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Original article

Activation of the Na^+/H^+ exchanger in isolated cardiomyocytes through β-Raf dependent pathways. Role of Thr^{653} of the cytosolic tail

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ABSTRACT

The mammalian Na^+/H^+ exchanger isoform 1 (NHE1) is a ubiquitous plasma membrane protein that is a key regulator of intracellular pH in isolated cardiomyocytes. A 500 amino acid membrane domain removes protons and is regulated by a 315 amino acid cytosolic domain. In the myocardium, aberrant regulation of NHE1 contributes to ischemia reperfusion damage and to heart hypertrophy. We examined mechanisms of regulation of NHE1 in the myocardium by endothelin and β-Raf. Endothelin stimulated NHE1 activity and activated Erk-dependent pathways. Inhibition of β-Raf reduced NHE1 activity and Erk-pathway activation. We demonstrated that myocardial β-Raf binds to the C-terminal 182 amino acids of the NHE1 protein and that β-Raf is associated with NHE1 in intact cardiomyocytes. NHE1 was phosphorylated *in vivo* and the protein kinase inhibitor sorafenib reduced NHE1 phosphorylation levels. Immunoprecipitates of β-Raf from cardiomyocytes phosphorylated the C-terminal 182 amino acids of NHE1 and mass spectrometry analysis showed that amino acid Thr^{653} was phosphorylated. Mutation of this amino acid to Ala resulted in defective activity while mutation to Asp restored the activity. The results demonstrate that Thr^{653} is an important regulatory amino acid of NHE1 that is activated through β-Raf dependent pathways by phosphorylation either directly or indirectly by β-Raf, and this affects NHE1 activity.

1. Introduction

In mammalian cells, a key regulator of pH homeostasis is isoform one of the Na^+/H^+ exchanger (NHE1). The 500 N-terminal amino acids of NHE1 make up an integral membrane domain that facilitates movement of one intracellular proton is exchange for a single extracellular sodium ion. The 315 C-terminal amino acids comprise a cytoplasmic domain that regulates activity of the membrane domain [1]. NHE1 is important in promoting cell growth, proliferation, differentiation, and apoptosis. It is ubiquitously expressed in mammalian cells and transport is regulated allosterically. At low intracellular pH, (pH_i) (high intracellular proton concentrations), protons allosterically activate NHE1 triggering proton extrusion. With a return to homeostatic pH_i the NHE1 protein becomes inactive. NHE1 activity is altered by hormonal regulation that changes the proton sensitivity of the protein, causing a shift of the proton activation curve to a more alkaline range. This results in more activity at a given alkaline pH_i [2,3]. It is generally believed that about half the regulation of NHE1 is mediated through phosphorylation of the cytosolic tail while the other half is through protein-protein interactions [4,5].

The regulation of intracellular pH is crucial in the myocardium for proper contractile function. In addition NHE1 plays a critical role in pathology of cardiovascular disease. It is implicated in ischemia reperfusion injury and cardiac hypertrophy. In ischemia reperfusion injury, elevated NHE1 activity leads to a reversal of activity of the Na^+/Ca^{2+} exchanger and results in an increase in intracellular calcium, triggering deleterious pathways that lead to cell damage and death [6,7]. NHE1 is also important in cardiac hypertrophy with increased activity of NHE1 demonstrated in various animal models of hypertrophy [8]. Inhibition of NHE1 has been shown to prevent hypertrophy [9,10]. Hormonal regulation of NHE1 has been demonstrated (reviewed in [5]) but the pathways downstream from receptor coupling are not well characterized. Endothelin 1 (ET-1) is one hormonal regulator of NHE1 [5,6,11]. Activation of NHE1 by ET-1 is part of an autocrine and paracrine loop that may ultimately lead to a rise in NHE1 activation and elevated intracellular Na, leading to reverse mode sodium calcium exchanger activity [12]. We recently demonstrated that in CHO cells ET-1 treatment leads to enhanced association of calmodulin with the NHE1 protein [13]. Additionally, we also have recently shown that the kinase β-Raf is an important regulator of NHE1 in HeLa cells, in human embryonic kidney cells and in malignant melanoma cells [14]. However, in the myocardium details of the mechanism of these two regulators of NHE1 are not well characterized.

Abbreviations: ET-1, endothelin 1; NHE1, Na^+/H^+ exchanger isoform 1; NRVM, neonatal rat ventricular myocytes; pH_i, intracellular pH.

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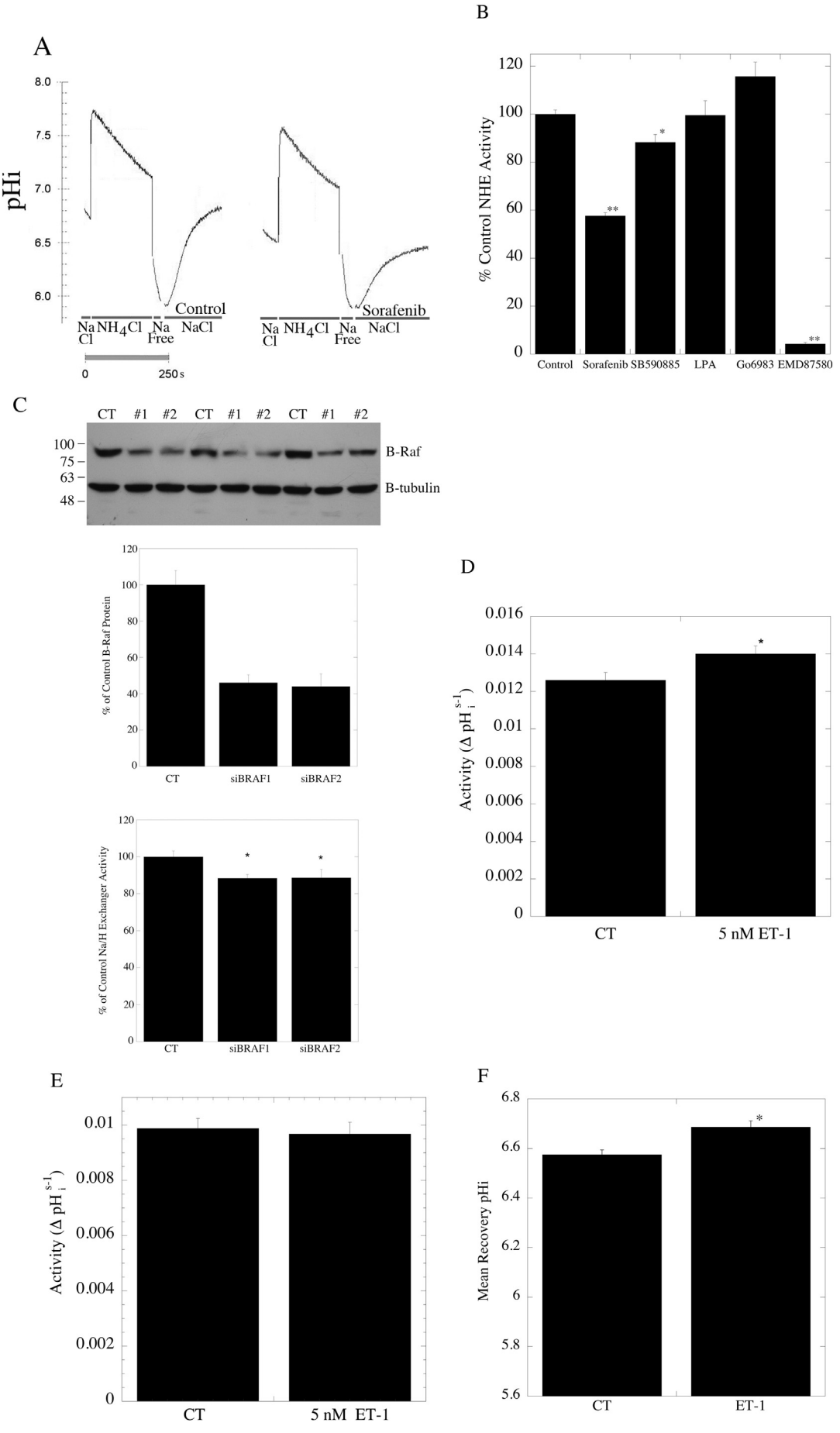
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In this study we examine downstream mechanisms of regulation of NHE1 in the myocardium. We previously have demonstrated that β-Raf can bind to NHE1 and regulate its activity in some cell types [14]. Here we explore its mode of action in the myocardium. We demonstrate that β-Raf can act through phosphorylation of amino acid Thr^{653} of the NHE1 regulatory tail to affect NHE1 activity and cellular function.

