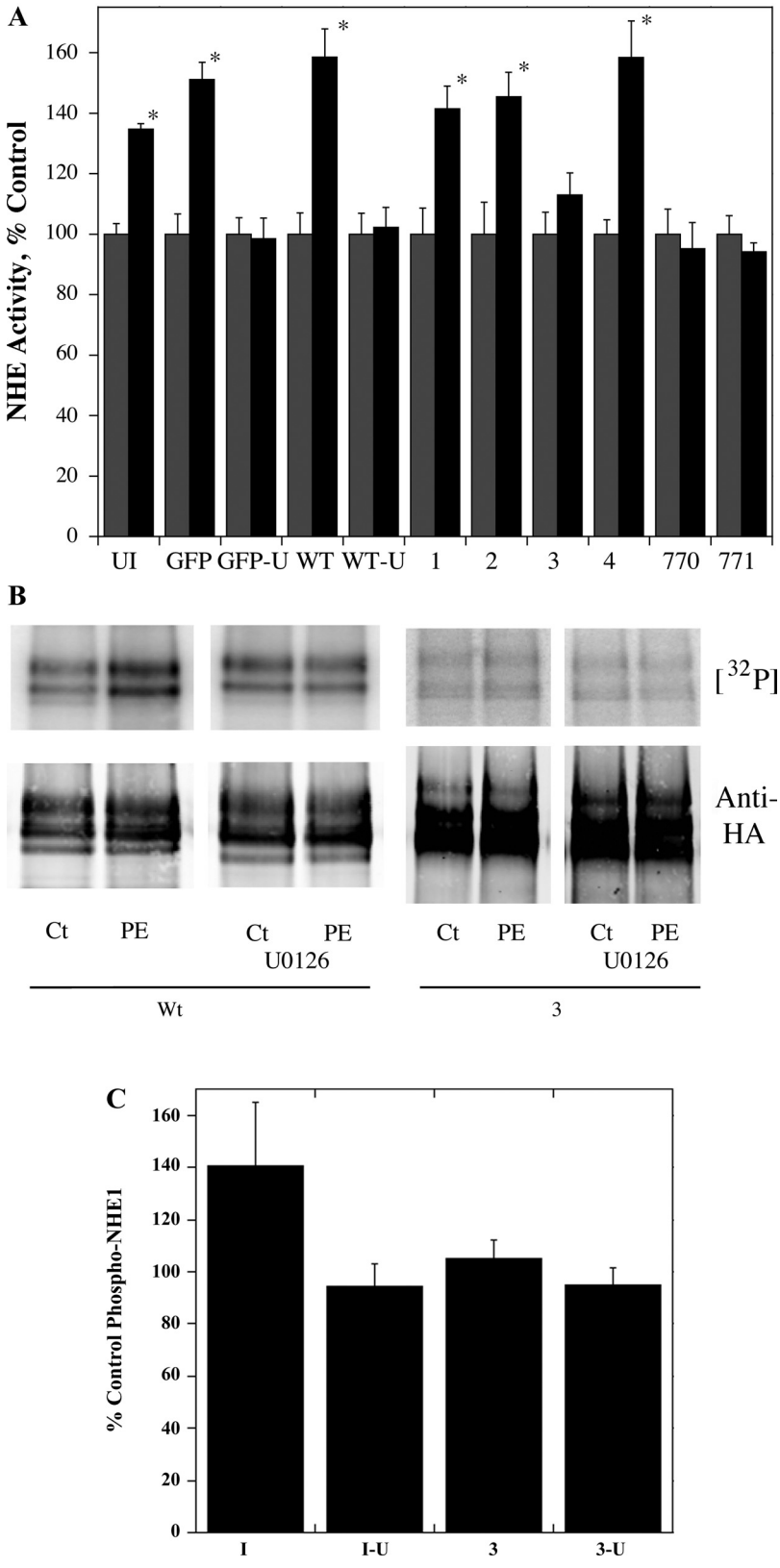


Fig. 6. Effect of 100 μM phenylephrine on activity and phosphorylation of WT and mutant NHE1 proteins. *A*: effect of phenylephrine on NHE1 activity of isolated cardiomyocytes. UI, uninfected cells; 770 and 771, cells infected with mutant NHE1 proteins containing the S770A and S771A mutations, respectively. For UI and GFP-infected cells, activity was in the absence of EMD-87580. For other cells, activity was measured in the presence of 10 μM EMD-87580. Grey bars, relative values of cells stimulated in the absence of phenylephrine; black bars, values after phenylephrine stimulation. Cells were subjected to a single pulse of ammonium chloride in the presence or absence of phenylephrine, and treated cells were compared with controls prepared simultaneously and measured on the same day. $*P < 0.001$, significantly different from the control value. *B*: examples of NHE1 protein immunoprecipitated from cardiomyocytes incubated with [^{32}P] ± phenylephrine treatment. *B*, *top*: phosphorylated protein immunoprecipitated with anti-HA tag to obtain exogenous NHE1 protein. *B*, *bottom*: anti-HA Western blot used to correct for the amount of immunoprecipitated HA-NHE1 protein. Ct, immunoprecipitated NHE1 protein from control cardiomyocytes; PE, immunoprecipitated NHE1 protein from cardiomyocytes treated with phenylephrine; 3, *mutant 3* NHE1 protein with mutations in Ser^{770} and Ser^{771}. Exposure times for *mutant 3* were increased relative to those of the WT. *C*: summary of effects of phenylephrine on phosphorylation levels of WT and *mutant 3* NHE1 proteins in isolated cardiomyocytes. Values are means ± SE of 4 experiments. $*P < 0.05$, significantly elevated over the level of control. Values are of NHE1 protein from cells stimulated in the presence of phenylephrine and were obtained using paired unstimulated controls.

these types of stimulation act through a common region in the carboxyl terminal of the NHE1 protein.

In addition to effects on NHE1 activity, the mutation of amino acids Ser^{770} and Ser^{771} also abrogated the increase in phosphorylation that was observed with the stimulation of NHE1 by sustained acidosis or phenylephrine. The mutation of this region and these two amino acids were the only mutations that had this effect. This indicated that these particular amino

