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Sustained acidosis and phenylephrine activate the myocardial Na^+/H^+ exchanger through phosphorylation of Ser^{770} and Ser^{771}

by

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To my husband Costas whose love and support is endless
To my mother, father and brother, who have always encouraged my love of science

Abstract

The mammalian Na^+/H^+ exchanger isoform 1 (NHE1) is a ubiquitously expressed membrane protein that regulates myocardial intracellular pH. Inhibition of NHE1 prevents hypertrophy and reduces ischemia-reperfusion (I/R) injury in animal models. To understand the regulation of NHE1 in the myocardium by phosphorylation we constructed adenoviruses, which express wild type or mutant cDNA for NHE1. Additionally, wild type and mutant NHE1 had mutations Leu163Phe/Gly174Ser, which increases NHE1 resistance to EMD87580 (NHE1 inhibitor) by 100-fold. This allowed measurement of exogenous NHE1 activity while inhibiting endogenous NHE1 activity. We examined the effects of a series of mutations of phosphorylation sites in the cytosolic domain of NHE1. Sustained intracellular acidosis and phenylephrine caused an ERK-dependent activation of NHE1 activity and phosphorylation levels. We demonstrated that amino acids Ser^{770} and Ser^{771} were essential for activation of NHE1 activity in isolated rat cardiomyocytes by sustained intracellular acidosis and phenylephrine. Furthermore, mutation of Ser^{770} and Ser^{771} to Ala prevented increased NHE1 phosphorylation by sustained intracellular acidosis and phenylephrine. This was found to occur in an ERK-dependent manner. Taken together, our results demonstrate that both sustained intracellular acidosis and phenylephrine rapidly activate the NHE1 protein in isolated cardiac cells via an ERK-dependent pathway that acts on the common amino acids Ser^{770} and Ser^{771} of the C-terminal tail of NHE1.

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Abbreviations

aa	Amino acid
α-MEM	Modified Eagle's medium, α-modification
Ad	Adenovirus
AE1	Anion exchanger isoform 1
Amiloride	3,5 diamino-6-chloro-N-(diaminomethylene)pyrazine-cardoxamide
Ang II	Angiotensin II
AP-1	Chinese hamster ovary cell line deficient in plasma membrane Na^+/H^+ exchanger
AP-2	Activator protein-2
AR	Adrenergic receptor
ARVM	Adult rat ventricular cardiomyocytes
ATP	Adenosine 5'-triphosphate
BCECF-AM	2'-7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxy methyl ester
bp	base pair
CAII	Carbonic anhydrase II
CaM	Calmodulin
cDNA	Complementary deoxyribonucleic acid
CFTR	Cystic fibrosis transmembrane conductance regulator
CHP	Calcineurin B homologous protein
CHP1	Calcineurin B homologous protein isoform 1
CHO	Chinese hamster ovary cell line
COUP-TFs	Chicken ovalbumin upstream promoter transcription factors
DMA	5-(N,N-dimethyl) amiloride
D-MEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
EIPA	5-(N-ethyl-N-isopropyl) amiloride
EL	Extracellular loop
EMD87580	N-(4,5-Bis-methansulfony-2-methyl-benzoyl) guanidine
ERK	Extracellular signal-related kinase
ERM	Ezrin, radixin, and moesin
ET-1	Endothelin-1
FMK	Fluoromethylketone
GPCR	G protein coupled receptors
HA	Hemagluttinin
HEK293A	Human embryonic kidney 293A cell line
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HSP70	Heat shock protein 70 kDa
IC_{50}	Half maximal inhibitory concentration

IgG	Immunoglobulin G
IL	Intracellular loop
IRM	Inhibitor resistant mutant
I/R	Ischemia/reperfusion
kb	kilobase
kDa	kilodalton
LF2000	Lipofectamine™ 2000 reagent
LPA	Lysophosphatidic acid
MAPK	Mitogen activated protein kinase
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
Mutant 1	NHE1 mutant S693A
Mutant 2	NHE1 mutant T718A and S723/726/729A
Mutant 3	NHE1 mutant S766/770/771A
Mutant 4	NHE1 mutant T779A and S785A
NHE	Na^+/H^+ exchanger
NHE1	Na^+/H^+ exchanger isoform 1
NHE (#)	Na^+/H^+ exchanger isoform (#)
NIK	Nck-interacting kinase
NMR	Nuclear magnetic resonance
NRVM	Neonatal rat ventricular cardiomyocytes
p90RSK	p90 ribosomal S6 kinase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phenylephrine
pHi	Intracellular pH
PIP2	Phosphatidylinositol 4,5-bisphosphate
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PP2B	Protein phosphates 2B
SA	Sustained acidosis
SDS-PAGE	Sodium dodecyl polyacrylamide gel electrophoresis
SE	Standard error
SIA	Sustained intracellular acidosis
TM	Transmembrane segment

Chapter 1:

Introduction

1.1 Regulation of intracellular pH in mammalian cells

The family of mammalian Na^+/H^+ exchange (NHE) proteins are membrane proteins that catalyze the electroneutral exchange of one intracellular proton (H^+) for one extracellular sodium ion (Na^+). These ion exchanging proteins play an essential role in the maintenance of pH in mammalian cells. Alterations in cellular pH trigger a multitude of events such as cell growth and differentiation. In addition, intracellular acidosis inhibits the activity of a variety of enzymes and therefore various aspects of cell metabolism. Thus, it is critical to maintain intracellular pH in order to maintain cellular homeostasis. The Na^+/H^+ exchangers are the major pH regulatory system in most mammalian cells (1). Na^+/H^+ exchangers also regulate cellular volume by uptake of Na^+ and are stimulated by hypertonic conditions (2). Therefore, Na^+/H^+ exchangers play an important role in many basic cellular functions.