2. Materials and methods

2.1. Materials

2′, 7-bis (2-Carboxyethyl)-5(6) carboxyfluorescein acetoxymethyl ester (BCECF-AM) was from Molecular Probes, Inc. (Eugene, OR). Anti-HA-antibody (Y-11) and anti-β-Raf polyclonal or monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MAPK (ERK1/2), anti-phospho ERK1/2, anti-phospho-p38 and anti-p38 were supplied by Cell Signaling Technology Inc. (Beverly, MA). Anti-NHE1 antibody was purchased from BD biosciences. DSP (dithiobis(succinimidylpropionate)) was purchased from Pierce, (Rockford, IL). Sorafenib was obtained from LC Laboratories (Woburn, MA). SB590885 was obtained from Sellechem.com. Anti-Phospho-Thr polyclonal antibody was from New England Biolabs. LPA (1-oleolyl lysophosphatidic acid) and Go6983 were purchased from Sigma Chemicals. EMD87580 was a generous gift of Dr. N. Beier of Merck KGaA, Frankfurter, Germany.

Other chemicals used were of high analytical grade and were purchased from Fisher Scientific (Ottawa, ON, Canada), Sigma (St. Louis, MO, USA) or BDH (Toronto, ON, Canada).

2.2. Expression and purification of fusion proteins

The carboxyl-terminal 182 amino acids of the human Na^+/H^+ exchanger (NHE1) was expressed and purified as a fusion protein with a C-terminal hexahistidine tag (His182) using the plasmid pDest 14 and the Gateway Cloning System as described earlier [15]. The *E. coli* strain BL21-SI was induced with 0.3 M NaCl for 5 h. The His182 protein was harvested using standard conditions, and the protein was purified from the supernatant of *E. coli via* Ni-NTA affinity chromatography according to the manufacturer's directions (Qiagen). Residues 2–183 of this protein correspond to amino acids 634–815 of the NHE1 isoform of the human NHE1. The hexahistidine tag was at the C-terminus for protein purification and an initiator methionine residue was at the N-terminus.

2.3. Overlay procedure

To examine wild type β-Raf binding to the Na^+/H^+ exchanger we expressed and purified C-terminal tail regions of the NHE1 protein as described earlier and as above [16]. These His tagged proteins were of the human NHE1 C-terminus and contained the distal 182 (His182, amino acids 634–815) and 239 (His239, amino acids 577–815) amino acids. A control was His-tagged calcineurin homologous protein (HisCHP) produced as described earlier [17]. Protein samples were separated on 12% SDS-PAGE and then transferred to nitrocellulose membranes. Nitrocellulose membranes were then blocked with 10% (w/v) skim milk powder in TBS (20 mM Tris, pH 7.4, 137 mM NaCl) for 5 h at room temperature. Afterwards they were then incubated with NRVM cell extracts. The membranes were rocked gently overnight at 4 °C then washed with TBS for 4 × 15 min at room temperature. The membrane was then incubated with anti-β-Raf antibody (1:2000) in TBS with 1% skim milk powder for 2 h at room temperature. This was followed by washing for another hour with TBS. Detection was achieved by secondary antibody coupled to horseradish peroxidase antibodies. Reactive bands were visualized by the Amersham Biosciences Enhanced Chemiluminescence system or autoradiography and the experiment was repeated four times.

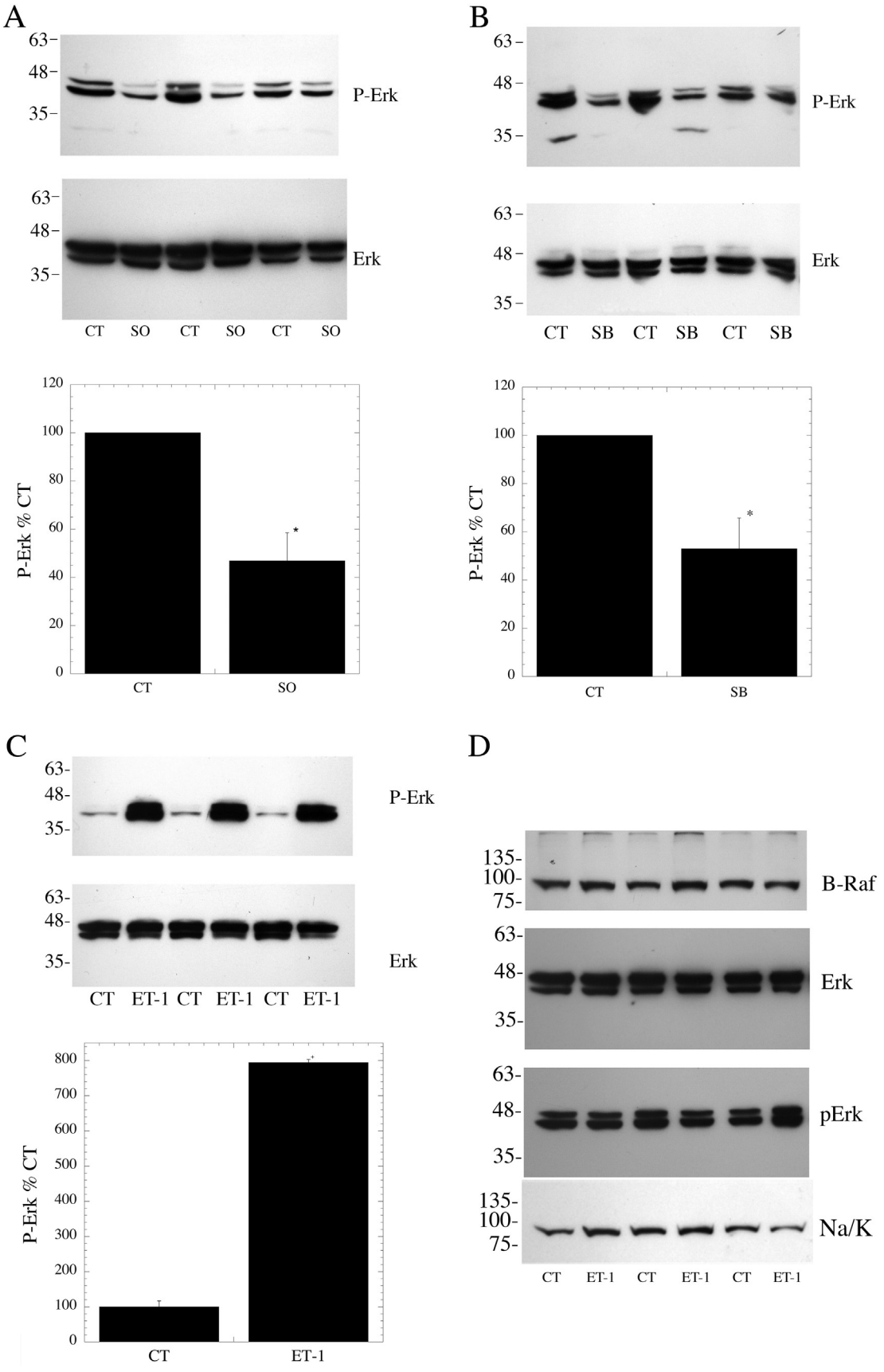
2.4. Isolated neonatal rat ventricular cardiomyocytes (NRVM)

NRVM were made essentially as described earlier [18]. Briefly, primary cultures were made from neonatal Sprague Dawley rat heart ventricles by dissociation with collagenase. Primary myocytes were plated onto Primaria™ (Falcon) culture dishes or flasks. Myocytes were maintained for 4–5 days in prior to immunoprecipitation experiments 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) non-essential amino acids, 10% MEM vitamin, 0.1 mM bromodeoxyuridine, and 30 mM HEPES, pH 7.4. Cell extracts from isolated NRVM were made by lysis the cells with RIPA buffer or lysis buffer provide in Pierce Crosslink IP kit (Thermo Scientific, Rockford, IL, USA).

