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Sustained intracellular acidosis activates the myocardial Na^+/H^+ exchanger independent of amino acid Ser^{703} and $p90^{rsk}$

Pratap Karki, Ersilia Coccaro, Larry Fliegel *

Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

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ABSTRACT

The mammalian Na^+/H^+ exchanger isoform 1 (NHE1) is a ubiquitously expressed pH-regulatory membrane protein that functions in the myocardium and other tissues. It is an important mediator of the myocardial damage that occurs after ischemia–reperfusion injury and is implicated in heart hypertrophy. Regulation of NHE1 has been proposed as a therapeutic target for cardioprotection. We therefore examined mechanisms of control of NHE1 in the myocardium. Several different amino acids have been implicated as a being critical to NHE1 regulation in a number of tissues including Ser^{703}, Ser^{770}, and Ser^{771}. In the myocardium, NHE1 is activated in response to a variety of stimuli including activation by an ERK-dependent sustained intracellular acidosis. In this study, we determined whether Ser^{703} and $p90^{rsk}$ activity are critical in activation of NHE1 by sustained intracellular acidosis. *In vitro* phosphorylation of NHE1 C-terminal fusion proteins determined that ERK-dependent phosphorylation of the cytoplasmic region was not dependent on Ser^{703}; however, phosphorylation by $p90^{rsk}$ required Ser^{703}. A Ser703Ala mutation decreased basal NHE1 activity in CHO cells but not in cardiomyocytes. NHE1 with a Ser703Ala mutation was activated in response to sustained intracellular acidosis in CHO cells. In addition, sustained intracellular acidosis also activated the Ser703Ala mutant protein in isolated cardiomyocytes and phosphorylation levels were also increased by acidosis. The presence of a dominant-negative $p90^{rsk}$ kinase also did not prevent activation and phosphorylation of NHE1 by sustained intracellular acidosis in isolated cardiomyocytes. We conclude that Ser^{703} and $p90^{rsk}$ are not required for activation by sustained intracellular acidosis and that $p90^{rsk}$ phosphorylation of Ser^{703} is independent of this type of activation.

1. Introduction

The Na^+/H^+ exchanger isoform-1 (NHE1) is a plasma membrane glycoprotein that protects cells from intracellular acidification by removing one intracellular proton in exchange for one extracellular sodium ion [1]. There are 10 isoforms of Na^+/H^+ exchanger (NHE1–NHE10), and the first isoform discovered (NHE1) is the only type that is ubiquitously distributed [2,3]. NHE1 also plays important roles in cell volume regulation, cell differentiation, and proliferation and in growth of some tumor cells [1,4,5]. The NHE1 isoform is critical in the myocardium in mediating the damage that occurs with myocardial ischemia/reperfusion injury [6,7] and is an important mediator of heart hypertrophy [8]. Chronic inhibition of NHE1 attenuates development of cardiac hypertrophy and can cause regression of cardiac hypertrophy [9].

NHE1 levels and activity are elevated in several models of heart disease [10,11]. NHE1 activity is also elevated in patients with end-stage heart failure [12]. We demonstrated that protein kinases that regulate NHE1 activity are activated in the myocardium by ischemia and reperfusion of intact hearts or isolated cardiomyocytes [13]. In addition, NHE1 is activated by sustained intracellular acidosis (SIA) [14,15]. To delineate which amino acids are critical for activation of NHE1, we earlier made mutations of NHE1 proteins and found that amino acids Ser^{770} and Ser^{771} are required for activation of NHE1 by sustained acidosis in CHO cells [15] and in isolated cardiomyocytes [16]. However, a number of studies have implicated Ser^{703} as being critical to NHE1 function. Takahashi et al. [17] demonstrated the importance of Ser^{703} in $p90^{rsk}$-dependent activation of NHE1 by growth factors in 293 cells. Furthermore, phosphorylated Ser^{703} was shown to be important in binding of the adaptor protein 14-3-3 to NHE1, and mutation Ser703Ala abolished this binding [18,19].

While extensive preclinical work has indicated that inhibition of the Na^+/H^+ exchanger affords significant protection to the myocardium subjected to ischemia and reperfusion, this has been contrasted by clinical studies that have given largely poor results when using NHE1 inhibitors. It has recently been suggested that the NHE kinase RSK might be an appropriate therapeutic target for indirectly

Abbreviations: CHO, Chinese hamster ovary; ERK, extracellular signal-regulated kinase; NHE1, Na^+/H^+ exchanger type 1 isoform; GSK3β, glycogen synthase kinase-3beta; HA, hemagglutinin; RSK, ribosomal S6 kinase; SIA, sustained intracellular acidosis

* Corresponding author. Tel.: +1 780 492 1848; fax: +1 780 492 0886.
E-mail address: lfliegel@ualberta.ca (L. Fliegel).

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suppressing cardiac NHE activity and that this pathway should be further investigated [20]. Therefore, in this study, we examined the importance of Ser^{703} and the $p90^{rsk}$ pathway in the activation of NHE1 in the myocardium. Several pathways of activation of NHE1 by different stimuli such as hormones and apoptosis stimulate NHE1. These stimuli have been suggested to act through phosphorylation of different amino acids to activate NHE1 in different cell types [17,21,22]. Stimulation of NHE1 activity and NHE1-directed protein kinases by ischemia and chronic acidosis may be important in NHE1-mediated damage in the cardiovascular system [13,23]. Chronic acidosis is an important clinical problem in many diseases and notably occurs quickly and persistently after coronary artery occlusion in the myocardium [24]. We therefore studied activation of NHE1 by sustained intracellular acidosis (SIA) in isolated cardiomyocytes [14,15]. Our results suggest that Ser^{703} and $p90^{rsk}$ are not essential for the activation of NHE1 by SIA in isolated cardiac myocytes and CHO cells. These suggest that acute acidosis could still activate the NHE1 protein in the clinical setting, independent of blockade of $p90^{rsk}$ activity.

2. Materials and methods

2.1. Materials

Routine chemicals were of analytical grade and were purchased from BDH (Toronto, ON), Fisher Scientific (Ottawa, ON), or Sigma (St. Louis, MO, USA). Other chemicals were collagenase type 2 (Worthington Biochemical Corporation (Lakewood, NJ)) and BCECF-AM (2′,7-bis(2-carboxyethyl)-5(6) carboxyfluorescein-AM) from Molecular Probes (Eugene, OR, USA). Also, platinum R Taq Polymerase was from Invitrogen (Burlington, Ontario, Canada), and EMD87580 was a generous gift of Dr. N. Beier of Merck KGaA, Frankfurter, Germany. PWO DNA polymerase was obtained from Roche Applied Science, and Lipofectamine™ 2000 reagent was from Invitrogen Life Technologies (Carlsbad, CA, USA). Mouse anti-NHE1 antibody was from BD Biosciences (San Jose, CA, USA). The monoclonal antibody for alpha-myosin heavy chain (MF20) was from the Developmental Studies Hybridoma Bank of the University of Iowa, (Iowa City, IA). Anti-phospho glycogen synthase kinase-3beta (GSK3β) and anti-$p90^{rsk}$ antibody were from Cell Signaling Technology (Beverly, MA, USA). Anti-hemagglutinin (HA) antibody Y-11 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein-A sepharose beads were from Sigma (St. Louis, MO, USA), and [^{32}P]orthophosphate was from Perkin Elmer Life and Analytical Sciences (Boston, MA, USA).

2.2. Exchanger fusion proteins

The carboxyl-terminal 178 amino acids of the rabbit cardiac Na^+/H^+ exchanger (amino acids 639–816) were expressed as described previously [25] as a fusion protein with GST (Gst178) using the plasmid pGEX-3X. The *Escherichia coli* TOPP 2 strain was induced with 1 mM isopropylthio-β-D-galactoside. GST-Na^+/H^+ exchanger fusion protein was purified via glutathione Sepharose 4B affinity chromatography as described earlier [13]. A series of related fusion proteins of GST were also made and purified using the same procedures. Gst703 was the same as Gst178 but with amino acid Ser^{703} mutated to alanine. Δ662–794 had amino acids 662 to 794 deleted from Gst178. Gst702 was the same as Gst178 but terminated at amino acid 702. For some experiments, we expressed and purified the C-terminal 182 amino acids of NHE1 as a fusion protein with a histidine tag (as opposed to a GST tag). This protein was produced and purified as described earlier [26].

2.3. Preparation of cell extracts of isolated myocytes and in-gel kinase assays

Tissue extracts were made from isolated perfused hearts or isolated myocytes essentially as described earlier [13,25]. Heart tissue was homogenized at a high setting with a Polytron homogenizer for 30 s in 2.5% (vol./wt.) of extraction buffer containing 50 mM tetra-sodium pyrophosphate, 50 mM sodium fluoride, 50 mM sodium chloride, 5 mM ethylene-diaminetetra-acetic acid disodium salt (EDTA), 5 mM ethylene glycol-bis (β-aminoethyl ether) *N,N,N,N′*-tetraacetic acid (EGTA), 0.1 mM sodium orthovanadate, 0.1% Triton X-100, 10 mM HEPES pH 7.4, and a cocktail of protease inhibitors. The homogenate was centrifuged at 6000 $g \times$ 60 min at 4 °C. To identify protein kinases that phosphorylated the Na^+/H^+ exchanger fusion proteins, cell extracts (80 μg protein) from isolated myocytes or isolated perfused hearts were prepared and run on 10% SDS–PAGE in a gel containing 1 mg/mL of substrate. In-gel kinase assays were performed as previously described [25]. The gels were dried for autoradiography and visualization of phosphorylation. Protein concentrations were measured using the Bio-Rad DC protein assay.