1.2 Mammalian Na^+/H^+ exchanger family of proteins

The family of mammalian Na^+/H^+ exchanger (NHE) proteins are a class of membrane proteins that exchange one proton (H^+) for one sodium ion (Na^+). The family of NHE proteins includes ten isoforms (NHE1-10) each with distinct gene products, unique tissue distribution and physiological roles (reviewed in (3)). The protein identity of the NHE isoforms varies from 25-70%, with calculated molecular masses between 74,000 and 93,000 Da. All NHE isoforms share

common predicted secondary structure, with 12 conserved transmembrane segments and a divergent cytoplasmic domain (4).

1.2.1 NHE subtypes and distribution

The first NHE cloned was NHE1 by Sardet *et al.*, in 1989; they also were the first to describe the primary structure of NHE1 (5). NHE1 was found to be ubiquitously expressed in the plasma membrane of mammalian cells and is thus considered the "housekeeping" isoform. Furthermore, the NHE isoform 1 is the most extensively studied protein of the Na^+/H^+ exchanger family. NHE1 was also identified as the predominant isoform in the myocardium (6-8). This isoform was found to be the most sensitive to NHE inhibitors, such as amiloride, amiloride derivatives, and more recently to specific NHE1 inhibitors including the benzoylguanidine inhibitors (reviewed in (9)). The NHE family of proteins has many important physiological roles including the maintenance of pH_i and cell processes such as growth, proliferation, differentiation, migration and apoptosis (10-12). NHE also plays pathophysiological roles in both heart disease and cancer (reviewed in (13,14)).

Other plasma membrane isoforms of NHE including NHE2-4 are expressed predominantly in the kidney and gastrointestinal tract. Specifically NHE2 and 3 localize to the apical membrane of epithelia in the kidney and intestine, whereas NHE4 is most abundant in the stomach but is also expressed in the intestine, kidney, brain, uterus, and skeletal muscle (15-17). NHE2 has been shown to play an important role in secretory processes, whereas NHE3 is

responsible for the majority of renal and intestinal Na^+ absorption. Both NHE3 and 4 are insensitive to amiloride and non-amiloride NHE inhibitors (18,19). NHE5 is expressed in the brain and shows similarity in structure to both NHE1 and 3, 39% and 53%, respectively (20). Furthermore, NHE5 shows similar NHE inhibitor sensitivities as NHE3 (21). However, no physiological role of NHE5 in the brain has yet been demonstrated.

The NHE isoforms 6-9 are the most divergent from plasmalemmal NHEs (1-5); they are expressed ubiquitously amongst tissues but are restricted to intracellular compartments. Specifically, NHE6-9 are localized to intracellular organelle membranes, including endosomes and the Golgi network (22,23). These intracellular NHEs likely play a role in organelle and luminal pH and cation maintenance (24). NHE6 is expressed in early recycling endosomes of metabolically active tissues such as skeletal muscle, heart and brain (25). NHE7, however, is localized to the trans-Golgi network where it functions to mediate the exchange of Na^+ or K^+ for H^+ (26). NHE8 expression is localized to the mid- to trans-Golgi network in skeletal muscle and the kidney (24,27). NHE9 is localized to the late recycling endosomes (24). NHE10, an osteoclast-specific NHE, has been shown to play an important role in osteoclast differentiation and survival (28). Recently, a gene was found in the spermatozoa to express a sperm NHE. This sperm-specific NHE appears to be essential for sperm motility and male fertility (29,30); however, its ability for Na^+/H^+ exchange has not been shown.

1.2.2 Physiological roles of NHE1

NHE proteins have many important physiological roles in addition to pathological ones (briefly described here, see (4,31-33) for reviews). Some of these physiological roles apply to NHE in the myocardium while others are tissue specific. As an ion transporter, its most basic function in all cell types is the maintenance of pH_i and volume control. NHE is involved in normal cell processes such as growth, proliferation, differentiation, migration, apoptosis and cell survival (3,10-12,34). NHE1 deficient cells display pH dependent cell growth, and growth is inhibited at neutral and acidic pHs (10). This study was the first to show that NHE1 activity and intracellular pH play a crucial role in cell growth. NHE1 deficient mice were found to have decreased post-natal growth, while exhibiting ataxia and epileptic-like seizures (35,36). Furthermore, NHE1 has been implicated in cell-cycle progression either directly by activating progression of cells from the G2 to M phase of the cell cycle or indirectly by stimulating the expression of genes involved in cell-cycle progression (37,38). NHE1 is also permissive in many types of cellular differentiation (11,39,40). Taken together, the role of NHE1 in cell growth, proliferation and differentiation indicates that NHE1 is a critical protein in normal cellular processes.

NHE1 association with ezrin, radixin and moesin (ERM) proteins is involved with its role in cell migration (12,41). Association of NHE1 with ERM also acts as an anchor for the cytoskeleton. Studies implicate NHE1 activity in promoting apoptosis, but this varies according to cell type studied (42,43). In

mouse pro β cells, apoptosis is activated by NHE1 upon trophic factor withdrawal and this occurs via phosphorylation of the cytoplasmic tail of NHE1 by the p38MAPK (43). In contrast, NHE1 has also been shown to activate the pro-survival kinase Akt in tumor cells (44).