2.5. Immunoprecipitations

NRVM were treated with DSP (dithiobis(succinimidylpropionate) essentially as described earlier [19]. Briefly, cells were washed twice with phosphate buffered saline (PBS, 150 mM NaCl, 5 mM sodium phosphate, pH 7.4), then 2 mM DSP was added in 2 ml cross-link buffer (20 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 150 mM NaCl) for 30 min at room temperature. 10 mM Tris was used to quench the reaction for 15 min at room temperature. Further washes were twice with PBS and cells were frozen in 2 ml RIPA buffer with detergent (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 80 mM NaF, 5 mM EDTA, 1 mM EGTA, 1 mM Na-orthovanadate, 1% NP-40, 0.5% deoxycholate and proteinase inhibitor cocktail) by placing cells on dry ice. After defrosting, cells were sonicated for 15 s centrifuged (35,000 × *g* for 1 h) and the supernatant was collected. The supernatant was rocked for 4 h with 10 μl rabbit anti-β-Raf polyclonal antibody. Protein A Sepharose was added and the sample was incubated overnight at 4 °C. The resin was washed with RIPA buffer and protein bound to the resin was solubilized with

Fig. 1. Characterization of NHE1 activity in NRVM or H9C2 cardiomyocytes. A, Example of recovery from an acute ammonium chloride induced acid load in NRVM. NHE1 protein activity was assayed in NRVM cells as described in the "Materials and methods". Left panel, example of recovery from control cells. Right panel, recovery of cells treated with 10 μM sorafenib. NH_4Cl, treatment period with ammonium chloride added to NaCl containing media. After NH_4Cl treatment there was a brief "Na Free" treatment period to induce acidosis. NaCl, cells were in NaCl containing buffer. The recovery period from acidosis was in NaCl containing buffer. Time scale is indicated. The example is from one experiment which is typical of at least 7 measurements. B, Summary of effect of Sorafenib, SB590885, LPA, Go6983 and EMD87580 treatment on NHE1 activity of NRVM. Cells were treated with an ammonium chloride pulse in the absence (control) or presence of treatments. Control values were set to 100%. Treatments were; 10 μM sorafenib treatment (30 min), 10 μM SB590885 (1 h), 10 μM LPA (10 min), 2 μM Go6983 (1 h) and 10 μM EMD87580 (1 min). N = 7–12. * significantly different from control at $P < 0.05$. ** significantly different from control at $P < 0.001$. C, Effect of control or β-Raf siRNA on NHE1 activity of H9C2 cardiomyocytes. H9C2 cells were treated with siRNA sets #1 or #2 as described in the "Materials and methods". Upper panel, western blot of cell lysates simultaneously blotted for β-Raf and β-tubulin. Middle panel, summary of effect of siRNA on β-Raf levels, n = 6. Lower panel, summary of effect of siRNA on Na^+/H^+ exchanger activity. * significantly different from control at $P < 0.05$. D, Effect of ET-1 on NHE1 activity. Cells were grown in reduced serum (0.5%) for 18 h and then were treated for 25 min with 5 nM ET-1. NHE1 activity was measured after an ammonium chloride induced acid load as described in the materials and methods. N = 14, * significantly different from control at $P < 0.001$. E, as in D but cells were treated for 24 h with 5 nM ET-1. F, resting pH_i of cells after ammonium chloride treatment ± 5 nM ET-1 for 25 min.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SDS-PAGE sample buffer. After SDS-PAGE and transfer to nitrocellulose anti-NHE1 antibody or anti-β-Raf antibody was used to probe the blots [20]. For some experiments immunoprecipitation followed the instructions of the Pierce Crosslink IP kit (Thermo Scientific, Rockford, IL, USA).

2.6. Phosphorylation of NHE1 by β-Raf

The standard reaction conditions for phosphorylation of His182 contained 10.0 μg of His182, 20 μl of β-Raf NRVM immunoprecipitate, 12.5 mM 3-(4-morpholino)propanesulfonic acid (MOPS) pH 7.2, 0.5 mM EGTA, 2 mM DTT, 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 in a final volume of 30 μl. Samples were incubated at 30 °C for 90 min., and the reaction was terminated by the addition of SDS-PAGE loading buffer [15]. Samples were run on a 12% SDS-PAGE gel, dried, and exposed for autoradiography. The levels of phosphorylation were estimated on autoradiograms using the program Image J.

In vivo phosphorylation of NHE1 was examined in AP-1 cells containing HA-tagged NHE1 protein and grown as described below. For *in vivo* phosphorylation assays, the cells were grown in phosphate free medium for 3 h with 100 μCi/ml [^{32}P]. Sorafenib (10 μM × 1/2 h) or SB590885 (10 μM × 1 h). After washing, NHE1 protein was immunoprecipitated using the HA tag on the protein as described earlier [21]. Phosphorylated NHE1 was examined by exposure of nitrocellulose transfers to X-ray film. Results were repeated four times.

2.7. Determination of β-Raf phosphorylation site

To determine the site phosphorylated by β-Raf immunoprecipitates we did an experimental and mock immunoprecipitation of β-Raf from NRVM. These two samples were used to phosphorylate 10 μg of His182 protein as described above, but the [γ-^{32}P]ATP was replaced with unlabeled ATP. For determination of the phosphorylation site of the β-Raf immunoprecipitate on NHE1 we used in-gel microwave-assisted acid hydrolysis as described earlier [22]. Briefly, the control and experimental bands of His182 were cut out SDS-PAGE and washed with 1 ml of water 2 × 5 min. The gel band was cut into small pieces and dehydrated 2 x using 500 μl of acetonitrile for 5 min. Gel pieces were dried in a vial in a SpeedVac for 15 min. 120 μl of 12 mM DTT and 40 μl of trifluoroacetic acid were then added. Then the sample vial was placed in a water bath and was irradiated for 10 min in a domestic 1200 W (2450 MHz) microwave oven. The peptide mixture generated was extracted using 300 μl of 85% acetonitrile/0.1% trifluoroacetic solution twice. The extracted peptide solution was dried in a SpeedVac. Reduction and alkylation of peptide mixture was then performed by treating with 20 μl of DTT (90 mM) for 1 h at 37 °C and 50 μl of iodoacetic acid (200 mM) for 1 h at room temperature. The peptide mixture was then desalted as described earlier [23]. The desalted peptide mixture was subjected to immobilized metal ion affinity chromatography (IMAC) phosphopeptide enrichment using a Fe-IMAC resin (Phos-Select iron affinity gel; Sigma, Ontario, Canada). The desalted and dried hydrolysate was re-suspended in 30% acetonitrile mixed with 250 mM acetic acid and the peptides were loaded onto the Fe-IMAC resin. The resin was washed three times with 30% acetonitrile/250 mM acetic acid, two times with water after overnight incubation at 4 °C. The phosphopeptides were then released from the resin with 400 mM ammonium hydroxide. Both the non-phosphorylated peptides in the wash buffer and the released phosphopeptides were collected for desalting and LC-MS/MS analysis. LC-MS/MS analysis of peptide mixture and database search was as described earlier [24].