2.4. In vitro phosphorylation of the Na^+/H^+ exchanger fusion proteins

The standard reaction conditions for phosphorylation of heart extract fractions contained 3.0–8.0 μg of substrate, 8 μl of heart extract, 12.5 mM 3-(4-morpholino) propane sulfonic acid (MOPS) pH 7.2, 0.5 mM EGTA, 2 mM DTT and 8.5 mM magnesium chloride, 6 μM okadaic acid, 0.24 mM sodium fluoride, 500 μM ATP, and 1 μl of 10 μCi/μl of [γ-^{32}P]ATP (3000 Ci/mmol) in a final volume of 24 μl (modified after Moor and Fliegel [25]). Samples were incubated at 30 °C for 90 min, and the reaction was terminated by the addition of SDS loading buffer. Samples were run on a 12% SDS gel, dried, and exposed for autoradiography. The appropriate bands were identified by autoradiography. For some experiments, we used immunoprecipitated ERK or $p90^{rsk}$ kinase to phosphorylate the Na^+/H^+ exchanger fusion proteins. This was prepared with commercial antibody as described earlier [13]. For other experiments, we used commercially available active ERK2 to phosphorylate Na^+/H^+ exchanger fusion proteins (Cell Signaling Technologies, Danvers, MA, USA). The reaction contained 0.38 ng of ERK2 in a buffer as described above.

2.5. Western blotting and immunoprecipitation

SDS–PAGE and immunoblotting were performed essentially as described earlier [27]. 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, Quebec, Canada) or anti-NHE1 monoclonal antibody (BD Biosciences). Second antibody was peroxidase-conjugated goat anti-mouse antibody (Bio/Can, Mississauga, Ontario, Canada) and visualized using a chemiluminescence detection system. To visualize and quantify immunoreactive proteins, we used a Li-COR fluorescence labeling and detection systems (LI-COR Biosciences, Lincoln, NE, USA).

2.6. Site-specific mutagenesis

Site-specific mutagenesis of Ser^{703} to Ala was as described earlier [28]. The primers used were 5′-GGCCCGCATCGGC**g**C**g**GAtCCACTGGCC-TATGAG-3′ (forward) and 5′- CTCATAGGCCAGTGGaTCcGcGCC-GATGCGGGCC-3′ (reverse). The mutated nucleotides are in lower case letters and bold. The new restriction site *Bam*HI was created by silent mutation. The mutated hemagglutinin (HA)-tagged NHE1 cDNA was transferred to pAdTrack-CMV, and the virus was produced using the pAdEasy system as described earlier [16]. DNA sequencing confirmed the mutation and fidelity of the cDNA after mutation.

2.7. Cell culture and stable transfection

Stable cell lines expressing the wild type HA-tagged NHE1 protein and the mutant NHE1 with a Ser703Ala mutation were made in AP-1

cells as described earlier using the plasmid pYN4$^+$ [29]. AP-1 cells are a Chinese hamster ovary cell line that lacks an endogenous Na^+/H^+ exchanger. Briefly, the cells were grown in a humidified atmosphere of 5% CO_2 and 95% air in α-MEM supplemented with 10% (vol./vol.) bovine growth serum, 25 mM HEPES, penicillin (100 U/mL), and streptomycin (100 μg/mL), pH 7.4 at 37 °C. Transfection was with LIPOFECTAMINE™ 2000 reagent, and selection of stable cell lines was carried out with 800 μg/mL G418 as described previously [29]. After initial selection, single clones of transfected cells were obtained, and stable cell lines were maintained in 400 μg/mL G418 and were regularly reestablished from frozen stocks at passage numbers between 5 and 15 where necessary. Cell lysates from AP-1 cells were made as described earlier [29].

2.8. Preparation and treatment of isolated cardiomyocytes

Five- to six-day-old neonatal Sprague-Dawley rat heart ventricles were used to prepare primary cultures of cardiomyocytes as described earlier [25]. Isolated primary cardiomyocytes were plated onto Corning culture dishes or flasks when harvesting cell extracts or onto glass coverslips for intracellular pH measurements. Myocytes were maintained for 48 hours before adenoviral infection in a medium containing Dulbecco's modified Eagle's medium 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.

When indicated, isolated cardiomyocytes were infected with adenovirus containing HA-tagged NHE1 gene. The basis of the adenoviral constructs (pADTRack-CMV-NHE1) was described earlier [16,30], and they contain full-length cDNA of a hemagglutinin (HA)-tagged NHE1 isoform of the Na^+/H^+ exchanger. The NHE1 cDNA expression contains a double-mutation Leu163Phe/Gly174Ser that increases the resistance to inhibition by EMD87580 and other NHE1 inhibitors [16,30]. This allows detection of its activity while inhibiting the endogenous NHE1 protein activity with EMD87580. Infection by adenovirus was routinely monitored by GFP expression, and more than 99% of cells were infected. Control cells were infected with an adenovirus that expressed GFP alone. Cells were routinely infected at a multiplicity of infection of 20 (PFU/cell). Control or experimental adenoviruses were maintained on cells for a period of 24 hours before characterization of activity. The S770/771A adenovirus contains the inhibitor-resistant NHE1 with the mutations of Ser^{770} and Ser^{771} to Ala as described earlier [16,30].

2.9. Intracellular pH measurement

Intracellular pH measurement in AP-1 cells was as described earlier using monolayers of coverslips of cells loaded with BCECF-AM [15]. NHE1 activity was examined in isolated cardiomyocytes that were mock-infected and infected with pAd-NHE1 or pAd-GFP (which is a control adenovirus expressing only GFP) as described earlier [16]. Cells were infected for approximately 24 hours before intracellular pH measurement. Isolated cardiomyocytes were grown on coverslips, and pH changes were measured using a PTI Deltascan spectrofluorometer. Coverslips were held in a holder that was precisely machined to fit cuvettes and retain coverslips. Solutions were changed rapidly by removing coverslips in the holder from one solution and placing them in a new solution. All solutions were at 37 °C and were stirred constantly during pH measurement. The initial rate of recovery was measured during the first 20 s after return of NaCl at 37 °C. A calibration curve was done with nigericin at the end of every experiment to calibrate intracellular pH to fluorescence as described earlier [28]. To examine NHE1 activity in isolated cardiomyocytes in response to SIA, we used an assay we have recently described [15,16]. Briefly, cells were treated with ammonium chloride two times, and allowed to recover in NaCl containing medium after each treatment. After the first treatment with ammonium chloride, cells were rinsed with Na^+-free medium for 10–20 s and then the decline in pH_i was allowed to stabilize. After the pH_i was no longer declining and stabilized, NaCl was added to allow for intracellular pH recovery. The second pulse was done similarly except cells were maintained in Na^+-free medium for 3 min before reintroduction of 135 mM NaCl to cause SIA. Where appropriate, the value obtained for the second pulse in the presence of SIA was compared to that obtained for a second pulse in the absence of SIA. This activity, in the absence of SIA, was referred to as basal NHE1 activity. For experiments with cells infected with adenovirus, in some cases, assays were done in the presence of 10 μM EMD87580 where indicated. This inhibited >95% of endogenous NHE1 but did not inhibit the exogenously expressed NHE1 because it contained two mutations (Leu163Phe/Gly174Ser) that made it resistant to inhibition [16]. All results are shown as the mean ± SE and statistical significance was determined using the Wilcoxon–Mann–Whitney rank-sum test.

2.10. Immunoprecipitation of phospho-NHE1 protein

Cell lysates from isolated cardiomyocytes incubated with [^{32}P] were used for the immunoprecipitation of exogenously expressed NHE1. The procedure was as essentially described earlier and exogenous NHE1 was immunoprecipitated using the HA tag [13]. RIPA buffer contained 50 mM Tris (pH 8.0), 150 mM NaCl, 80 mM NaF, 5 mM EDTA, 1 mM EGTA, 25 mM Na-pyrophosphate, 1 mM Na-orthovandate, and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 40 μM ALLN) plus detergents at concentrations of 1% NP-40, 0.1% SDS, and 0.05% deoxycholate. After incubating in [^{32}P]-containing medium, cells were washed in phosphate-free buffer and treated with ammonium chloride to induce SIA as described above. Ice-cold RIPA buffer (3 mL without detergents) was added, and cells were placed on dry ice to freeze them. After thawing on wet ice, cells were scraped into centrifuge tubes and sonicated for 10 s. Cells were centrifuged at 35,000 rpm × 1 hour and 1 mL of RIPA buffer with detergents was added to the pellet to solubilize NHE1. 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 as described earlier [16]. After SDS–PAGE, 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 routinely 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, USA). Results are the mean ± SE of at least three experiments.