Apart from pathological functions in the myocardium (section 1.5.2), NHE1 plays a role in malignant transformation, tumor cell migration, and invasion (45,46). Specifically, malignant cells display alkaline intracellular pH and the maintenance of this altered pH*i* by NHE1 has been shown to be a key mechanism in oncogenic transformation and contributes to maintaining cancer cells in a transformed state (46). NHE1 is also important in breast cancer cell invasion by regulating cytoskeletal organization via the RhoA/p160ROCK/p38MAPK signaling pathway (47). The role of NHE1 in cancer has led to NHE1 inhibition as a potential source for anti-oncologic therapies (reviewed in (14)). Overall, these studies, and others, have shown that NHE1 is an important regulatory protein within and outside the myocardium.

1.3 Na^+/H^+ exchanger structural domains

NHE is a multi-spanning transmembrane protein with a large cytoplasmic domain (5). Wakabayshi *et al.* used cysteine accessibility analysis to reveal that NHE has 12 transmembrane spanning segments (TM I-XII), with one extracellular and two intracellular membrane associated loops (re-entrant loops) (48) (Figure 1.1). NHE1 has approximately 815 amino acids. The first 500 amino

acids comprise the membrane domain, which acts as the ion transporter, and the remaining 315 amino acids are part of the regulatory cytoplasmic domain.

NHE is post-translationally modified with *N*- and *O*-linked glycosylations on extracellular loop 1 (49,50). Two forms of NHE1 are observed when separated by SDS-PAGE, a 110 kDa and a 85 kDa band representing the fully glycosylated mature protein and an immature partially or non-glycosylated protein, respectively. However, *N*-linked glycosylation can be removed without affecting NHE1 function (49).

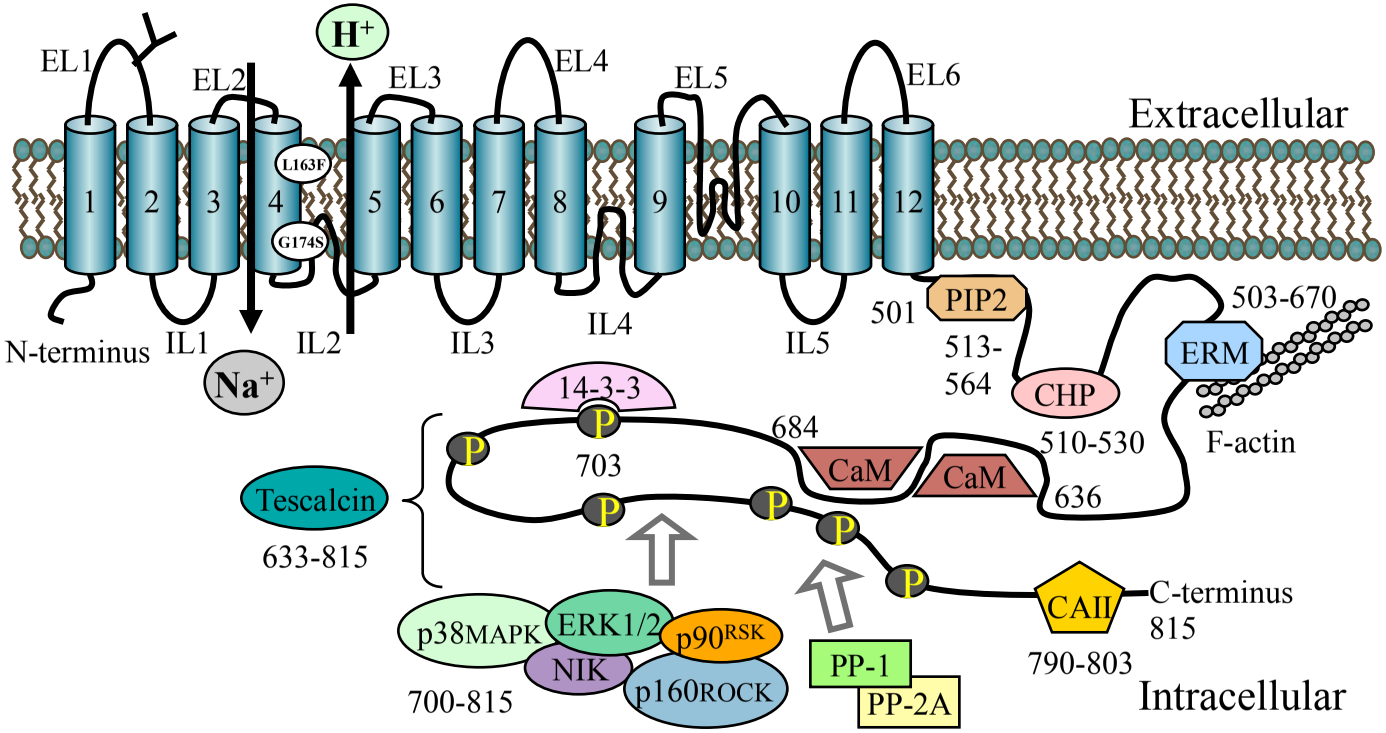

Figure 1.1 Topology model of NHE1. Topology model of NHE1 displaying the membrane and cytosolic domains. The cytoplasmic tail displays the major protein-protein interactions and kinases which have been shown to regulate NHE1. PIP2, phosphatidylinositol 4,5-bisphosphate; CHP, calcineurin homologous protein; ERM, ezrin, radixin, moesin; CaM, calmodulin; CAII, carbonic anhydrase II; ERK1/2, extracellular signal regulated kinase 1/2; $p90^{RSK}$, p90 ribosomal S6 kinase; NIK, nck interacting kinase; p38 MAPK, p38 mitogen activated protein kinase ; p160ROCK, Rho-associated coiled coil forming kinase 1; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A.