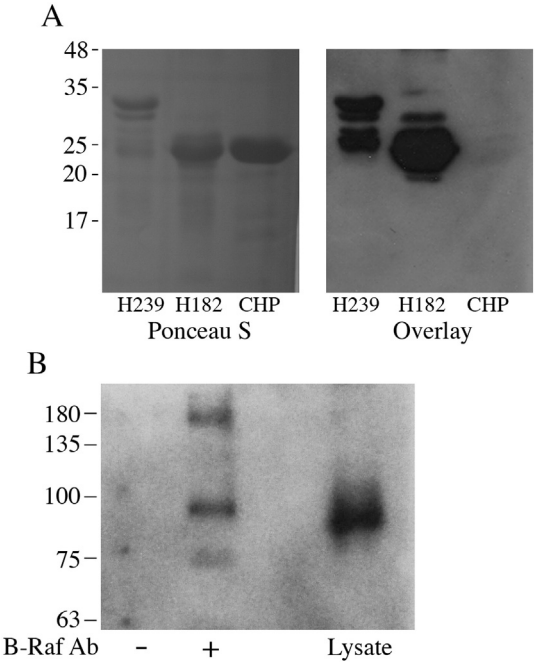

Fig. 3. Analysis of association of myocardial β-Raf protein with NHE1. A, Association of β-Raf with NHE1 *in vitro*. Left panel, nitrocellulose transfer of NHE1 His tagged proteins His239 and His 182 and a control protein His CHP. The transfer was stained with Ponceau S prior to experimentation to demonstrate the presence of the proteins. Right panel, overlay assay using myocardial cell lysate as described in the "Materials and methods". After extensive washes, the blot was incubated with anti-β-Raf antibody. The results are typical of 4 experiments. B, Association of β-Raf with NHE1 in intact NRVM. Samples were immunoblotted with anti-NHE1 antibody. β-Raf was immunoprecipitated with anti-β-Raf antibodies as outlined in the "Materials and methods". "+" indicates that β-Raf antibody was used in the immunoprecipitation from isolated NRVM. "−" indicates no β-Raf antibody was present in the mock immunoprecipitation. "Lysate" indicates a sample of NRVM lysate.

2.8. Intracellular pH

Intracellular pH and Na^+/H^+ exchanger activity in isolated NRVM was measured essentially as described earlier [20]. The pH_i of an entire population of isolated NRVM 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 rate of recovery in the initial 20 s of recovery beginning after change of NaCl was used with a linear fit as described earlier [20,25]. 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. A calibration curve was done with 10 μM nigericin at the end of every experiment to calibrate pH_i to fluorescence as described earlier [26]. When comparing differing stable cell lines, a correction for variations in the buffering capacity of the

Fig. 2. Effect of various treatments on NHE regulatory pathways in NRVM. A, Effect of 10 μM sorafenib (30 min) treatment on phospho-Erk levels of NRVM. Upper panel, immunoblotting with anti phospho-Erk antibody. Middle panel, immunoblot with anti Erk antibody. Lower panel, summary of results. B, Effect of 10 μM SB590885 (60 min) on NRVM. Upper panel, immunoblotting with anti-phospho-Erk antibody. Middle panel, immunoblot with anti Erk antibody. Lower panel, summary of results. C, Effect of ET-1 (1 nM, 5 min) on phospho-Erk levels. Upper panel, phospho-Erk protein; middle panel, Erk protein levels; lower panel summary of phospho-Erk levels corrected for Erk protein levels. + significantly different from control at $P < 0.01$. D, Effect of long-term ET-1 treatment on phospho-Erk and β-Raf protein levels. Upper panel, β-Raf protein levels, second panel, Erk protein levels, third panel, phospho-Erk levels, lower panel, Na^+/K^+ ATPase levels. * significantly different from control at $P < 0.05$.