3. Results

3.1. Phosphorylation of NHE1 by heart extracts

We have previously demonstrated that ERK is involved in the regulation of NHE1 in the myocardium and that ERK can directly phosphorylate a fusion protein containing the C-terminal region of NHE1 [13,25]. Initial experiments were to confirm this observation and to delineate which amino acids are critical for regulation of NHE1 via phosphorylation by ERK. For this purpose, we made a number of fusion proteins of the C-terminus of the protein (Fig. 1). In-gel kinase assays [25] were initially done with heart cell extracts to make a gross assessment of the regions of the NHE1 C-terminus that heart cell extracts can phosphorylate. The results are shown in Fig. 2. In-gel kinase assays of heart extracts with the C-terminal 178 amino acids of

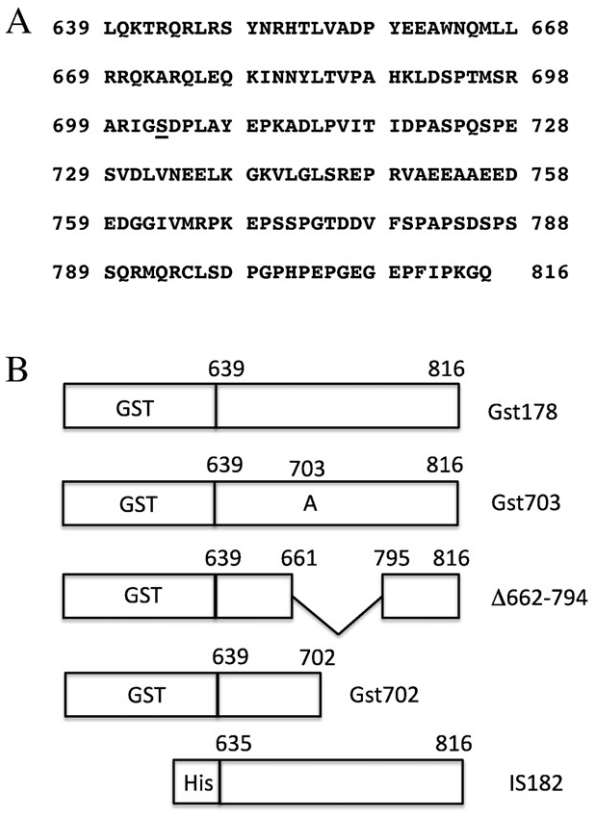


Fig. 1. (A) Amino acid sequence of C-terminal amino acids 639–816 of rabbit NHE1. Ser^{703} is underlined. (B) Mutant NHE1 fusion proteins used in this study. GST, glutathione *S*-transferase; His, histidine tag. Numbers indicate amino acid numbers of the NHE1 protein.

NHE1 revealed that several kinases were active in phosphorylating the NHE1 protein (lanes 1 and 2). A prominent kinase was of molecular weight of approximately 44 kDa, the same size as ERK. A duplicate experiment also used heart extracts to phosphorylate the NHE1 C-terminus (lanes 3 and 4) except that it used a fusion protein (Δ662–794) that had amino acids 662–794 deleted. While some kinases were still active toward this fusion protein, there was a very noticeable absence of kinase activity in the 44-kDa range. In-gel kinase assays with either the entire C-terminus or with Δ662–794 did not show strong phosphorylation in the 90-kDa region.

3.2. Localization of NHE1 phosphorylation sites

To localize the region of the Na^+/H^+ exchanger that was phosphorylated by ERK we used *in vitro* phosphorylation of Na^+/H^+ exchanger proteins expressed and purified from *E. coli*. In initial experiments, we used a whole heart extract and examined the ability of the extract to phosphorylate either a GST fusion of the C-terminal 178 amino acids of the Na^+/H^+ exchanger or a histidine-tagged fusion of the C-terminal 182 amino acids of the Na^+/H^+ exchanger. Lane 1 (Fig. 3A) is a control showing that, under the conditions used, without the presence of a substrate, little autophosphorylation of the cell extract occurred. Lanes 2 and 3 show that cell extracts phosphorylate the Na^+/H^+ exchanger–GST and Na^+/H^+ exchanger–His-tagged (His182) fusion proteins that contain the C-terminal of the Na^+/H^+ exchanger.

We then used immunoprecipitated ERK1/2 from heart cell extracts to examine if this kinase could directly phosphorylate the Na^+/H^+ exchanger fusion proteins. Fig. 3B shows the results. Lane 1 is a control without any substrate added to the *in vitro* phosphorylation reaction. There was no evidence of autophosphorylation of the immunoprecipitate. Lane 2 is a second control whereby we used the whole extract of

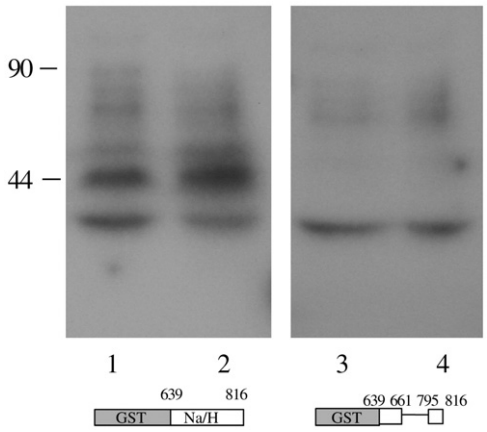


Fig. 2. Na^+/H^+ exchanger-directed protein kinase activity of heart extracts using Gst178 or Δ662–794 as a substrate. The schematic diagram below each gel indicates the substrate used for the in-gel kinase assay. Heart extracts were used for in-gel kinase assay as described in Materials and methods. Lanes 1 and 2 are duplicate myocyte extracts run in a gel using the Na^+/H^+ exchanger fusion protein as a substrate (1 mg/mL) (Gst178). It contains the terminal 178 amino acids of the rabbit NHE1 protein. Lanes 3 and 4 were the same cell extracts from hearts that used for in-gel kinase assays containing the same region of the Na^+/H^+ exchanger except that amino acids 662 to 794 were deleted (Δ662–794).

intact hearts to phosphorylate the His182 fusion protein. Lane 3 shows that immunoprecipitated ERK1/2 could phosphorylate the GST-tagged fusion protein of the Na^+/H^+ exchanger. Lane 4 shows that the histidine-tagged fusion protein (not containing GST) is also phosphorylated by immunoprecipitated ERK1/2.

To determine whether Ser^{703} could be phosphorylated by ERK or $p90^{rsk}$, we made a series of fusion proteins that contained truncations, mutation, or deletions of the Na^+/H^+ exchanger C-terminus. We then used these proteins as a substrate of commercially available ERK2 or

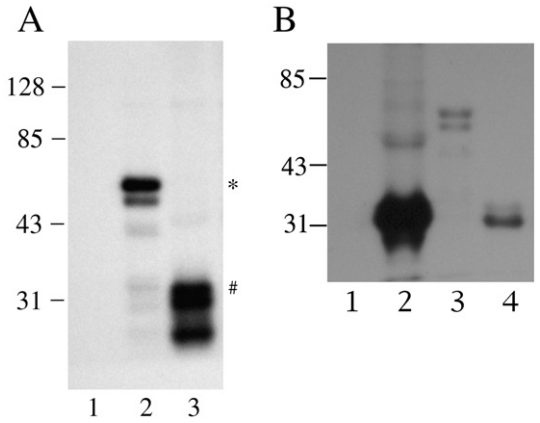


Fig. 3. Phosphorylation of the Na^+/H^+ exchanger by heart cell extracts and immunoprecipitated ERK1/2. (A) Cell extracts from intact hearts were incubated with Na^+/H^+ exchanger fusion proteins in a buffer containing [γ^{32}P]ATP as described in Materials and methods. Lane 1 is a control reaction with no Na^+/H^+ exchanger fusion protein. Lane 2 is a reaction containing cell extracts and GST–Na^+/H^+ exchanger fusion protein containing the C-terminal 178 amino acids of the protein. Lane 3 is a reaction with cell extracts and His-tagged Na^+/H^+ exchanger containing the C-terminal 182 amino acids of the Na^+/H^+ exchanger. * and # indicate the position of the full-length GST and histidine-tagged Na^+/H^+ exchanger fusion proteins respectively. (B) Phosphorylation of the Na^+/H^+ exchanger by immunoprecipitated ERK1/2. ERK1/2 was immunoprecipitated from intact hearts and used to phosphorylate Na^+/H^+ exchanger fusion proteins as described in the Materials and methods. Lane 1 is a control reaction with no Na^+/H^+ exchanger fusion protein. Lane 2 is a reaction containing cell extracts and as in A and His182 (His-tagged Na^+/H^+ exchanger with the C-terminal 182 amino acids of the Na^+/H^+ exchanger). Lane 3 is a reaction with ERK1/2 immunoprecipitated from heart cell extracts phosphorylating GST–Na^+/H^+ exchanger fusion protein containing the C-terminal 178 amino acids of the protein (Gst178). Lane 4 is a similar reaction to lane 3, except containing His182 and immunoprecipitated ERK1/2.

p90^{rsk} immunoprecipitated from rat heart extracts. ERK2-purified kinase was used as a source rather than immunoprecipitated ERK from the myocardium because it was possible to obtain larger amounts of the kinase that gave consistent results. The effect of these mutations and deletions is shown in Fig. 4. ERK kinase (Fig. 4A) phosphorylated Gst178 and Gst703 (which had amino acid 703 mutated to alanine). In contrast, there was no phosphorylation of Δ662–794 or Gst702. Another similar experiment showed that the His182 fusion protein (C-terminal 182 of NHE1 with histidine tag) was also phosphorylated by ERK2, while additionally, another histidine-tagged fusion protein with amino acids 639 to 763 deleted was phosphorylated to an equal or greater extent (not shown). Fig. 4B shows that p90^{rsk} phosphorylated the Gst178 (lane 1) protein, but this phosphorylation was almost eliminated (10%–15%) by mutation of Ser^{703} to alanine (lane 2). In addition, Δ662–794 and Gst702 were not phosphorylated by p90^{rsk}. These experiments showed that amino acid Ser^{703} was not critical for the phosphorylation of the NHE1 C-terminus by ERK *in vitro*; however, *in vitro* phosphorylation by p90^{rsk} was virtually eliminated by mutation or deletion of Ser^{703}.