1.3.1 Structure of the membrane domain

Little is known about the tertiary structure of the mammalian NHE. Topology models of NHE1 have been based on hydropathy analysis, cysteine accessibility analysis, and more recently, using phylogenetic and empirical data. However, to date only the structures of mammalian NHE transmembrane segments IV, VII, IX and XI have been characterized by high-resolution nuclear magnetic resonance (NMR) analysis (51-54). Transmembrane segment IV was shown by NMR to start with a series of β-turns, an extended flexible region and then a helix (52). TM VII of NHE1 is mostly α-helical with a break near the middle of this helix at functionally important residues (53). The NMR structure of TM IX revealed two α-helical regions separated by a kink around a pivot point (51). Recently published studies of TM XI showed two α-helical regions connected by a poorly defined region (54). Further analysis of the quaternary structure of NHE1 revealed that NHE1 forms isoform specific homodimers, that contain independently active subunits (55). This dimerization has been shown to be essential for NHE1 in maintaining physiologic pH*i* sensitivity (56).

1.3.2 Structure of the cytoplasmic domain

The C-terminal cytosolic domain regulates the membrane domains' ion exchange activity and is the site of phosphorylations and protein and cofactor interactions. Circular dichroism analysis of the C-terminal tail of NHE1 revealed ~17% ß-turn, ~35% α-helix and the remaining 48% in random coil (57). Our

laboratory has shown that the distal region of the C-terminus of NHE1 can change conformation depending on pH and calcium ion concentration (58). The membrane proximal region is more compact whereas the distal region is more flexible, thereby potentially enabling structural changes due to protein binding or phosphorylation.

1.3.3 Structure of the NhaA

Recently the structure of the *E.coli* Na^+/H^+ antiporter (NhaA) was solved by high resolution crystallography (59). The NhaA structure revealed similar basic topology to NHE1, consisting of 12 transmembrane segments, and N- and C- terminal cytoplasmic domains. Furthermore, ten of the twelve transmembrane segments are classical α helices, however two, specifically TMIV and XI, differ in that they contain a short α helix followed by an extended region, and another short α helix. This was similar to what was observed by the NMR analysis of the mammalian NHE1 TM IV and XI structures (52,54). Although the eukaryotic NHE isoform exhibits little similarity to the *E.coli* isoform, they both retain the same function, and therefore provide insight into the tertiary structure of NHE1. Recent studies have used the structure of NhaA as a template to predict the structure of the mammalian NHE1, even though there is only 10% amino acid sequence similarity. Furthermore, this new three dimensional model of NHE1 based on phylogenetic and empirical data has revealed similar inhibitor binding sites (60).

1.3.4 Important amino acids of NHE1

The N-terminal membrane domain contains amino acids, which are specifically involved in ion affinity, transport and NHE inhibitor sensitivity. Transmembrane segment IV contains many amino acids that are important in ion transport and inhibitor sensitivity. The amino acids Leu^{163} and Gly^{174} in TM IV are essential for NHE1 inhibitor sensitivity and when these residues are mutated to Phe and Ser, respectively, NHE is 100 times more resistant to inhibition (61,62). Additionally, NHE1 mutants Leu163Phe and Gly174Ser display a two-fold decrease in Na^+ affinity, implicating these amino acids in ion binding and transport. The amino acid Phe^{161} in TM IV is a pore-lining residue important in ion transport, and mutation of this residue resulted in NHE1 loss of function (52). Previous studies revealed that Pro^{167} and Pro^{168} in TM IV are required for normal NHE1 activity, as they are important residues in NHE1 expression and membrane targeting (63).

The amino acids in the loop regions around TM IV, EL2 and IL2 are also important in NHE1 function. Specifically, in EL2, Gly^{148}, Pro^{153} and Pro^{158} are implicated in sensitivity to inhibitor drugs as well as catalytic activity of the exchanger (64). Additionally, amino acids Arg^{180} and Gln^{181} when mutated to Cys in IL2 severely inhibited NHE1 transport. In addition, one study showed accessibility of these residues in IL2 from both sides of the membrane and thus it was postulated that IL2 interacts with the pore of NHE1 (48).

Amino acids in TM VII, including residues Glu^{262} and Asp^{267}, are both important in NHE1 activity and mutation of these residues to Gln and Asn,

respectively, abolished exchanger activity, whereas conservative mutations that retained the negative charge of Glu and Asp did not affect activity. This suggested a role of these amino acids in coordination of the sodium ion (65).

Amino acid residues in transmembrane segment IX also play a role in inhibitor sensitivity and ion transport. His^{349}, when mutated to either Gly or Leu, increases NHE1 resistance to the NHE inhibitor, amiloride (66). Also Glu^{346} and Gly^{352} are important in inhibitor sensitivity and Na^+ affinity, and it is postulated that Glu^{346} coordinates Na^+ in a site that interacts with NHE inhibitors (64,67).

Other important residues in the N-terminal domain reside in TM IX and the adjacent intracellular loop 5 (IL5). The amino acid residues in TM IX, Arg^{440}, Gly^{455} and Gly^{456} are involved in sensing protons. Mutations of Gly455/456 to Cys shifted the steady state intracellular pH of the exchanger (pK ~7). In contrast, mutation of Arg440Cys shifted the steady state pH*i* to pK<6.2 (68), thereby suggesting that altering these amino acids in TM IX and IL5 alters the sensitivity of the exchanger to hydrogen ions and they are therefore part of the proton sensor of NHE1 (69). Furthermore, altering the steady state pH*i* to a more alkaline pH results in increased NHE1 activity without affecting ion affinity or inhibitor sensitivity. More recent studies by Hisamitsu *et al*. have shown that the negatively charged residue Glu^{131} of intracellular loop 1 is important in the regulation of pH*i* by NHE1 (70).