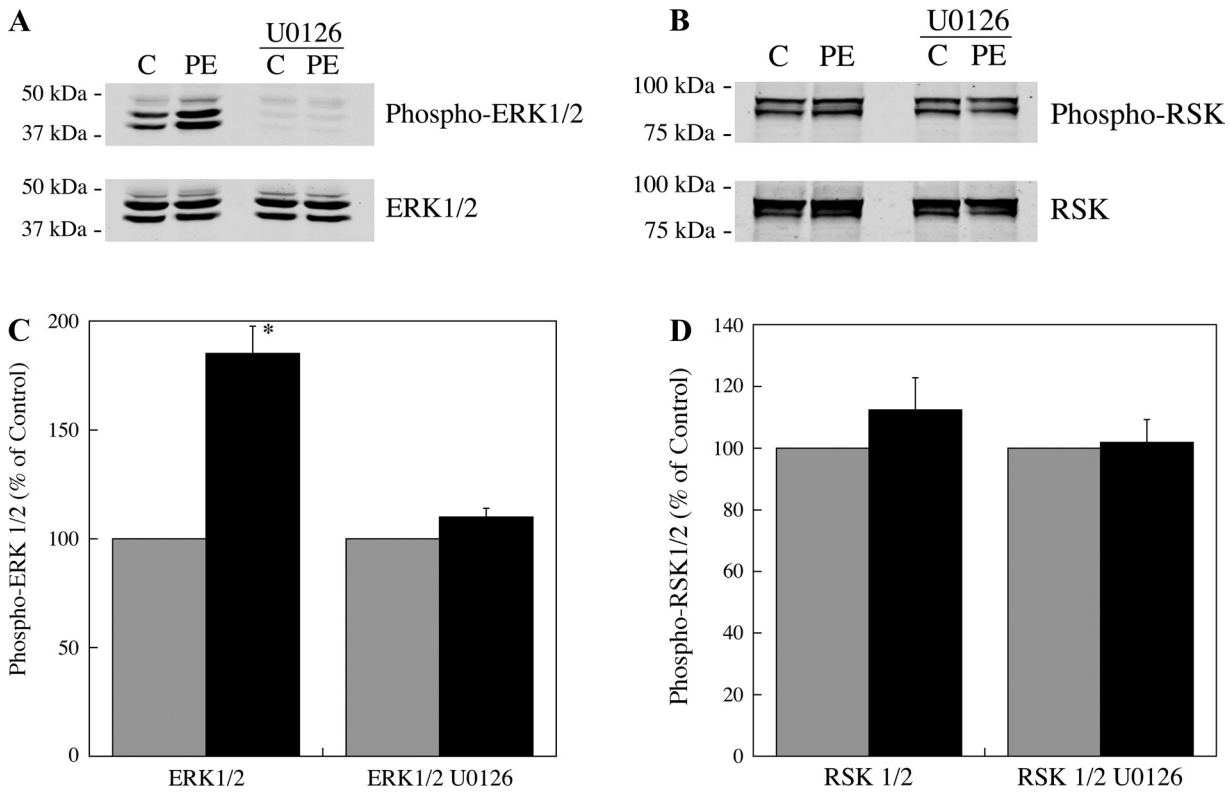


Fig. 7. Effects of phenylephrine on activation of ERK and $p90^{rsk}$ of isolated cardiomyocytes. Isolated cardiomyocytes were treated with 100 μM phenylephrine as described in pH_i measurement. *A*: Western blot with ERK (*bottom*) and anti-pERK (*top*) antibodies. Where indicated, cells were treated in the presence of 10 μM U-0126. PE, cells were treated with 100 μM phenylephrine for 6 min. *B*: Western blot with anti-Western blot with $p90^{rsk}$ (*bottom*) and anti-phospho-$p90^{rsk}$ (*top*) antibodies. Where indicated, cells were treated in the presence of 10 μM U-0126. *C*: summary of effects of phenylephrine on ERK1/2 phosphorylation levels. The level of pERK was corrected for the level of protein and was measured in the presence or absence of 10 μM U-0126. *D*: summary of effects of phenylephrine on $p90^{rsk}$ phosphorylation levels. The level of phospho-$p90^{rsk}$ was corrected for the level of protein and was measured in the presence or absence of 10 μM U-0126. Grey bars, relative values of cells stimulated in the absence of phenylephrine; black bars, values after phenylephrine stimulation. $*P < 0.01$, significantly different from control value.

acids were responsible for mediating the increase in NHE1 activity. It was notable that with mutation of these two amino acids, the absolute level of phosphorylation of NHE1 was always much less than that of wild-type NHE1 or NHE1 with a mutation at other sites. We found that the mutation of these two sites caused a decrease of over 90% in the level of phosphorylation of the NHE1 protein. This indicated that at least in isolated neonatal cardiomyocytes, within the time frame we used, these two sites account for a majority of the rapidly accessible pool of phosphorylatable amino acids on NHE1. This does not preclude the role of other amino acids that