3.3. Effect of Ser^{703} mutation on NHE1 activity in AP-1 cells

To characterize the role of Ser^{703} in the ability of NHE1 to be activated by SIA, we mutated this residue to Ala and examined the effects on activation of NHE1 by SIA in AP-1 cells. AP-1 cells are a CHO cell line with the endogenous NHE1 protein absent [29]. Fig. 5A confirms that we stably expressed the NHE1 protein in these cells. Western blotting demonstrates that both the fully glycosylated and the partially or deglycosylated form of the protein were present, similar to earlier results [15,29]. Partially or deglycosylated NHE1 represents mostly intracellular protein [15,29].

To characterize the activity of the NHE1 protein in response to SIA, we used a two-pulse assay [15]. Cells were given two ammonium chloride pulses. After the first ammonium chloride treatment, cells are allowed to recover immediately. After the second ammonium chloride treatment, cells are maintained in sodium free medium for 3 min to create a sustained acidosis. This stimulates NHE1 activity. The second pulse of SIA can be maintained by either sodium-free medium or alternatively, sodium-containing medium in the presence of NHE1 inhibitor [16]. In a separate two-pulse assay, a second pulse without a sustained acidosis is used as a control. Examples of the effects of SIA on control and Ser703Ala mutant NHE1 protein are shown in Figs. 5B–E. For clarity, only the second pulses are shown. With two ammonium chloride pulses without SIA, the second recovery was equivalent to the first (Fig. 5B). With SIA, the activity of NHE1 after the second pulse was increased (Fig. 5C). Figs. 5D and E show that the same phenomenon occurred with the Ser703Ala mutant NHE1 protein. SIA stimulated the second recovery from SIA (Fig. 5E). A summary of these results is shown in Fig. 5F. For cells with wild type NHE1 treated without SIA, the activity after the second pulse was not changed significantly. When treated with SIA, the wild type NHE1 activity after the second pulse was greatly and significantly increased. This is consistent with our previous results [15]. Interestingly, similar results were found with the Ser703Ala mutant, and the activity with SIA was also significantly elevated (Fig. 5F). The absolute level of activity of the wild type NHE1 protein was 1.08 ± 0.018 ΔpH/min. The level of activity of the Ser703Ala mutant was reduced about 25% in comparison to the wild type to a value of 0.85 ± 0.036 ΔpH/min. Overall, these results show that the Ser703Ala mutant NHE1 protein, while slightly reduced in activity, was still significantly activated by SIA in AP1 cells in the same manner as controls.

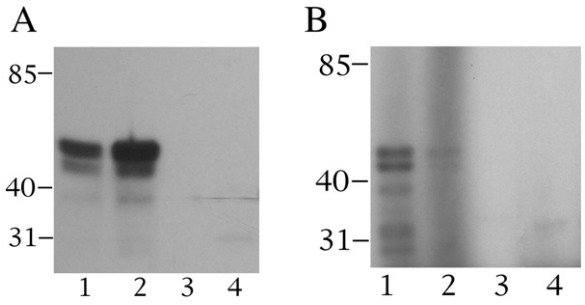


Fig. 4. Phosphorylation of various fusion proteins of the Na^+/H^+ exchanger by the protein kinases ERK2 and p90^{rsk}. A series of GST fusions of the Na^+/H^+ exchanger were made and used for *in vitro* phosphorylation as described in Fig. 3. The fusion proteins used were as follows: lane 1, Gst178 (GST and amino acids 639–816); lane 2, Gst703, C-terminal 178 amino acids but with serine 703 mutated to alanine; lane 3, Δ662–794 same protein as lane 1 except amino acids 661–794 were deleted; lane 4, Gst702, GST fusion expressing amino acids 639–702. (A) ERK2 was used to test if a variety of Na^+/H^+ exchanger fusion proteins were susceptible to *in vitro* phosphorylation. (B) Immunoprecipitated p90^{rsk} was used for *in vitro* phosphorylation of the same samples as in A.

3.4. Expression of exogenous NHE1 in cardiomyocytes

To examine the importance of Ser^{703} and p90^{rsk} in isolated cardiomyocytes, we expressed the mutated NHE1 protein in isolated cardiomyocytes. Because isolated cardiomyocytes have their own NHE1 protein, we inhibit the endogenous NHE1 protein with an NHE1 inhibitor. We express an "exogenous" NHE1 protein that has an HA tag for detection and mutations that leave it functional, but resistant to NHE1 inhibitors (Leu163Phe and Gly174Ser) [16]. Either a wild type exogenous NHE1 protein was expressed, or one which had Ser^{703} changed to alanine. Fig. 6A shows a Western blot of isolated cardiomyocytes that had been infected with HA-tagged NHE1 proteins. Both the wild type NHE1 and Ser703Ala mutant expressed NHE1 to similar levels in isolated cardiomyocytes. GFP-infected cells had only a slight background staining that was often present with the anti-HA antibody used. Probing with anti-myosin heavy chain antibody (MF20, lower panel) confirmed that equal amounts of sample were present on the blot.

To further confirm that we had expressed NHE1 protein, we used an antibody against NHE1 (as opposed to the NHE1 HA tag). The results (Fig. 6B) show that the wild type adenovirus and the Ser703Ala mutant both increased the level of expression of NHE1 in isolated cardiomyocytes (lanes 4 and 5). AP-1 cells transfected with wild type NHE1 plasmid were used as a positive control (lane 1).

3.5. Expression of dominant-negative p90^{rsk} in cardiomyocytes

We used a p90^{rsk} dominant-negative adenovirus that expresses an HA-tagged p90^{rsk} protein with a mutation at Lys100Ala, which renders it catalytically inactive (a gift from Dr. M. Avkiran, St. Thomas Hospital, London). To characterize this virus, we infected cells and examined expression of the resultant protein. Western blotting using anti-HA antibody (Fig. 6C) showed that infection with this virus produced the resultant tagged protein. Uninfected cells only showed a nonspecific background staining that was present with this antibody.

We confirmed that exogenous p90^{rsk} protein was expressed using an antibody against the p90^{rsk} protein itself (Fig. 6D). Immunoblotting GFP-infected cells (lane 1) showed the characteristic double band of p90^{rsk} (indicated by the asterisk), while cells infected with the dominant-negative p90^{rsk} showed an increased amount of slightly larger protein (its increased size likely due to the HA tag).

To confirm that the dominant-negative p90^{rsk} adenovirus was functioning appropriately, we used Western blotting against phosphorylated GSK3β (Ser^{9}). In experiments similar to those done by others, we examined the effectiveness of phorbol 12-myristate 13-acetate (PMA)-induced phosphorylation which is mediated by RSK [31]. The results are shown in Figs. 7A and B. Stimulation of either mock- or GFP-infected cells resulted in increased levels of phosphorylation of GSK3β. However, when cells were treated with dominant-negative p90^{rsk}, there was no increase in GSK3β phosphorylation with