1.3.5 NHE inhibitors

Due to its many pathophysiological roles in the myocardium, the Na^+/H^+ exchanger has been widely studied as a target for the development of NHE specific inhibitors. NHE inhibitors have different affinities for the various NHE isoforms. However, the majority of these inhibitors exhibit high affinity for the NHE isoform 1, the predominant plasma membrane isoform in the myocardium. Their affinity for other isoforms is much lower.

The first NHE inhibitor discovered was amiloride. Amiloride was not very specific and inhibits the Na^+ channel and the Na^+/Ca^{2+} exchanger in addition to NHE1 and 2. To a lesser extent it inhibited NHE5 but did not inhibit NHE3, 4 and 7 very well (71). Other pyrazine derivatives were then developed from amiloride to be more potent and selective for NHE1, including DMA and EIPA. Both DMA and EIPA are more effective NHE inhibitors and display a relatively low efficacy in inhibition of the Na^+ channel or the Na^+/Ca^{2+} exchanger. Their potencies for the various isoforms are as follows: NHE1>NHE2>NHE5>NHE3 (9). The pyrazine derivatives were not ideal as they had a number of side effects in humans (72), and thus another class of NHE inhibitors was developed, the benzoylguanidine derivatives. Figure 1.2 shows the chemical structures of some of the NHE inhibitors, including the pyrazine and benzoylguanidine derivatives.

The benzoylguanidine derivatives display even greater potency in NHE1 inhibition, slight efficacy in NHE2 inhibition and do not inhibit NHE3 or 5. Some NHE inhibitors in this class include HOE694, cariporide (HOE642), and

EMD87580 (9). The potency of these drugs is influenced by the ionization of the guanidinium group. Thus, at low intracellular pH, the guanidinium group is protonated and this makes these drugs more potent (for example, the IC_{50} of cariporide at pH 6.2 = 22 nM, and at pH 6.7 = 120 nM) (9). Several benzoylguanidine derived NHE1 inhibitors have been used in clinical trials, and although they show potential for use in treatment of ischemia/reperfusion injury, they also displayed unexpected side effects, resulting in increased mortality (section 1.5.2) (13,73). Based on these clinical findings, more research is required to better understand the regulation of NHE1 in pathophysiological conditions and to potentially develop new strategies to modify NHE1 activation rather than completely abolish its activity (74).

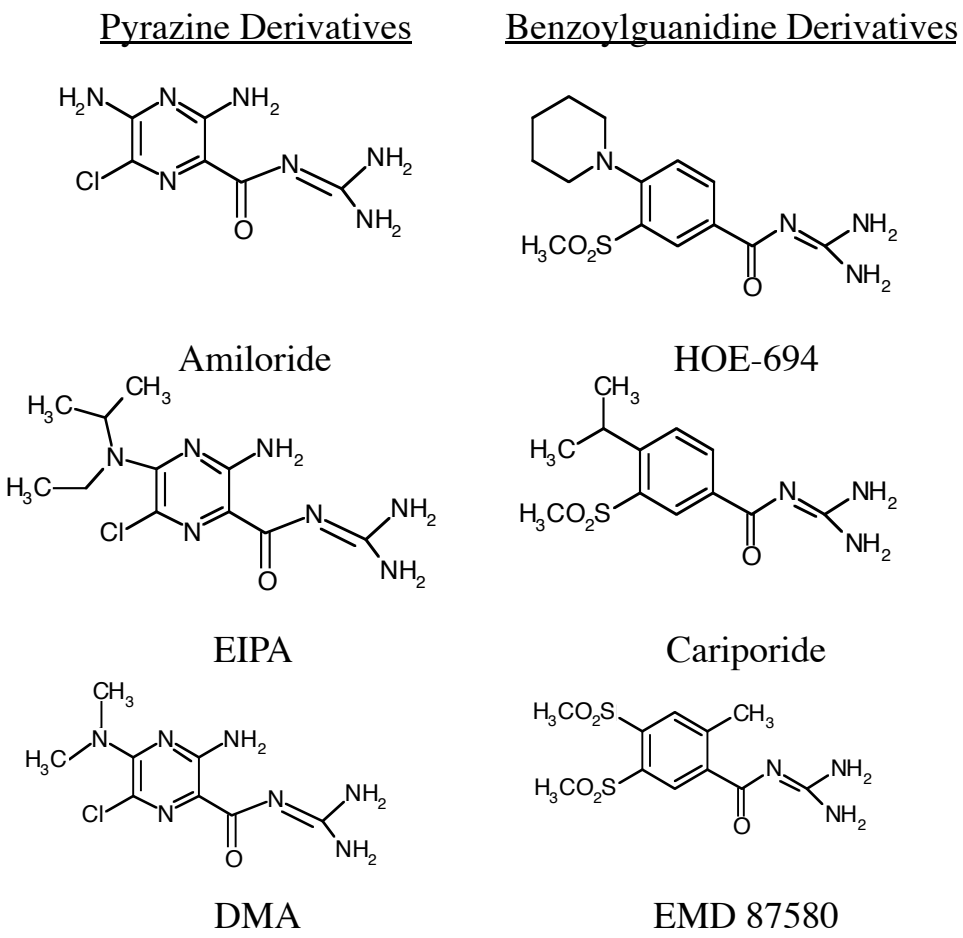

Figure 1.2 Chemical structures of some Na^+/H^+ exchange inhibitors. The chemical structures of some Na^+/H^+ exchanger inhibitors. The pyrazine derivatives include 3,5 diamino-6-chloro -N-(diaminomethylene)pyrazine-carboxamide (amiloride), and its derivatives 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA) and 5-(*N*,*N* -dimethyl) amiloride (DMA) are shown on the left. The benzoylguanidines compounds include HOE-694, Cariporide (HOE 642) and EMD-87580 (*N*-(4,5-Bis-methansulfony-2 -methyl-benzoyl) guanidine) are shown on the right.