exchange phosphates over a slower period of time or with other types of physiological stimuli. But within this time frame and with these type of stimuli, Ser^{770} and Ser^{771} are the key regulatory amino acids of NHE1 and the major amino acids that are responsive to this stimulation in neonatal isolated cardiomyocytes. Other amino acids have been suggested to be important in the regulation of NHE1 in other cell types, such as Ser^{726} and Ser^{729} (14). However, we did not find these amino acids critical in our experiments, and our earlier studies defining ERK-dependent phosphorylation of the carboxyl terminal region did not indicate that any other amino acids were phosphorylated by this pathway (28, 45). This indicates that NHE1 can be regulated by different amino acids in different systems. In the latter study, the stimulation of NHE1 was mediated by apoptosis, which acted through a different stimulatory pathway (14, 24). Thus NHE1 regulation varies significantly depending on the cell type and possibly on the type of stimulus used.

It was interesting to note that the mutation of either Ser^{770} or Ser^{771} decreased the phosphorylation and activation of NHE1 completely. It is possible that only one of these two sites is phosphorylated and that the mutation of the other residue makes the other residue nonphosphorylatable by the alteration of the consensus sequence. This could account for the lack of increased phosphorylation that occurred with the individual mutations of these amino acids. Ser^{771} precedes Pro^{772}, and this forms a typical consensus sequence of ERK (42). It may be that the mutation of Ser^{770} disrupts the consensus sequence and thus disrupts the phosphorylation of Ser^{771}. It is possible that Ser^{771} is the critical phosphorylatable residue; however, further experiments are necessary to confirm this possibility.