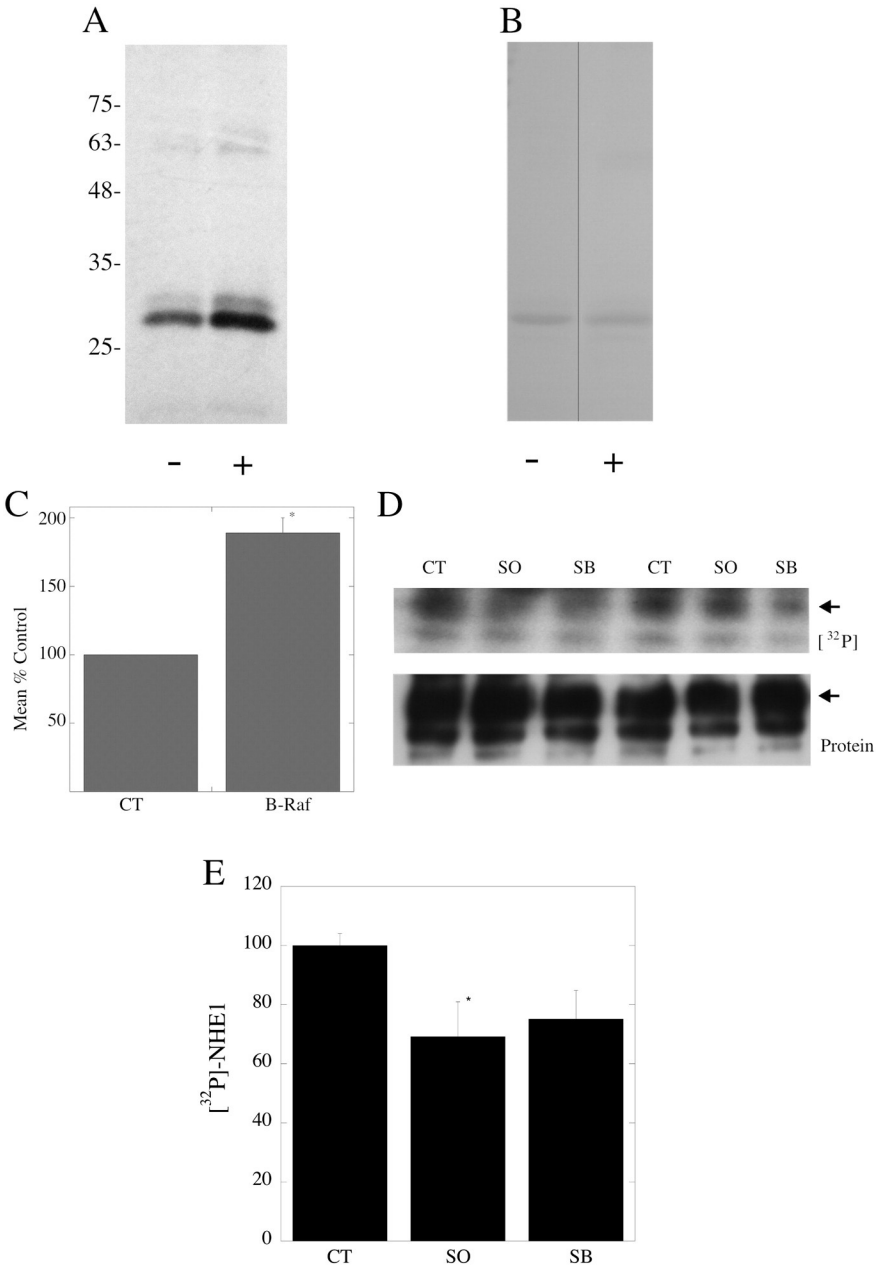

Fig. 4. Phosphorylation of NHE1 by β-Raf *in vitro* (A–C) and *in vivo* (D, E). A–C Phosphorylation of purified NHE1 C-terminus by β-Raf immunoprecipitate. β-Raf was immunoprecipitated from NRVM as described in Fig. 3. The immunoprecipitate was then incubated with a histidine tagged fusion protein containing the C-terminal 182 amino acids of the NHE1 protein in a phosphorylation reaction containing [γ-^{32}P]ATP. A, Autoradiogram indicates example of phosphorylated proteins. "−" indicates a mock immunoprecipitation in the absence of β-Raf antibody. "+" indicates immunoprecipitation using β-Raf antibody. B, Coomassie blue stain of samples of reaction mixture examined to ensure equivalent amounts of protein in assay. C, Summary of typical results of three experiments (mean ± SE) comparing results with mock immunoprecipitate to results with β-Raf immunoprecipitate. * significantly different from control at $P < 0.05$. D, Autoradiogram of phosphorylated immunoprecipitated NHE1 protein. AP-1 cells containing HA-tagged NHE1 protein were incubated with [^{32}P] in the presence or absence of 10 μM sorafenib (SO) treatment (30 min) or 10 μM SB590885 (SB, 1 h) as described in the "Materials and methods". Immunoprecipitated proteins were examined by autoradiography (D, upper panel) and levels of NHE1 phosphorylation were corrected for the amount of protein immunoprecipitated (D, lower panel). Arrows indicate location of full-length ≈ 100 kDa NHE1 protein. E, Summary of four experiments. * significantly different from control at $P < 0.05$.

stable cell lines was done as described earlier [14,26]. There was no difference in the buffering of cells with acute treatments with drugs or hormones as indicated by the degree of acidification caused by ammonium chloride. Cells were routinely incubated in low-serum media (0.5% FBS) overnight prior to hormonal treatments. For some experiments using treatments with various compounds we used a two-pulse assay with two treatments of ammonium chloride as described earlier [20]. In these cases we compared the value of the second pulse to that of first in control assays with no inhibitors. Then an independent two-pulse assay, in which the second pulse contained inhibitor, was compared to the control two pulse assay. All results are shown as means ± SE, and statistical significance was determined using the Wilcoxon-Mann-Whitney rank sum test.

2.9. Site-specific mutagenesis

Mutagenesis of NHE1 was using the plasmid pYN4+ described above [27,28] which contains full length NHE1 with a HA (hemagglutinin) tag for detection of NHE1 which does not affect activity [29]. Mutations created a restriction enzyme site for use in screening for mutants. The primers used

to mutate Thr653 to Ala and Asp were T653A, GCTGCGGTCC TACAACAGgCAtgCGCTGGTGGCAGACCCCTAC (its opposite strand) and T653D, GGCTGCGGTCCTACAACcGtCAtgatCTGGTGGCAGACCCCTACG and its opposite strand. (Lower case letters indicate mutations and underlined residues indicate new restrictions sites introduced for screening for mutations.) DNA sequencing confirmed the fidelity of DNA amplification and the presence of the mutation.

2.10. Cell culture and stable transfection

AP-1 cells are a mutant cell line derived from Chinese hamster ovarian cells that does not have an endogenous NHE1 [27]. Stably transfected cells were established using Lipofectamine™ 2000 Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) as described earlier [28]. Plasmids carrying a neomycin resistance gene, allowed the selection of transfected cells using geneticin (G418) antibiotics. pYN4 + plasmid which expresses the human NHE1 cDNA, HA tagged was used as a source of NHE1 [19]. Where indicated, cells were transfected with the plasmid described. Stable cell lines for experiments were regularly re-established from frozen stocks at passage numbers between 4 and 11. Results are typical of at least two stable cell lines.

For some experiments, where indicated, H9C2 cardiomyocyte cells were grown in DMEM/F12 1:1 culture media supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere (95% O_2-5% CO_2) as described earlier [30]. Intracellular pH determination and transfection with Lipofectamine™ 3000 were as described above and below respectively.

2.11. siRNA transfection

For siRNA reduction of β-Raf levels the following sets of RNA oligonucleotides were used to reduce wild type β-Raf. A set of double stranded oligonucleotides from the company Invitrogen based on the sequence 5′-GAUUUGCUGUUUGUCUCCAAGUUCA-3′ (referred to as #1) and another set of double stranded oligonucleotides from IDT (Integrated DNA Technologies) based on the sequence 5′-GUAAUCAGAAUGCAAGAUAAAAACC-3′ referred to as #2. The transfection was done with Lipofectamine™ 3000 following the manufacturer's protocol 200 nM siRNA (anti-β-Raf or control) was used for 48 h. A set of non-specific double stranded oligonucleotides was used as a control as described earlier [14].

A.

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1     MILRNNLQ KTRQRLRSYN RHTLVADPYE EAWNQMLLRR QKARQLEQKI 680

49  NNYLTVPAHK LDSPTMSRAR IGSDPLAYEP KEDLPVITID PASPQSPESV 730

99  DLVNEELKGK VLGLSRDPAK VAEEDEDDDG GIMMRSKETS SPGTDDVFTP 780

149  APSDSPSSQR IQRCLSDPGP HPEPGEGEPF FPKGQHHHHH H          815
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B.

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1     MILRNNLQ KTRQRLRSYN RHTLVADPYE EAWNQMLLRR QKARQLEQKI 680

49  NNYLTVPAHK LDSPTMSRAR IGSDPLAYEP KEDLPVITID PASPQSPESV 730

99  DLVNEELKGK VLGLSRDPAK VAEEDEDDDG GIMMRSKETS SPGTDDVFTP 780

149  APSDSPSSQR IQRCLSDPGP HPEPGEGEPF FPKGQHHHHH H          815
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Fig. 5. His182 protein sequence mapping results. A, Results from protein mock phosphorylated with immunoprecipitate from neonatal cardiac myocytes. B, Results from protein phosphorylated with β-Raf immunoprecipitate from neonatal cardiac myocytes. Underlined residues indicate coverage in mass spectrometry. Residues struck through were expressed in the His182 fusion protein but are not part of the NHE1 sequence. Residues are numbered according to the size of His182 on the left. Numbering on the right is the residue number in the original NHE1 full-length sequence. The residue found phosphorylated T^{653}, is bold.

2.12. Cell surface expression

To determine targeting of the NHE1 protein to the cell surface, cell surface proteins were labeled with sulfo-NHS-SS-biotin and cells were solubilized [27]. Cell surface proteins (and cell surface NHE1 protein) were bound to immobilized streptavidin resin. Equivalent amounts of unbound and total protein were separated on SDS-PAGE. Western blotting was used to check for immunoreactive (HA-tagged) Na^+/H^+ exchanger (NHE1). It was not possible to efficiently and reproducibly elute NHE1 bound to the immobilized streptavidin resin because of the high affinity of streptavidin for biotin. Both the upper and lower HA-immunoreactive species of NHE1 protein were compared for plasma membrane targeting estimations. Quantification of the total and unbound NHE1 protein was using Image J and the percentage of the protein targeting on the cell membrane was calculated with the equation: (Total-unbound) / Total × 100%.

2.13. SDS-PAGE and immunoblotting

NHE1 expression in transfected AP-1 cell lines was confirmed by immunoblotting using antibodies against the HA tag on the C-terminus of the NHE1 protein. Cell lysates (100 μg) were run on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. Monoclonal anti-HA antibody was the primary antibody to identify HA-tagged NHE1. Peroxidase-conjugated goat anti-mouse antibody was the secondary antibody (Bio/Can, Mississauga, Canada). Reactive protein was visualized with X-ray film using the Amersham enhanced chemiluminescence western blotting and detection system. Quantification of the NHE1 protein was using Image J 1.35 software (National Institutes of Health, Bethesda, MD, USA).