1.4 NHE1 regulation

NHE1 regulation occurs at multiple levels, ranging from transcriptional regulation to binding of proteins and cofactors, as well as covalent modifications such as phosphorylation. Understanding how NHE1 is regulated is of particular importance as it aids in the elucidation of mechanisms that affect NHE1 for both normal cellular functions as well as pathological ones.

1.4.1 Transcriptional regulation of NHE1

The human NHE1 gene is composed of 12 exons and 11 introns. There have been several studies that have examined the regulation of the NHE1 gene in mouse, rat, rabbit, porcine, and human tissues. The NHE1 promoter has been shown to be responsive to a variety of transcription factors as well as reactive oxygen species. Transcriptional regulation of NHE1 affects both mRNA and protein production and ultimately affects the exchanger's activity in the cell. Studies in animal models examining NHE1 transcription during ischemia/reperfusion injury have shown that NHE1 mRNA levels increase under ischemic conditions (75,76).

There are very few studies examining the regulation of the NHE1 promoter in the myocardium. However, deletion of the activator protein-2 (AP-2) binding site (bp -95 to -111) resulted in a four-fold decrease in NHE1 promoter activity in comparison to control wild type NHE1 (77). Furthermore, mutation of the AP-2 site combined with deletion of distal regions of the NHE1 promoter

abolished promoter activity, thereby showing that the AP-2 binding site is an essential transcription factor for NHE1 gene activity. The chicken ovalbumin upstream promoter transcription factor (COUP-TF) binds to and enhances NHE1 promoter activity (78). Protein binding in the COUP-TF region of -841 to -800 has been shown to increase upon treatment of myocardial cells with thyroid hormone. Interestingly, in the NHE1 promoter there is a thyroid hormone receptor (TRα) binding site that also lies in the enhancer region of the promoter but at a distinct site from COUP-TF (79).

The NHE1 promoter has been shown to be responsive to reactive oxygen species (ROS). Increases in NHE1 mRNA levels have been associated with superoxide production and both NHE1 promoter activity and superoxide production were inhibited by the oxidase inhibitor, diphenyleniodinium (80). Two different human cell lines have been used to show that superoxide targets the NHE1 promoter (80,81). The role of superoxides and NHE1 transcription in the myocardium remains unknown. However, ROS are known to be important signaling molecules under various conditions in the myocardium such as ischemia/reperfusion injury as well as hypertrophy. Understanding the role of transcriptional regulation of the NHE1 gene is of great interest, as the diseased myocardium has increased NHE1 expression.

1.4.2 Binding proteins of NHE1

The regulation of NHE1 occurs by a combination of proteins and phosphorylation dependent reactions involving protein kinases such as extracellular regulated kinase 1 and 2 (ERK1/2) and p90 ribosomal S6 kinase ($p90^{RSK}$) (Figure 1.1 and Table I) (82,83). The capacity of growth factors to stimulate NHE1 is reduced by approximately 50% when the distal region of the cytoplasmic tail is removed (84). Thus, the cytoplasmic tail of NHE1 acts as the major site of NHE1 regulation.

Calmodulin (CaM) is a calcium binding protein that plays an important role in modulating NHE1 activity in response to intracellular Ca^{2+}. After binding Ca^{2+}, CaM binds to two CaM binding domains in the cytoplasmic tail of NHE1. There are both high and low affinity sites located between amino acids 636-700 (85). Binding of CaM to the high affinity site blocks the auto-inhibitory interaction and activates NHE1 (86). The autoinhibitory interaction involves the amino acids Leu^{639}, Lys^{651}, and Tyr^{652} (87). Additionally, there are seven conserved amino acids in the distal region of NHE1's cytoplasmic tail 753-759 (EEDEDDD) that play a role in modulating CaM binding (88). Mutation of these acidic residues results in decreased NHE1 activity and decreased CaM binding.

Phosphatidylinositol 4,5 – bisphosphate (PIP_2) is a phospholipid signaling molecule in eukaryotic plasma membranes. PIP_2 can be converted to the important

second messenger signaling molecules inositol 1, 4, 5-triphosphate (IP_3) and diacylglycerol. PIP_2 has been shown to activate NHE1 activity. Although NHE1 does not consume ATP directly, its activity is altered by ATP depletion (89). Specifically, it has been shown that PIP_2 depletion is concurrent with ATP depletion and this resulted in NHE1 inhibition (90). There are two PIP_2 binding motifs in the cytoplasmic tail of NHE1 between amino acids 509-516 and 552-560 and PIP_2 has been shown to bind to the cytoplasmic tail of NHE1 *in vitro* in these regions (90).

Calcineurin homologous protein (CHP) exists in two isoforms, 1 and 2, which share 61% amino acid identity (91,92). CHP possesses four EF-hand motifs, although only EF 3 and 4 bind and coordinate Ca^{2+}. Mutation of EF 3 or 4 results in reduced NHE1 activity, and mutation of both EF 3 and 4 greatly decreases binding to NHE1 (93). CHP1 is found in the myocardium and binds to NHE1 between amino acids 510-530, and mutations that inhibit binding of CHP1 to NHE1 result in decreased NHE1 activity (94,95). Deletion of CHP1 in chicken B lymphoma DT40 cells was recently shown to destabilize NHE1 and results in decreased plasma membrane NHE1, and reintroduction of CHP1 restored NHE1 expression (96). Thus, it has been suggested that CHP1 is an essential cofactor for NHE1.