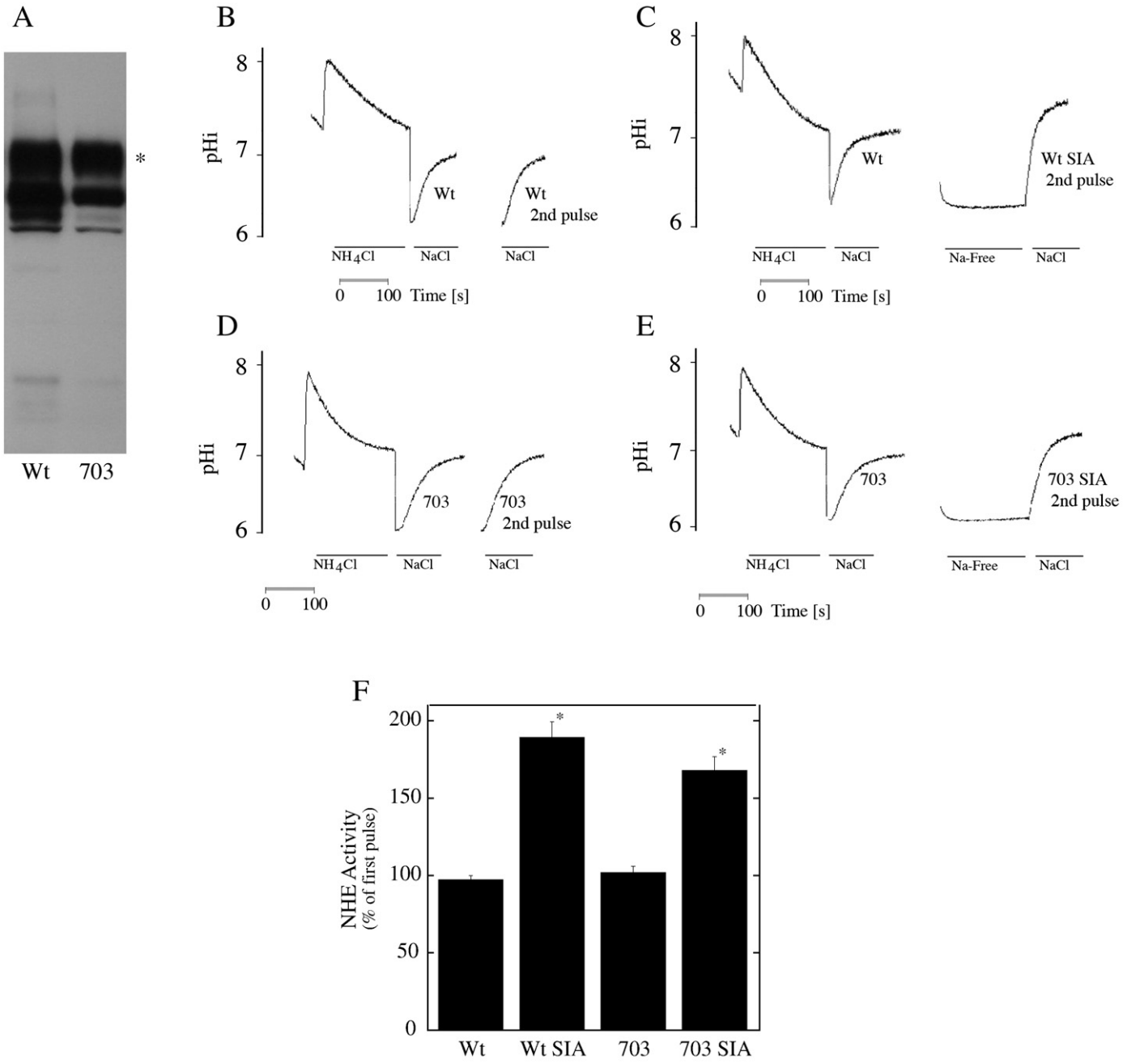


Fig. 5. Analysis of wild type and mutant (Ser703Ala) NHE1 protein in AP-1 cells. (A) Western blot analysis (anti-HA antibody) of NHE1 expression in stable cell lines transfected with pYN4$^+$ plasmid expressing NHE1 protein. Wt, cells transfected with wild type NHE1 protein; 703, cells transfected with NHE1 containing the Ser703Ala mutation. * indicates the position of the fully glycosylated ≈105 kDa NHE1 protein. (B–F) Effect of SIA on wild type and mutant NHE1 activity of stable cell lines of AP-1 cells. Cells were subjected to two pulses of ammonium chloride with recovery periods. Where indicated, the second pulse contained a period of SIA as described in the Materials and methods. (B–E) Examples of measurement of Na^+/H^+ exchanger activity after ammonium chloride-induced acid loads. Lines indicate the presence of ammonium chloride and NaCl -containing or Na-free solutions. SIA indicates that the recovery was after a SIA. Traces are shown for the entire initial ammonium chloride treatment and recovery. For clarity, only the acidosis and recovery are shown for the second ammonium chloride pulse (second pulse). Wt, cells expressing wild type NHE1 protein. 703, cells expressing NHE1 protein with the Ser703Ala mutation. (F) Summary of the effects of SIA on AP-1 cells expressing either wild type (Wt) NHE1 or NHE1 with the Ser703Ala mutation (703). * indicates significantly elevated from the first pulse at $P<0.05$. Values are mean ± SE of at least six experiments.

treatment with PMA. These results confirmed that the adenoviral expression of dominant-negative protein specifically suppressed the activity of endogenous p90^{rsk} in the isolated cardiomyocytes.

3.6. Effect of p90^{rsk} and Ser703Ala mutation on NHE1 activity in cardiomyocytes

We then characterized NHE1 activity in isolated cardiomyocytes. We used a two-pulse assay described earlier for isolated cardiomyocytes [16] and compared the activity of the second pulse with that of the first. There was no difference in buffering capacity of cells infected with the various adenoviruses. Cells subjected to two-pulse assays displayed an increase in NHE1 activity, when the second pulse had an SIA treatment. The absolute level of activity of the wild type inhibitor-resistant NHE1 protein (in the presence of 10 μM EMD87580 to inhibit endogenous NHE1) was 0.302 ± 0.06 ΔpH/min in cardiomyocytes; this was not significantly different from that of NHE1 with the Ser703Ala mutation 0.296 ± 0.03 ΔpH/min. The results of activity

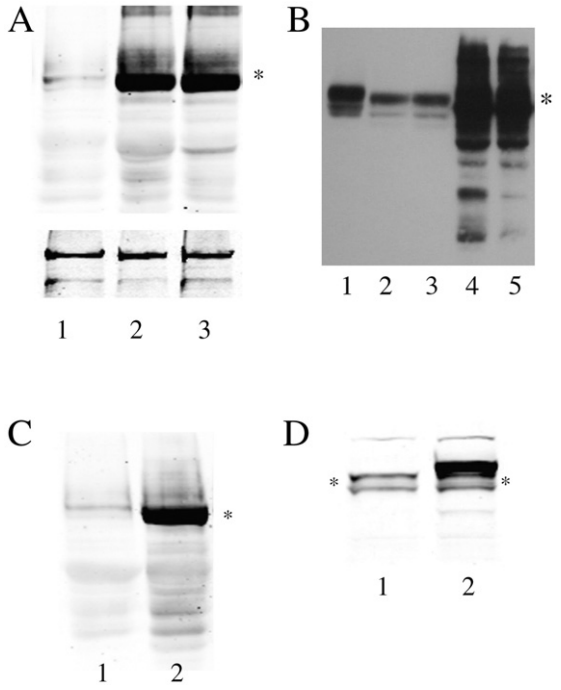


Fig. 6. Analysis of adenoviral expression of NHE1 protein in isolated cardiomyocytes. (A) Western blot analysis of NHE1 expression of isolated cardiomyocytes transfected with adenovirus containing NHE1 protein (pAdNHE1), mutant NHE1 protein (Ser703Ala), or with adenovirus expressing GFP protein alone. Isolated, cardiomyocytes were made from neonatal rat hearts as described in Materials and methods. Equal numbers of cells were then infected with pAdNHE1 containing the HA-tagged inhibitor-resistant NHE1 protein. Upper panel: Probed with anti-HA tag antibodies. Lower panel: Loading control probed with anti-MF20 antibody. Lane 1, GFP-infected cells; lane 2, cells infected with wild type NHE1; lane 3, cells infected with NHE1 with the Ser703Ala mutation. (B) Western blot of samples using anti-NHE1 antibody. Lane 1, positive control consisting of CHO cells stably transfected with HA-tagged NHE1 cDNA; lane 2, extract from mock infected isolated cardiomyocytes; lane 3, cells infected with adenovirus expressing GFP protein alone; lane 4, cells infected with adenovirus with wild type NHE1; lane 5, cells infected with adenovirus with NHE1 with the Ser703Ala mutation. * indicates NHE1 protein of size approximately 100 kDa. (C) Western blot of samples using anti-HA antibody. Isolated cardiomyocytes cells were infected with adenovirus expressing GFP (lane 1) or a dominant-negative $p90^{rsk}$ protein (with an HA tag, lane 2). * denotes the location of 90-kDa immunoreactive band. (D) Western blot of samples using anti-$p90^{rsk}$ antibody. Isolated cardiomyocytes cells were infected with adenovirus expressing GFP (lane 1) or a dominant-negative $p90^{rsk}$ protein (lane 2). * denotes the location of $p90^{rsk}$ double band.