3. Results

We characterized the effect of a number of inhibitors and stimulators of pathways related to Na^+/H^+ exchanger activity. Sorafenib is an inhibitor of β-Raf kinase activity [14]. NRVM were treated with 10 μM sorafenib for 30 min and we examined the effect on the rate of recovery from an acute acid load. The results (Fig. 1A, B) demonstrate that sorafenib causes a large and significant decrease in Na^+/H^+ exchanger activity. SB590885 is another more specific inhibitor of the β-Raf kinase that is also effective in preventing cardiac hypertrophy [31]. Treatment of NRVM with SB590885 resulted in a smaller but significant decrease in the rate of recovery from an acute acid load (Fig. 1B). Since LPA has been reported to stimulate NHE1 in fibroblast cells [32] and protein kinase C has been reported to regulate NHE1 in some cell types [33], we also examined the effect of LPA and the protein kinase C inhibitor Go6983 on cardiomyocytes (Fig. 1B). Cells were treated with LPA after being maintained overnight in low serum (0.5%) medium. LPA treatment had no effect on NHE1 activity. Go6983 had no significant effect on activity, though a small insignificant stimulatory effect was observed. For comparative purposes NRVM were treated with the selective NHE1 inhibitor 10 μM EMD87580 which inhibited >90% of NHE1 activity (Fig.

Table 1
Phosphopeptides identified by LC-MS/MS of in-gel MAAH of His 182-2. Numbering refers to that of the His182 peptide. Observed: observed peptide *m/z*; Mr(expt): observed peptide mass; Mr(calc): predicted peptide mass; ppm: mass delta between observed peptide mass and predicted peptide mass; miss: number of missed trypsin cleavage sites; deamidated (NQ): deamidation of N or Q residue; Phospho (T): phosphorylation of T residue; ions score: a measure of how well the observed MS/MS spectrum matches to the stated peptide.

Start–end	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
16–23 Deamidated (NQ); Phospho (T) (ions score 32)	535.7528	1069.4910	1069.4594	30	0	R.SYNRHTLV.A
16–24 Deamidated (NQ); Phospho (T) (ions score 26)	571.2651	1140.5156	1140.4965	17	0	R.SYNRHTLVA.D

1B). In another set of experiments we treated H9C2 cardiomyocytes with two different siRNA directed against β-Raf (Fig. 1C). The β-Raf siRNA reduced β-Raf levels approximately 50% (upper and middle panel). Treatment with β-Raf siRNA also significantly reduced Na^+/H^+ exchanger activity 10–15%. The extent of the reduction was not as great as with inhibitors of β-Raf, however this might be due to the incomplete reduction in β-Raf protein levels.

We also examined the effect of treatment of NRVM with ET-1. Fig. 1D shows that a 25 minute treatment with 5 nM ET-1 resulted in a small, but significant increase in the rate of recovery from an acute acid load. In contrast, long-term (24 h) treatment of the cells with ET-1 did not increase the rate of recovery from an acute acid load (Fig. 1E). There was no difference in the degree of acidification caused by ammonium chloride when comparing the control groups to the ET-1 treated groups, indicating there was no changing in buffering between controls and experimentals. We also measured the pH_i after 3 min of recovery from the ammonium chloride induced acid load. We found, that cells treated with ET-1 for 25 min recovered to a higher rate of pH_i than controls after 3 min (Fig. 1F).

To examine the mechanism of regulation of NHE1 by ET-1 and related pathways we examined the phosphorylation status of Erk, which has earlier been demonstrated to be important in regulation of NHE1 activity [21,34]. Sorafenib treatment caused a marked decrease in the level of phospho-Erk1/2 (Fig. 2A) while SB590885 also caused a decrease in the level of phospho-Erk (Fig. 2B). In contrast, treatment of cells with 1 nM ET-1 for 5 min resulted in a large increase in the level of phospho-Erk compared to unstimulated cells (Fig. 2C). Long-term treatment of NRVM had different effects. When NRVM where treated for 24 h with 5 nM ET-1, there was no increase in the level of phospho-Erk. The level of β-Raf protein increased slightly relative to either Erk protein levels (Fig. 2D, 14%) or relative to another control, the level of Na^+/K^+ ATPase protein (10%) but this increase was not statistically significant.

To determine if β-Raf from isolated NRVM could interact with the C-terminal tail of NHE1 we tested the binding of two His-tagged proteins containing the C-terminal 239 and 182 amino acids of the NHE1. A control His-tagged protein (CHP, calcineurin homologous protein) was used. These samples were transferred to nitrocellulose and incubated with NRVM cell extracts. Fig. 3A demonstrates Ponceau S staining (left panel) that confirms that the proteins all properly transferred. After incubation with NRVM cell extracts, immunoblotting with anti-β-Raf antibodies confirmed that β-Raf was bound to the NHE1 protein. Both the His239 and His182 protein bound β-Raf. There was no binding to the negative control.

We had previously demonstrated that β-Raf protein, which is present in heart cell extracts, could bind to an affinity column containing the C-terminal region of NHE1 [14]. To determine if this binding also occurs *in vivo* in isolated NRVM we immunoprecipitated β-Raf and checked for the presence of associated NHE1 protein. The results are shown in Fig. 3B. Anti-β-Raf antibody was used to immunoprecipitate β-Raf and associated proteins. Immunoblotting with anti-NHE1 antibody showed that the immunoprecipitate contained NHE1 protein (+). A mock immunoprecipitation in the absence of β-Raf antibody did not show any associated NHE1 protein (−). A dimeric form of NHE1 was present in the immunoprecipitate. We have previously shown that NHE1 can dimerize and that this varies with sample conditions and environment [35,36]. Western blotting of the intact lysate confirmed the presence of NHE1 in the sample (right lane).

We tested if β-Raf from the myocardium could phosphorylate the C-terminal 182 amino acid residues of NHE1. β-Raf was immunoprecipitated from NRVM and incubated with a histidine tagged C-terminal fusion protein in the presence of [γ-^{32}P]ATP as described in the "Materials and Methods". The results are shown in Fig. 4. Incubation of the C-terminal region of NHE1 with an immunoprecipitate resulted in marked phosphorylation of the C-terminal of NHE1 with results summarized in Fig. 4C. A mock immunoprecipitation from NRVM lysate in the absence of β-Raf antibody resulted in a background level of phosphorylation, but this was greatly reduced relative to that of the experimental. Samples of reactions mixtures were run to ensure equivalent amount of proteins in each reaction (Fig. 4B).

In vivo phosphorylation of NHE1 by β-Raf was examined in AP-1 cells containing HA-tagged NHE1 protein. The amount of immunoprecipitate was corrected for the amount of protein present (Fig. 4D, E). We found that the mean level of NHE1 phosphorylation was reduced by about 30% by both sorafenib and SB590885, in the case of sorafenib the decrease was significant.

We next determined the phosphorylation site of β-Raf immunoprecipitates on the His182 fusion protein. *In vitro* phosphorylation of NHE1 was followed by mass spectrometry analysis. The results are shown in Fig. 5. For the control, a mock immunoprecipitation in the absence of β-Raf antibody, 541 peptides were identified when we searched the LC-MS/MS data against the sequence of His 182. The sequence coverage was 93% (Fig. 5A). No phosphopeptide was found in this protein. For the His182 protein phosphorylated by β-Raf immunoprecipitate, 613 peptides were identified when we searched the LC-MS/MS data against the sequence of His182. The sequence coverage was 95% (Fig. 5B). Both phosphorylated and non-phosphorylated T^{21} were identified from this gel band, which corresponds to T^{653} in the full length NHE1 sequence. Two phosphopeptides, R.SYNRHT^{P}LV.A and R.SYNRHT^{P}LVA.D were found from this protein (Table 1). For these two phosphopeptide matches, manual inspection of the MS/MS spectra and peak assignment was performed to confirm the phosphorylation site assignment.