Expression of CHP2 is restricted to normal intestinal epithelial cells (97). However, CHP2 is also highly expressed in tumor cells and the association of CHP2 with NHE1 was shown to protect cells from serum deprivation induced

death (92). Thus, it is proposed that the NHE1-CHP2 interaction maintains tumor cells in a malignant state. The role of CHP2 in the myocardium remains unknown, although CHP2 mRNA has been detected (92).

Tescalcin is a Ca^{2+} binding protein that is homologous to CHP1. Tescalcin is strongly expressed in the myocardium where it binds to the cytoplasmic tail of NHE1 in a Ca^{2+}-dependent manner (58,98). Specifically, tescalcin binds to amino acids 505-571, the same region that binds CHP1 and CHP2 (99). Binding of tescalcin to the cytoplasmic tail of NHE1 results in conformational changes that promote NHE1 maturation and cell surface stability in CHO cells (58,99).

Carbonic Anhydrase II (CAII) is an enzyme which catalyzes the hydration of CO_2 producing HCO_3^- and H^+. CAII binds to NHE1 at amino acids 790-802, with amino acids Ser^{796} and Asp^{797} forming part of the binding site (100). Association of NHE1 with CAII results in increased NHE1 activity (101). This increase in NHE1 activity upon CAII binding is dependent on phosphorylation of the cytoplasmic tail (101). When the cytoplasmic tail of NHE1 is dephosphorylated there is reduced CAII binding (100). Binding of CAII to the cytoplasmic tail of NHE1 may serve to facilitate the removal of protons produced by hydration of CO_2. CAII has been shown to be expressed in the myocardium and CAII inhibitors have been shown to reduce myocardial NHE1 activity (100,102).

Ezrin, Radixin, and Moesin (ERM) are a family of proteins which form links between actin filaments of the cytoskeleton and integral proteins of the plasma

membrane (103). NHE1 has two ERM binding motifs in its cytoplasmic tail between amino acids 553-564, and this interaction directs proper localization of NHE1 and maintains cell shape (12). This interaction facilitates cellular events including cell migration and the formation of cell signaling complexes (12,41). In some cell types it has been shown that the interaction of NHE1-ERM is required for cell survival from apoptotic stress by activating the pro-survival kinase Akt (44).

14-3-3 adaptor protein binds to NHE1 when phosphorylated on Ser^{703} by the kinase, $p90^{RSK}$. The binding of 14-3-3 activates NHE1 by protecting Ser^{703} from dephosphorylation (104).

1.4.3 Phosphorylation of NHE1

The regulation of NHE1 occurs by a combination of proteins and phosphorylation dependent reactions involving protein kinases and phosphatases (Figure 1.1) (82,83). The capacity of growth factors to stimulate NHE1 is reduced by approximately 50% when the distal region of the cytoplasmic tail is removed (84).

In fibroblasts, the cytoplasmic tail of NHE1 is phosphorylated in response to growth factors, thrombin, phorbol esters, and serum. This stimulation favors NHE1 activity at a more alkaline pH (105). Furthermore, thrombin, epidermal growth factor, and okadaic acid (phosphatase PP-1 and PP-2A inhibitor) increase phosphorylation of NHE1 at a common set of sites (106). Deletion analysis of the

cytoplasmic tail of NHE1 further revealed that *in vivo* phosphorylation sites map to amino acids 636-815 of the NHE1 cytoplasmic tail and deletion of this region results in a 50% decrease of growth factor induced stimulation of NHE1 (107). One theory is that phosphorylation induces a conformational change in the cytoplasmic tail of NHE1 enabling the proton sensor of the NHE1 membrane domain to be more sensitive and thus activating NHE1 (108).

A number of Ser/Thr protein kinases have been shown to phosphorylate and regulate NHE1 in non-myocardial cells. The mitogen activated protein kinase (MAPK) cascade, in particular ERK1/2, is critical in mediating NHE1 activation (109-112). Early studies in Chinese hamster ovary (CHO) cells showed that inhibition of the MAPK cascade by using a dominant negative form of ERK2, expression of a MAP kinase phosphatase MKP-1, or treatment with a MAPK cascade inhibitor, resulted in a 50-60% decrease in growth factor induced activation of NHE1 (112). This decrease in NHE1 activation was reminiscent of studies in which the cytoplasmic tail of NHE1 was removed. Further studies revealed that fractions of rabbit skeletal muscle were able to phosphorylate the cytoplasmic tail of NHE1 *in vitro*. Western blot analysis revealed that three of the four fractions were ERK1/2 dependent phosphorylation reactions and the stoichiometry of ERK1/2 to NHE1 was 1 phosphate to 1 mol of protein (110). Additionally both ERK1 and 2 were found to bind to the cytoplasmic tail of NHE1 (unpublished data Dr. Fliegel laboratory). $p90^{RSK}$, a kinase downstream of ERK1/2, has also been shown to directly phosphorylate NHE1 at Ser^{703} and stimulate NHE1 activity (82). Taken together, these results indicate that the