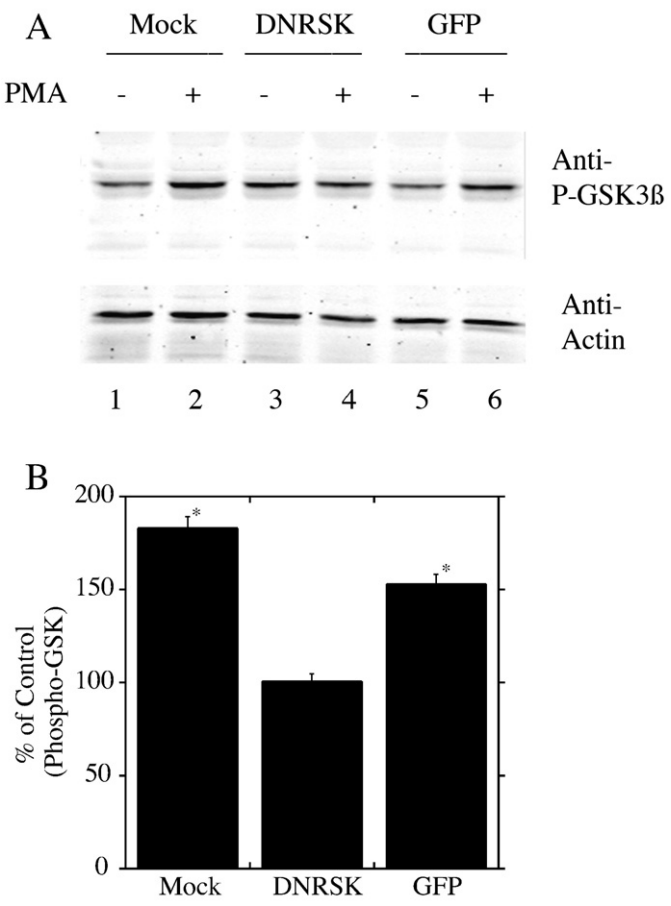


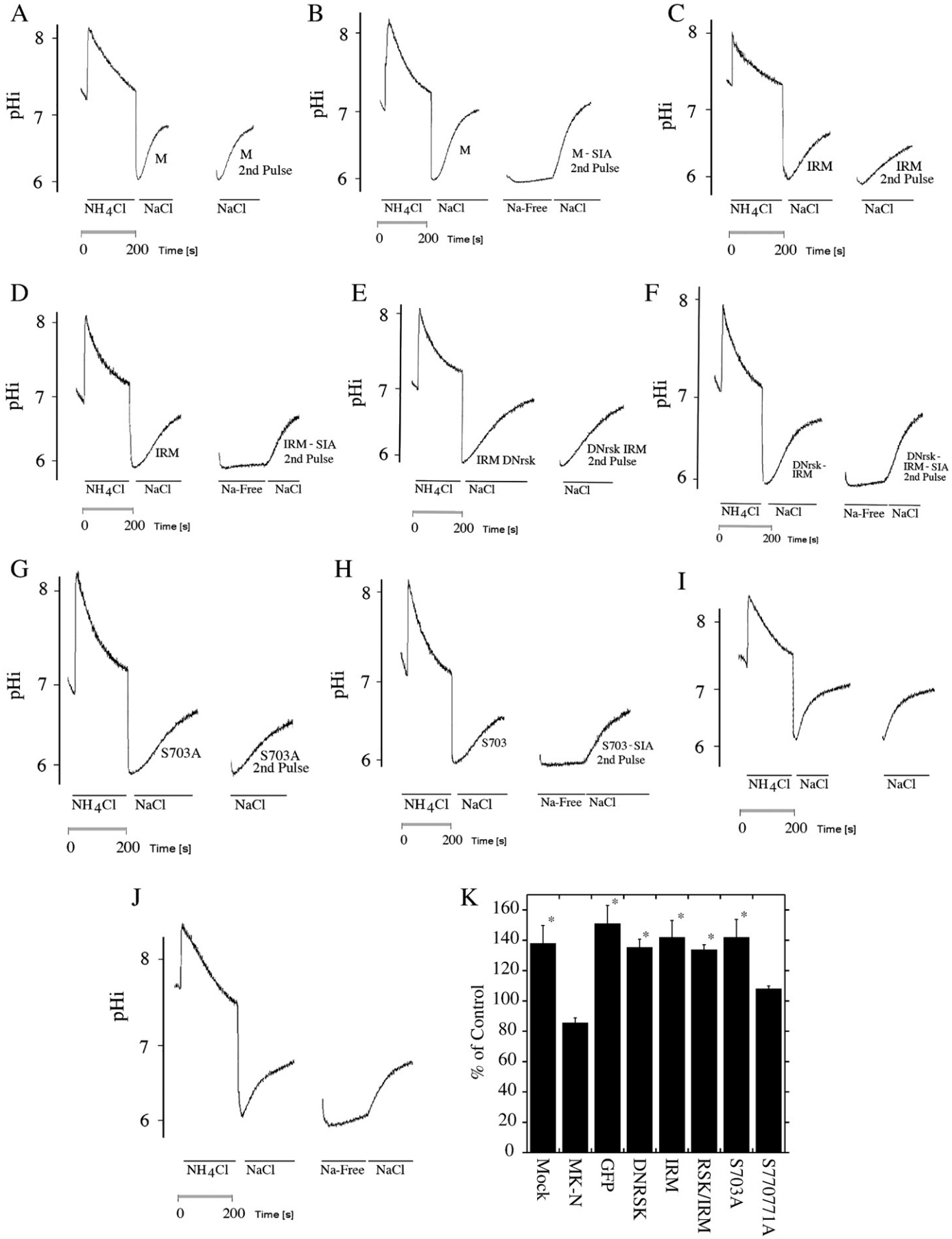
Fig. 7. Western blot analysis of GSK3β phosphorylation levels. (A) Isolated cardiomyocytes were infected with a dominant-negative $p90^{rsk}$ adenovirus (DNRSK) or control (GFP) adenovirus- or mock-infected. Cell extracts were made and immunoblotted with anti-phospho-GSK3β antibody to detect phosphorylation levels. Where indicated (lanes 2, 4, and 6), cells were treated with PMA (50 ng/mL) for 20 min before harvest. Lower panel: Cell extracts were probed with anti-actin antibody (Santa Cruz Biotechnology) to ensure equal loading of samples. (B) Summary of experiments examining the ability of PMA to cause GSK3β phosphorylation. Cells were prepared and assessed as described in A. Results are mean ± SE of at least three experiments. * indicates significantly different from GFP treated cells at $P<0.01$. Values of phosphorylation of GSK were assessed as a percentage of extracts not stimulated by PMA.

measurements are shown in Figs. 8A–K. SIA resulted in an increase in activity of NHE1 varying around 40% (Fig. 8K). In cells that were not treated with SIA (MK-N), the activity of NHE1 after the second pulse decreased slightly compared to the first. For mock-, GFP-, and DN-RSK-infected cells, this assay was done in the absence of EMD87580, and the endogenous NHE1 was measured. In the other cases, we used EMD87580 to inhibit endogenous NHE1 and measured exogenous NHE1 expressed from the adenovirus. SIA increased exogenous NHE1 activity even in the presence of the dominant-negative $p90^{rsk}$. This occurred when assaying either endogenous NHE1 activity or exogenous NHE1 activity of the inhibitor-resistant mutant. These results demonstrated that $p90^{rsk}$ was not required for the stimulatory effects of SIA.

Examination of wild type and Ser703Ala NHE1 expressed in isolated cardiomyocytes showed that SIA also increased exogenous NHE1 activity when the exogenous NHE1 contained the Ser703Ala mutation. This indicated that phosphorylation of Ser^{703} is not essential in the stimulation of NHE1 by sustained acidosis. In contrast to this observation, mutation of the amino acids Ser770/771Ala prevented increased activity with SIA. When the amino acids Ser^{770} and Ser^{771} were mutated to Ala, SIA did not cause an increase in the rate of recovery of the second pulse with SIA (Figs. 8I–K). These results show that, while Ser^{703} was not essential for stimulation by SIA, amino acids Ser^{770} and Ser^{771} were required. The results with amino acids Ser^{770} and Ser^{771} were in agreement with our earlier observations [16].

3.7. Effect of $p90^{rsk}$ and Ser703Ala mutation on NHE1 phosphorylation

To determine whether the dominant-negative $p90^{rsk}$ or the Ser703Ala mutation prevents phosphorylation of NHE1 in response to SIA, we examined the phosphorylation levels of the NHE1 protein. The inhibitor-resistant NHE1 protein was expressed in isolated cardiomyocytes that were incubated with [^{32}P] and treated with or without SIA, and then the NHE1 protein was immunoprecipitated. The amount of labeled NHE1 protein was measured relative to the amount of NHE1 immunoprecipitated so that [^{32}P] levels were always corrected by measuring the amount of HA-tagged immunoprecipitated protein. The results are shown in Fig. 9. For the wild type NHE1, treatment with SIA increased the phosphorylation levels relative to that of controls, even in the presence of the dominant-negative $p90^{rsk}$ protein. A similar increase in the phosphorylation levels occurred with the Ser703Ala mutant NHE1 protein. In both the case of treatment with the dominant-negative $p90^{rsk}$ protein, or with the



Ser703Ala mutation, the level of phosphorylation did not rise as high as with the wild type protein but was significantly elevated.

We have previously noted that mutation of the amino acids Ser^{770} and Ser^{771} to Ala resulted in a huge drop in the overall phosphorylation level of the NHE1 protein [16]. In this case, the Ser703Ala mutant did not show such a large drop in the level of phosphorylation. The phosphorylation level was 75.5% ± 4.5% (mean ± SE, $n = 3$) of the wild type NHE1, whereas earlier we saw approximately a 90% decrease in the phosphorylation level in NHE1 containing the Ser^{770} and Ser^{771} to Ala mutations [16].

4. Discussion

4.1. NHE1 regulation in the myocardium and other tissues

The NHE1 isoform of the Na^+/H^+ exchanger is involved in the damage that occurs during ischemia/reperfusion and in cardiac hypertrophy [32]. It has been demonstrated that while NHE1 activity is reduced during metabolic inhibition, NHE1 activity is highly activated (nearly doubled) during reperfusion following ischemia, and this activation may be key for the injurious rise in Na^+ [23]. Activation of NHE1-directed kinases in the ischemic myocardium may contribute to this increased activity of NHE1 during reperfusion [13]. While a simple approach to preventing the deleterious actions of NHE1 might be the use of inhibitors, clinical studies with NHE1 inhibitors have given disappointing results, partly because of their lack of effectiveness in humans and partly because of the detrimental side effects of these inhibitors [20]. Another alternative therapeutic approach might be alteration of regulation of the NHE1 protein in the myocardium [20]. To further this end, the molecular mechanisms involved in NHE1 kinase-mediated regulation were investigated.

A review of previous studies suggests that the kinases and amino acids involved in regulation of NHE1 vary with the tissue and type of stimulus. Earlier experiments showed that Ser^{726} and Ser^{729} are important in mediating the p38MAPK-induced apoptotic response. Mutation of Ser 726/729 to Ala protected cells from serum withdrawal-induced death [21,22]. However, a different and more proximal amino acid, Ser^{648}, is regulated by protein kinase B. Protein kinase B phosphorylates Ser^{648} of NHE1 and inhibits NHE1 activity in adult rat ventricular cardiomyocytes [33]. More confusingly, another even more recent study showed that phosphorylation of this same amino acid by protein kinase B stimulated NHE1 activity in fibroblasts, leaving open the prospect that phosphorylation of the same amino acid in two different tissues can have varying effects [34].

Another kinase, ERK, can directly phosphorylate the NHE1 C-terminus [25,35,36]. We have shown that ERK plays a role in NHE1 regulation, through Ser^{770} and Ser^{771}. In both CHO cells and in isolated cardiomyocytes, NHE1 is activated by SIA [15,16], and this activation was dependent on ERK kinase activation and phosphorylation of amino acids Ser^{770} and Ser^{771}.