Because we found that T^{653} was phosphorylated *in vitro* by a β-Raf immunoprecipitate, we examined the effect of mutation of this residue on the function of the NHE1 protein. This amino acid was mutated to both the non-phosphorylatable residue Ala, or to the phosphomimetic residue Asp. Initially we determined the effects on protein expression and targeting. Stable cell lines of the wild type and mutant NHE1 proteins were characterized. Fig. 6A shows that the wild type and mutant NHE1 proteins all were expressed, with the characteristic pattern of a fully glycosylated protein and a partial or de-glycosylated NHE1 protein [28]. There were some minor differences in the expression level between the mutants and the control protein. Further examination of the surface targeting of the control and mutant proteins, showed only very minor, non-significant differences in the level of NHE1 protein targeted to the cell surface (Fig. 6B).

We next characterized the activity of the NHE1 protein including a correction for the level of protein expressed. Here the relative rate of change of pH_i is illustrated. Fig. 6C illustrates a summary of the rate of recovery from an acute acid load, induced by ammonium chloride. The results demonstrated that the mutation T653A slightly reduced NHE1 activity in comparison to control. There was no significant difference between the activity of the T653D mutant protein and the wild type. To further characterize the activity of these proteins we examined the proton flux of these cells, including a correction for variations in the buffering capacity of the stable cell lines as described earlier [26]. The results (Fig. 6D) showed that the T653A mutant protein had reduced activity relative to the wild type. The T653D NHE1 mutant protein was similar in its activity to the wild type NHE1 protein.

4. Discussion

The Na^+/H^+ exchanger isoform 1 (NHE1) plays a critical role in myocardial ischemia reperfusion damage and in heart hypertrophy. Numerous studies have shown that in animal models, NHE1 inhibition is protective of myocardial ischemia reperfusion injury [6,37]. However, unfortunately, clinical studies have met with very limited success (reviewed in [37]). It is of note that in one clinical trial NHE1 inhibition did result in a protective effect on myocardial infarction, but off target effects resulted in termination of the trial [37]. Nonetheless, it has been suggested that NHE1 is still a viable clinical target [37,38] and that targeting regulation of NHE1 may be a useful approach [39]. This makes a better understanding of NHE1 regulation of prime importance.

We have earlier shown that the NHE1 protein is regulated by a MAPK-signaling pathway [16,20] however the NHE1 protein appears to be regulated by several hormones and has numerous potential sites of phosphorylation and protein binding sites [5]. Because regulation of NHE1 has been shown to vary and to affect the pathology of myocardial disease [8,40] further investigation into the mechanism of regulation may lead to insights into the prevention of pathology. In this study, we examined the mechanisms of regulation of NHE1 by β-Raf and putative links to ET-1. We initially demonstrated that the protein kinase inhibitor sorafenib reduces the rate of recovery from an acute acid load in NRVM (Fig. 1). Sorafenib is a known inhibitor of β-Raf kinase but is not a specific inhibitor of β-Raf. We therefore tested the effect of a more specific β-Raf kinase inhibitor SB590885 and of siRNA against β-Raf. These treatments also resulted in significant reduction in NHE1 activity, though less than with sorafenib. These results suggest that β-Raf does play a significant role in regulation of Na^+/H^+ exchanger activity in cardiomyocytes, though clearly other protein kinases also facilitate increases in activity. We have earlier shown that β-Raf is important in regulation of NHE1 in malignant melanoma cells [14]. Our present results confirm that this regulation also occurs in NRVM. To confirm that NHE1 in NRVM was coupled to receptors, we also tested the effect of short and long-term treatment of cells with ET-1 on activity. The results confirmed that short-term treatment with ET-1 elevates NHE1 activity, while long-term treatment did not. It is not clear why long-term treatment did not elevate NHE1 activity considering that long-term ET-1 treatment has been shown to induce NHE1-dependent hypertrophy [11]. Long-term treatment may also increase dephosphorylation of NHE1 by activating NHE1-directed phosphatases and ET-1 has been shown to activate calcineurin [41]. Additionally, long-term adaptive effects on the myocardium of ET-1 treatment have been noted earlier [42] so that secondary effects could be partially responsible for the hypertrophy. We have earlier found that activation of Erk1/2, a regulator of NHE1 in NRVM, can peak early and then decline rapidly with time [43]. In the present study, we also found that ET-1 activates Erk1/2 acutely (Fig. 2C) but not with long-term treatment (Fig. 2D). Further studies are necessary to determine the mechanism by which NHE1 dependent cardiac hypertrophy is mediated in the long term.

We did not find a stimulatory effect of LPA on NRVM (Fig. 1B). This is in contrast to the effect reported by Wallert et al. [32]. It is possible that the difference between the results of the two studies is because of the difference in cell type. Their study used fibroblasts while the present study examined cardiomyocytes.

Another finding was that after 3 min after recovery from ammonium chloride treatment the resting pH_i of cells was elevated with ET-1 treatment. This is in keeping with the acute effect of ET-1 on NHE1 activity. We also tested the effect of the PKC inhibitor Go6983 on NHE1 activity. This inhibitor did not affect NHE1 activity. Though protein kinase C inhibition has been shown to affect stretch induced activation of NHE1 in papillary muscle [44], in this system a role for PKC was not apparent. We have earlier [45] shown that the cytosolic tail of human NHE1 is not directly phosphorylated by PKC, which may partially explain these results.

To determine if the MAPK pathway could be involved in activation of NHE1 by ET-1 we examined phospho-Erk levels of NRVM in response to protein kinase inhibitors or hormonal stimulation. We found (Fig. 2) that the protein kinase inhibitor sorafenib greatly reduced the level of phospho-Erk in comparison to controls. Similarly, the more specific β-Raf inhibitor SB590885 also greatly reduced the levels of phospho-Erk in comparison to controls. These results demonstrate that protein kinases, and specifically β-Raf could have a regulatory effect on the pathway known to affect the NHE1 protein [14]. We have earlier demonstrated that Erk directly phosphorylates the cytoplasmic tail of the NHE1 protein with sites of phosphorylation at amino acids 770 and 771 of the regulatory tail [20,21,34]. Additionally, inhibitors of the Erk kinase (MEK) prevented activation of the NHE1 protein in NRVM, kidney and CHO cells [20,34]. The present results, confirm that another upstream element of this pathway β-Raf, is also involved in regulation of NHE1 in the myocardium.

Previously [14] we showed that a myocardial cell lysate that contains β-Raf could bind to the C-terminal tail of NHE1 that is immobilized on an affinity column. We also demonstrated that in Hela and HEK cells, β-Raf is associated with NHE1 and regulates activity. In the present study we demonstrate by two different methods, that myocardial β-Raf associates with NHE1. We show that β-Raf from myocardial cells associates with the immobilized C-terminal 182 amino acids of NHE1. Further, we demonstrate an *in vivo* association by co-immunoprecipitation of β-Raf and NHE1 from isolated NRVM (Fig. 3). These results confirm a regulatory role for β-Raf through binding to NHE1 in the myocardium by two independent methods.

To determine a possible mechanism by which β-Raf can regulate NHE1 activity, we used a β-Raf immunoprecipitate and examined the ability to phosphorylate the C-terminal 182 amino acids of NHE1. We found that the immunoprecipitate could in fact phosphorylate NHE1 significantly. There was a background level of phosphorylation. The cause of this is not yet known for certain but could be due to binding of ATP to the NHE1 C-terminal without specific amino acid phosphorylation. The C-terminal of NHE1 is known to contain an ATP binding site [46].

Using mass spectrometry we found that a β-Raf immunoprecipitate from the myocardium phosphorylated amino acid Thr^{653} of a NHE1 fusion protein. A control immunoprecipitation did not demonstrate phosphorylation of any peptides. This contrasts with a basal level of phosphorylation demonstrated in Fig. 4. The discrepancy supports the suggestion that the background level of phosphorylation observed in *in vitro* phosphorylation assays might be due to ATP binding, which would not remain attached to the protein and detectable during mass spectrometry analysis. Thr^{653} is a different site than reported earlier by the Erk dependent pathway. Previously Ser^{770} and Ser^{771} have been suggested to be directly phosphorylated by Erk along with some more distal amino acids [20,21,34].