A number of reports (7, 27, 47) have suggested that Ser^{703} and $p90^{rsk}$ play important roles in NHE1 regulation. In our experiments we found a rapid and large increase in the phosphorylation of ERK by both SIA and α-adrenergic stimulation. In contrast, we found only minor increases in the phosphorylation of $p90^{rsk}$ by these stimuli. Earlier we found that $p90^{rsk}$, which is downstream of ERK, has a slower time course of phosphorylation than ERK (33). We observed a much more rapid phosphorylation of ERK than $p90^{rsk}$. ERK was fully phosphorylated within 30–60 s of serum addition, whereas $p90^{rsk}$ required several minutes for activation (33). We found similar results in intact myocardial tissues

subjected to ischemia and reperfusion. Ischemia-reperfusion caused a much stronger activation of NHE1-phosphorylating protein kinases of 44–42 kDa in size compared with 90 kDa protein kinases (33). Similar results were found in the present study, where ERK1/2 were rapidly activated by SIA, and we found a minimal activation of $p90^{rsk}$. These results suggest to us that ERK may be more responsible for a shorter, rapidly responsive regulation of NHE1 at amino acids Ser^{770} and Ser^{771}, whereas $p90^{rsk}$ might be responsible for a longer-term regulation at Ser^{703}. Supporting this hypothesis is our observation that with mutation of Ser^{770} and Ser^{771}, there was a huge decrease in the rapidly exchangeable phosphate on the NHE1 protein. This suggests that a majority of the rapidly exchanging phosphate is incorporated in one or both of these amino acids. In addition, earlier we did not find that ERK phosphorylates Ser^{703} (28). We do not know the status of Ser^{703} in our study, whether it was phosphorylated or unphosphorylated. It may be that it was constitutively phosphorylated and thus did not contribute to the rapid responses seen under the conditions of our experiments. Pharmacological interventions (7) and treatment with a dominant-negative $p90^{rsk}$ (29) have confirmed that Ser^{703} is important in the NHE1 regulation in the myocardium. The difference between these studies and our own may be that a longer-term stimulation of NHE1 was used. In addition, adult tissues were principally used in these studies. With the time periods required for adenoviral expression of NHE1 and allowing for a recovery of cardiomyocytes after enzymatic dissociation, it was necessary to use neonatal cardiomyocytes in the present study, which can be maintained in culture more easily and for longer periods of time. Adult cardiomyocytes tend to dedifferentiate when maintained in culture more rapidly than neonatal cardiomyocytes (10, 20, 52). In addition, the regulation of NHE1 in the young myocardium is of significant interest physiologically. The ERK pathway is active in the neonatal myocardium (46) and has recently been suggested to play a key role in the gestational development of the myocardium (36). We (32, 40) have shown the ERK regulation of NHE1 in the neonate is critically involved in Ca^{2+} overload in the myocardium mediated through NHE1. We have also earlier shown that NHE1 expression is increased during myocardial development in utero (39) and that the newborn myocardium has elevated levels of NHE1 activity and expression (18). The expression of ERK is much higher in the neonatal rat myocardium compared with the adult (26), which may account for its greater importance in the present study. The newborn myocardium has been reported to be more vulnerable than the adult to ischemic injury (4, 38, 54), though this observation is controversial, varying between different groups observation and possibly depending on which species and which parameter are measured (34). Nevertheless, ischemia-reperfusion damage is an important clinical problem in children undergoing surgery and transplantation (53). Whereas it is known that the neonatal myocardium is not as adept as the adult's in buffering intracellular acidosis (55), it is not known whether the increased NHE1 activity and ERK activity of the newborn affect the regulation of NHE1 and are responsible for any of the sensitivity of the newborn to ischemic injury.

In summary, our study examined the mechanism of sustained acidosis and phenylephrine-mediated stimulation of NHE1 in isolated neonatal cardiomyocytes. We demonstrated for the first time, that in the myocardium, both mechanisms of activation of NHE1 rapidly affect NHE1 through a common mechanism, the phosphorylation of amino acids Ser^{770} and Ser^{771}. A number of other hormones activate NHE1 in the myocardium, including endothelin (16), angiotensin II (15), and thrombin (57). It is not known whether these agonists regulate NHE1 through the same pathway in the adult or the neonatal myocardium. Future studies are needed to explore whether these amino acids Ser^{770} and Ser^{771} are a common mechanism of activation of NHE1 in the myocardium.

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