It has been demonstrated that other kinases and amino acids are also involved in the regulation of NHE1 in some cell types of the cardiovascular system and in fibroblasts. Most notable is the amino acid Ser^{703} and the kinase $p90^{rsk}$, which is downstream of ERK. Ser^{703} was shown to be important in activation of NHE1 by growth factors in 293 cells and was additionally demonstrated to be important in binding of the regulatory protein 14-3-3 to NHE1 [18,19]. This amino acid was reported to be phosphorylated in response to growth factors by $p90^{rsk}$ [17], and its phosphorylation resulted in stimulation of NHE1 activity in PS120 cells [37]. However, the earlier study identifying the role of Ser^{703} [17] differs from the present one in that they did not examine activation of NHE1 by chronic acidosis and did not examine the role of Ser^{703} in isolated cardiomyocytes. Activation of NHE1 was by growth factors and serum, differing from the present study.

The kinase $p90^{rsk}$ was initially shown to be active towards NHE1 in vascular smooth muscle cells [37] and was a major NHE1-directed kinase detected in in-gel kinase assays. $p90^{rsk}$ from vascular smooth muscle was shown to phosphorylate a synthetic peptide containing the Ser^{703} sequence [17]. In addition, Maekawa et al. [18] showed that $p90^{rsk}$ was necessary in H_2O_2-mediated activation of NHE1 in ventricular myocytes. Use of the $p90^{rsk}$ inhibitor fmk also blocked activation of NHE1 by phenylephrine in adult cardiomyocytes [38]. However, activation by SIA was not examined in these studies, and we showed that amino acids Ser^{770} and Ser^{771} were necessary for activation of NHE1 by phenylephrine in neonatal rat ventricular cardiomyocytes [16].

Overall, it is clear that a number of different kinases can regulate NHE1 in various tissues. Some studies suggested that Ser^{703} and $p90^{rsk}$ were critical in the regulation of some systems. However, other studies suggested that Ser^{770} and Ser^{771} were necessary for ERK-mediated SIA activation of NHE1 in neonatal rat ventricular cardiomyocytes stimulated by SIA. For this reason, we investigated the role of Ser^{703} and $p90^{rsk}$ in SIA activation of NHE1 in the myocardium.

4.2. Mutation of Ser^{703} does not affect in vitro phosphorylation by ERK

The present study therefore attempted to clarify the role of the $p90^{rsk}$ and Ser^{703} pathway in activation of NHE1 by SIA. Initial experiments confirmed that ERK-mediated *in vitro* phosphorylation of the NHE1 C-terminus was not dependent on Ser^{703}. Mutation of Ser^{703} did not prevent ERK phosphorylation of NHE1 fusion proteins. In contrast, we confirmed the earlier observation [17] that $p90^{rsk}$-mediated phosphorylation *in vitro* is dependent on the amino acid Ser^{703}. Mutation of this residue largely abolished $p90^{rsk}$-mediated *in vitro* phosphorylation. In our case, we used $p90^{rsk}$ immunoprecipitated

Fig. 8. Effect of SIA on control and mutant NHE1 activity of isolated cardiomyocytes. Cells were subjected to two pulses of ammonium chloride with recovery periods. Where indicated, the second pulse contained a period of SIA as described in Materials and methods. (A–H) Examples of measurement of Na^+/H^+ exchanger activity after ammonium chloride-induced acid loads. Lines indicate the presence of ammonium chloride and NaCl-containing or Na-free solutions. After ammonium chloride treatment, there was either a brief treatment in Na-free medium (indicated by break in bars) or a long treatment in Na-free medium causing SIA (indicated by Na^+-free bar). SIA indicates that the recovery was after a long treatment in Na-free medium. Traces are shown for the entire initial ammonium chloride treatment and recovery. For clarity, only the acidosis and recovery are shown for the second ammonium chloride pulse (second pulse). (A and B) pH recovery of mock (M)-infected cells, in the absence of NHE1 inhibitor. A: Second pulse was in the absence of SIA. B: Second pulse was in the presence of SIA. (C, D) pH recovery of cells infected with inhibitor-resistant NHE1 (IRM). C: Second pulse was in the absence of SIA. D: Second pulse was in the presence of SIA. Assay was in the presence of 10 μM EMD 87580 to inhibit endogenous NHE1. (E and F) pH recovery of cells infected with adenovirus containing inhibitor-resistant NHE1 plus dominant-negative $p90^{rsk}$ adenovirus. E: Second pulse was in the absence of SIA. F: Second pulse was in the presence of SIA. Assay was in the presence of 10 μM EMD 87580 to inhibit endogenous NHE1. (G and H) pH recovery of cells infected with inhibitor-resistant NHE1 containing the Ser703Ala mutation. G: Second pulse was in the absence of SIA. H: Second pulse was in the presence of SIA. Assay was in the presence of 10 μM EMD 87580 to inhibit endogenous NHE1. (I and J) pH recovery of cells infected with inhibitor-resistant NHE1 containing the Ser770/771Ala mutation. I: Second pulse was in the absence of SIA. J: Second pulse was in the presence of SIA. Assay was in the presence of 10 μM EMD 87580 to inhibit endogenous NHE1. (K) Summary of NHE1 activity of isolated cardiomyocytes. Cells were subjected to two-pulse Na^+/H^+ exchanger activity assays, and the activity of the exchanger in the second pulse was compared to that of the first pulse. The second pulse was done after a 3-min period of SIA immediately before recovery. MK-N indicates cells that were mock infected and were given a two-pulse assay but without a period of SIA in the second pulse. Mock, mock-infected cells; GFP, isolated cardiomyocytes infected with GFP containing virus without NHE1; DNRSK, infected with dominant-negative $p90^{rsk}$ adenovirus; IRM, infected with wild type inhibitor-resistant NHE1 adenovirus; RSK/IRM, infected with dominant-negative $p90^{rsk}$ and IRM; Ser703Ala, infected with inhibitor-resistant NHE1 adenovirus containing the Ser703Ala mutation; Ser770/771Ala, infected with inhibitor-resistant NHE1 adenovirus containing the Ser770/771Ala mutation. For cells infected with adenovirus containing inhibitor-resistant NHE1 (IRM, DNRSK/IRM, Ser703Ala, and Ser770/771Ala), the assay was in the presence of 10 μM EMD 87580 to inhibit endogenous NHE1. Where appropriate, the values shown were compared to those obtained in the absence of SIA. * indicates significantly elevated from the values in the absence of SIA, $P<0.05$. Values are mean ± SE of at least six experiments.

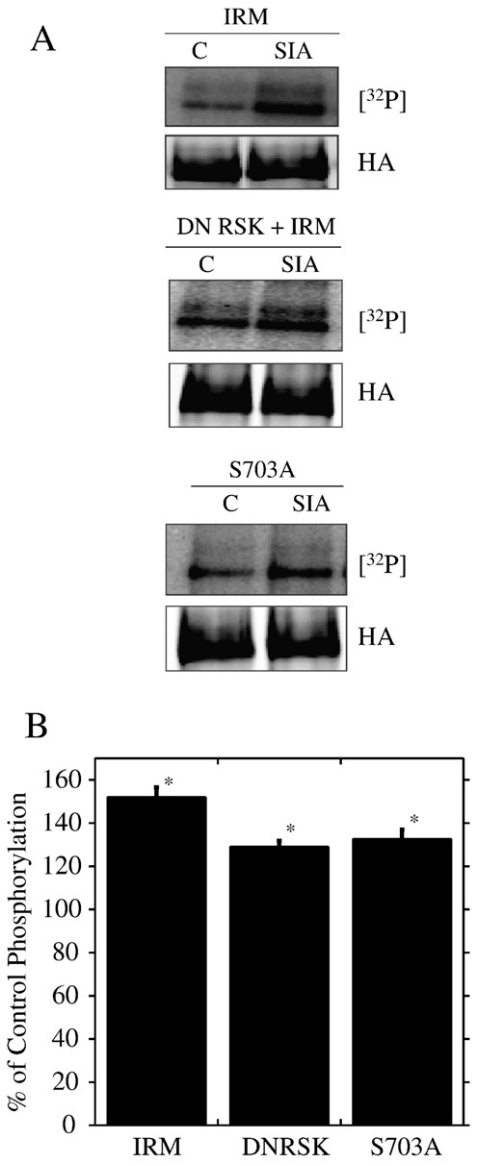


Fig. 9. Effects of SIA on phosphorylation levels of NHE1 protein in isolated cardiomyocytes. (A) Examples of NHE1 protein immunoprecipitated from cardiomyocytes incubated with [^{32}P]. Isolated cardiomyocytes were infected with various adenoviruses expressing wild type or mutant NHE1 protein. Exogenous NHE1 protein was immunoprecipitated with anti-HA antibody from cardiomyocytes incubated with [^{32}P] with or without SIA. Upper part of each panel illustrates autoradiogram of phosphorylated protein. Lower part illustrates anti-HA Western blot used to correct for the amount of immunoprecipitated protein. IRM, inhibitor-resistant NHE1 protein; DNRSK, dominant-negative p90^{rsk} adenovirus; Ser703Ala, inhibitor-resistant NHE1 protein with Ser703Ala mutation. (B) Summary of phosphorylation levels of immunoprecipitated NHE1 protein from isolated cardiomyocytes after stimulation by SIA. Levels of phosphorylation were corrected for the amount of NHE1 immunoprecipitated, as determined by the amount of HA tagged protein. Phosphorylation levels were compared to that of controls that were not stimulated by SIA and are the mean ± SE of at least three experiments. * indicates significantly elevated over the level of control at $P<0.05$.

from isolated cardiomyocytes as opposed to PS120 cells [17] confirming that the cardiomyocyte form of p90^{rsk} is specific for Ser^{703}.