When we mutated Thr^{653} we found varying results on intracellular pH regulation. Changing this residue to Ala caused a decrease in the rate of recovery from an acute acid load (Fig. 6). Mutation of this residue to an Asp residue restored the activity of the NHE1 protein. One might expect the T653D mutation to be more active than the wild type however phosphomimetic mutations do not always perfectly mimic a phosphorylated amino acid and the slightly different structure of negatively charged Asp *vs.* phospho-Thr might account for this discrepancy.

While this work was in progress, a report also showed that Thr^{653} is involved in NHE1 regulation [32]. However, there were significant differences between our report and this one. They found that in fibroblasts another kinase, Rock I and II, phosphorylates Thr^{653}. We examined if these kinases were present in our immunoprecipitate, but were unable to demonstrate their presence using antibodies against the Rock proteins (Supplementary figure). It has long been known that different protein kinases can phosphorylate the same protein at the same amino acids [47,48]. Thus it is possible that both Rock and β-Raf regulate NHE1 at this amino acid. The study of Wallert et al. [32] also found that the T653A mutation caused a defect in NHE1 activity. In this case they measured the ability of lysophosphatidic acid to activate the protein. Lysophosphatidic acid is known to activate the NHE1 protein, along with many other regulators of NHE1 [49]. Though we did not find LPA stimulated NHE1 in isolated cardiomyocytes, as noted above, this may be because of the difference in cell types. Other results are similar to the previous findings. We found that the T653A mutation was detrimental to the activation of the NHE1 protein. In our case we also examined the phosphomimetic mutation, T653D and found that it was more functional than the T653A mutation. While we did not find the same results as Wallert et al. [32], that reported that the T653D mutant was elevated in activity, this might be because the negatively charged Asp does not mimic phospho-Thr perfectly. An alternative explanation

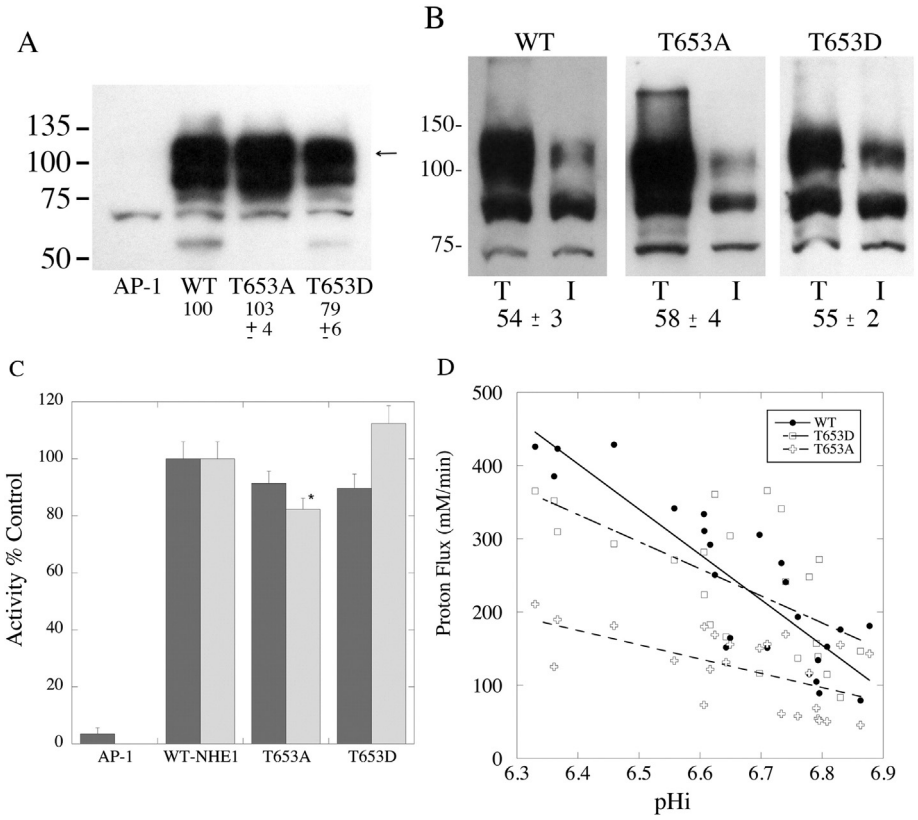

Fig. 6. Characterization of expression and activity of wild type (WT) and NHE1 mutant proteins. A, Western blot of whole cell lysates of stable cell lines expressing WT Na^+/H^+ exchanger or T653A or T653D mutants. The primary antibody was against the HA tag. The arrow indicates the position of full-length glycosylated NHE1 protein. AP-1 is a cell lysate from mock transfected AP-1 cells. Numbers indicate the expression levels of mutants in comparison to WT protein, mean ± SE. n = at least 3 determinations. B, Surface localization of NHE1 in AP-1 cells expressing wild type (WT) and T653A or T653D mutants. Equal amounts of the total cell lysate (T) and the unbound intracellular lysate (I) were examined by western blotting to identify the HA-tagged NHE1 protein. WT and mutant cell lysates expressing NHE1 proteins are shown. Numbers indicate the mean ± the SE of cell surface protein from at least 3 determinations. Arrow indicates the position of full-length glycosylated NHE1 protein. C, Summary of analysis of NHE1 activity of stable cell lines containing wild type (WT) and mutant NHE1 protein. NHE1 protein activity was assayed in stably transfected AP-1 cells with the mutants indicated as described in the "Materials and methods". Results are change in intracellular pH/s relative to control, WT NHE1 protein. The mean value of the control NHE1 activity was 0.021 ΔpH/s. n = at least 8 determinations. Grey bar indicates uncorrected activity (relative to control). Hatch bar, activity corrected for differences in surface processing and protein expression levels. AP-1, mock transfected AP-1 cells. Mutant cell lines are indicated. * indicates significantly different from control at $P < 0.05$. D, Analysis of proton flux of stable cell lines containing wild type (WT) and mutant NHE1 proteins indicated. NHE1 protein activity was assayed in stably transfected AP-1 cells with the mutants indicated as described in the "Materials and methods". Simple linear regression fit is shown.

is that amino acid residues surrounding T^{653} have varying influence under different conditions and in different tissues. NHE1 is a direct target of Erk1/2, and $p90^{RSK}$ [16,21]. Another amino acid, S^{648} has been shown to be an important regulator of NHE1 activity [50] and is in close proximity to T^{653}. Its phosphorylation or dephosphorylation may influence the phosphorylation of T^{653} and its effect on the protein. Future studies will examine the role of S^{648} in combination with T^{653} or other phosphorylation sites.

NHE1 in the heart is implicated in both myocardial damage from ischemia/reperfusion injury, and in promoting hypertrophy (reviewed in [5,51]). Activation of NHE1 through the calcium-calmodulin domain promotes heart hypertrophy [8]. Coincidently, β-Raf and Raf-1 are involved in cardiac hypertrophic pathways in response to pressure overload or hormonal activation by angiotensin II and ET-1 in the myocardium. Inhibitors or knock down of Raf proteins have been used to prevent some forms of cardiac hypertrophy in animal models [52–54]. At present, it is not clear if β-Raf mediated phosphorylation at amino acid T^{653} could stimulate heart hypertrophy but our data suggest there is a possible requirement for this amino acid. Our results do demonstrate that β-Raf in the myocardium is capable of binding to NHE1 and influencing its activity. Future studies could examine if the T^{653} residue can affect calcium-calmodulin mediated heart hypertrophy *in vivo*.

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Disclosures

The authors declare they have no conflicts of interest with the contents of this article.

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