It was notable that deletion of amino acids 662–794 from an NHE1 fusion protein resulted in a dramatic loss of a 44-kDa phosphorylated band. This mutation removed all of Ser^{703} and Ser^{770} and Ser^{771}, leaving open the possibility that one of these amino acids was the site of phosphorylation. This experiment also confirmed that the 44-kDa phosphorylated band was not due to phosphorylation of the GST part of the fusion protein because the GST part of the protein remained in this in-gel kinase assay. Other experiments with the histidine-tagged (as opposed to GST-tagged) NHE1 protein confirmed that both heart cell extracts and immunoprecipitated ERK from the heart phosphorylated either the GST- or the histidine-tagged NHE1 C-terminus. ERK readily phosphorylated an NHE1 fusion protein with the Ser703Ala mutation, suggesting that this amino acid was not the site of ERK-mediated phosphorylation of NHE1.

4.3. Role of Ser^{703} and p90^{rsk} in activation of NHE1

Preliminary experiments demonstrated that Ser^{703} was not necessary for SIA-induced activation of NHE1 in CHO (AP-1) cells. Mutation of this amino acid did not prevent SIA from activating the NHE1 protein. When we expressed the NHE1 protein in isolated cardiomyocytes with the Ser703Ala mutation, the activation of NHE1 by SIA was also not impaired. In addition, phosphorylation of NHE1 with the Ser703Ala mutation that was induced by SIA was also not impaired. This suggested that SIA mediates phosphorylation through another site or sites aside from Ser^{703}. We have earlier [16] shown that mutation of amino acids Ser^{770} and Ser^{771} to Ala abolished SIA activation and phosphorylation of NHE1. The present results are consistent with the idea that SIA mediates its effects through these two amino acids and does not require phosphorylation of Ser^{703} to activate NHE1 in isolated cardiomyocytes.

An interesting observation was that mutation of Ser^{703} to Ala reduced the basal activity of the NHE1 protein in AP1 cells by about 25%. In contrast this mutation did not affect the basal activity of NHE1 in isolated cardiomyocytes. This suggests some different mechanisms of regulation the protein in these cell types. In both cardiomyocytes and AP1 cells, acidosis could stimulate NHE1 activity, so that the regulation of basal activity was independent of regulation by acidosis. These data show that, in some cell types, Ser^{703} can affect basal activity of NHE1 but not in others.

In this study, we also addressed the kinases involved in the regulation of NHE1 in cardiomyocytes in response to SIA. We have earlier demonstrated that SIA activates NHE1 through ERK-dependent phosphorylation [16]. Because p90^{rsk} is downstream of ERK, and because p90^{rsk} was shown to regulate NHE1 at Ser^{703}, it was important to determine whether activation of NHE1 was directly through ERK or p90^{rsk}. Previous results have suggested to us that ERK is more quickly activated in our system than p90^{rsk} [16]. In our hands, working with neonatal rat ventricular cardiomyocytes, we found much slower increases in the activation of p90^{rsk} by acute stimulation via SIA or α-adrenergic stimulation [16]. Similarly, we found a relatively low level of phosphorylation of NHE1 by p90^{rsk} compared with ERK, in in-gel-kinase assays in the present study (Fig. 2B) or earlier using whole heart extracts or extracts of isolated cardiomyocytes [13]. Under the conditions used in these studies, it appeared as though ERK was more active toward NHE1 than p90^{rsk}. In contrast, studies using vascular smooth muscle cells show a relatively stronger 90-kDa kinase activity towards NHE1 [37]. In the present study, we expressed a dominant-negative form of p90^{rsk} adenovirus to eliminate activity of p90^{rsk} in our system. To confirm that this occurred, we stimulated cells with phorbol esters. In the presence of the dominant-negative p90^{rsk}, we were unable to stimulate p90^{rsk} (Fig. 6), confirming that the activation of p90^{rsk} was blocked. However, blockage of p90^{rsk} did not prevent activation of the Na^+/H^+ exchanger by SIA or increased phosphorylation of the protein. These results suggest that p90^{rsk} was not responsible for the regulation of NHE1 with this stimulation. Fig. 10 summarizes these results in a model. SIA can activate NHE1 in the absence of p90^{rsk} activity and in the absence of phosphorylation of Ser^{703}.

What then would be the role of Ser^{703} and p90^{rsk}-mediated regulation of NHE1 in the myocardium? We suggest that they may be more important in longer-term less-rapid regulation of NHE1 and in the regulation of NHE1 in adult cardiomyocytes. This is supported by the slower activation of p90^{rsk} that we observed earlier [16]. Further,

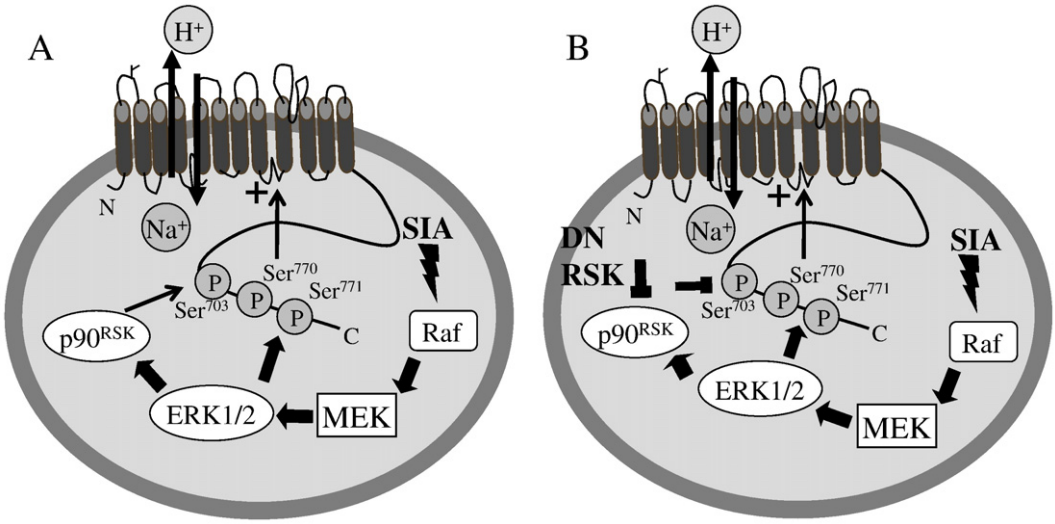


Fig. 10. Model of NHE1 regulation by sustained intracellular acidosis. Phosphorylatable residues Ser^{703}, Ser^{770}, and Ser^{771} are indicated. (A) Model of sustained intracellular acidosis (SIA) activating NHE1. (B) Model of SIA illustrating effects in the absence of $p90^{rsk}$ activity by use of a dominant-negative $p90^{rsk}$. Raf, mitogen-activated protein kinase kinase kinase; MEK, mitogen-activated protein kinase kinase; ERK1/2, extracellular-regulated kinase isoform 1 and 2; $p90^{rsk}$, p90 ribosomal protein S6 kinase.

we found that, with mutation of Ser^{770} and Ser^{771} to Ala, the overall rapidly exchangeable phosphorylation level of NHE1 dropped dramatically [16], while it did not in the present study with mutation of Ser^{703} to Ala. In our present study, it was a requirement to use neonatal cardiomyocytes, which can be maintained in culture more easily and for longer periods. The ERK pathway is very active in the neonatal myocardium [39] and plays a key role in gestational development of the myocardium [40]. ERK regulation of NHE1 in the neonate is critically involved in Ca^{2+} overload in the myocardium mediated through NHE1 [13,35], and the newborn myocardium has elevated levels of NHE1 activity and expression [41]. It is not suggested that Ser^{703} plays a more critical role in the adult, as opposed to neonatal myocardium.

Overall, our study suggests that with SIA stimulation, ERK mediates activation of NHE1 through phosphorylation of Ser770/771. $p90^{rsk}$ and Ser^{703} were not involved in this rapid method or regulation of NHE1. Although $p90^{rsk}$ can activate NHE1 through Ser^{703} [17], that activation was by serum and growth factors and was shown in other cell types. The present study suggests that in cardiomyocytes, activation by chronic acidosis is through a different mechanism. Our results suggest that in the clinical setting, chronic acidosis could still activate the NHE1 protein even if $p90^{rsk}$ were inhibited. Thus, NHE1 could still be activated and might still accentuate the damage that occurs to the myocardium under appropriate conditions.

Activation of NHE1 by different stimuli and in different cell types appears to be mediated by phosphorylation of different amino acids. In a different study, it was shown that p38-mediated phosphorylation of amino acids Ser^{726} and Ser^{729} also activates NHE1 in response to apoptotic stimuli [21]. How can phosphorylation at different sites by different protein kinases mediate activation of the NHE1 protein? A number of other proteins are regulated by dual phosphorylation by different kinases. In some cases, the initial phosphorylation can prime the phosphorylation of a second residue such as with CREB (cAMP-response element binding protein) [42] and with glycogen synthase [43]. In other cases, dual phosphorylation may act on amino acids that are widely separated such as with a K^+ channel [44]. Dual regulation of a protein can provide a mechanism of finely tuning the activity, sometimes being involved in mediating tissue-specific regulation. It has been suggested that the Na^+/H^+ exchanger has unique kinetic characteristics in the myocardium as compared to other tissues with a very steep relationship between pH_i and activity [45]. It may be that regulation of NHE1 in the cardiomyocyte varies in comparison to other cell types, perhaps due to regulation by ERK through Ser^{770} and Ser^{771}. Whether this occurs directly by phosphorylation or through phosphorylation modulated interactions with other regulatory proteins is not known but will be the subject of future investigations.

Acknowledgments

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