MAPK signaling pathway is key to NHE1 regulation and activation. *In vitro* studies in our laboratory have shown phosphorylation sites on the cytoplasmic tail of NHE1 include Ser^{693}, Ser^{766}, Ser^{770}, Thr^{779} and Ser^{785}, and are all phosphorylated by ERK2 (113). Further studies in our laboratory examined four groups of phosphorylation site mutants of NHE1, Ser693Ala, Thr718Ala and Ser723/726/729Ala, Ser766/770/771Ala, and Thr779Ala and Ser785Ala in CHO cells. Experiments in CHO cells revealed that the amino acids Ser^{766}, Ser^{770} and Ser^{771} are important in regulation of NHE1 activity, which occurs via a MAPK cascade-dependent pathway (114). More recently, studies in astrocytes from rat optic nerve showed that elevated hydrostatic pressure resulted in increased phosphorylation of ERK1/2, $p90^{RSK}$ and NHE1 and this was abolished by the MEK inhibitor U0126 (115). Furthermore, the rate of pH*i* recovery from an acid load was four-fold higher in pressure treated astrocytes and this increase was not shown when cells were treated with U0126 and/or NHE1 inhibitor (115). These results are consistent with NHE1 activation due to phosphorylation of ERK1/2, $p90^{RSK}$ and NHE1. A simplified cartoon of the MAPK cascade and its role in NHE1 regulation is illustrated in Figure 1.3.

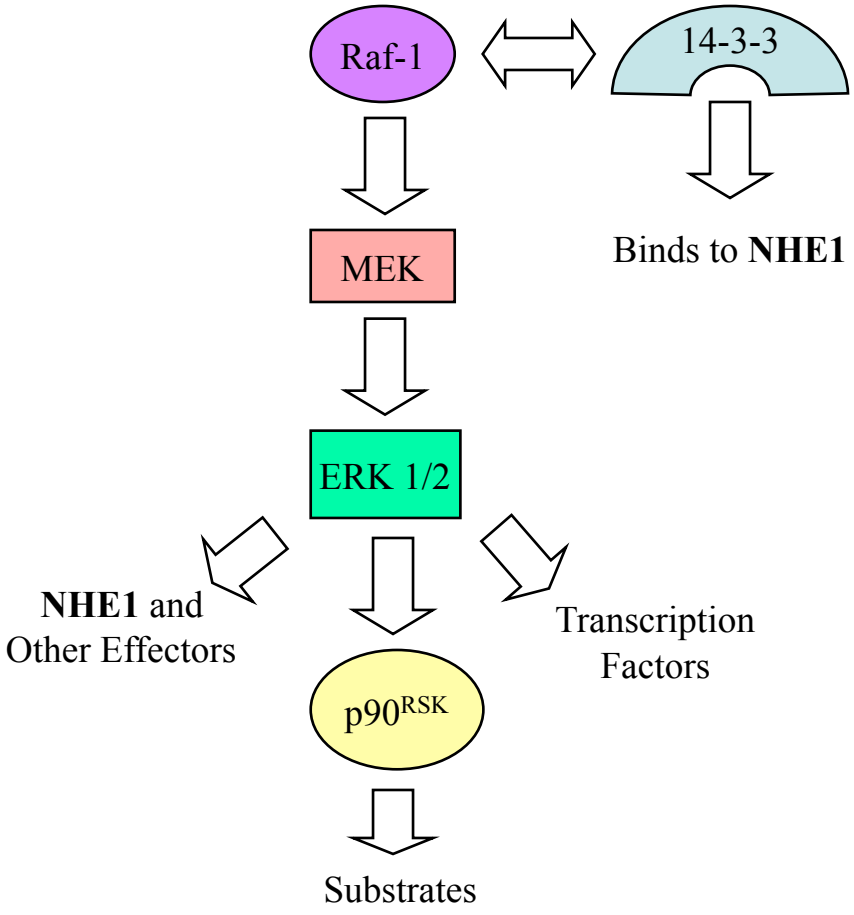

Figure 1.3 NHE1 regulation and the MAPK cascade. The various kinases in the cascade are indicated and the arrows denote the stepwise activation of the kinases in the MAPK cascade. Targets which relate to the Na^+/H^+ exchanger isoform 1 (NHE1) activity are shown. Raf-1, mitogen activated protein kinase kinase kinase. MEK, mitogen activated protein kinase kinase. ERK1/2, extracellular regulated kinase isoform 1 and 2. $p90^{RSK}$, p90 ribosomal protein S6 kinase. 14-3-3, an adaptor protein.

A number of other kinases also phosphorylate and regulate NHE1 in non-myocardial cells. The GTPase, RhoA, was shown to stimulate NHE1; this was mediated by lysophosphatidic acid and the GTPase, Gα13. The activation of NHE1 was important in RhoA induced cytoskeletal reorganization (116,117). RhoA activation of NHE1 was found to be mediated by the downstream target kinase p160ROCK, which has been shown, both *in vitro* and *in vivo*, to directly phosphorylate the cytoplasmic tail of NHE1 (636-815). These findings suggest NHE1 activity plays a role in cytoskeletal functions such as cell adhesion and contractility (118).

Early studies with p38MAPK and NHE1 revealed that angiotensin II stimulates NHE1 in vascular smooth muscle cells by p38MAPK phosphorylation and that this pathway is ERK-dependent (119). *In vitro* analysis by mass spectrometry performed by Khaled *et al.* revealed four p38MAPK phosphorylation sites on NHE1, Thr^{718}, Ser^{723}, Ser^{726} and Ser^{729} (human sequence) (43). *In vivo* analysis in pro-β cells demonstrated that these sites were important in causing apoptosis-induced alkalinization (43). Follow-up studies have shown that the amino acids Ser^{726} and Ser^{729} of NHE1 are critical in mediating the p38MAPK-induced apoptotic response (120). Mutation of Ser726/729 to Ala in NHE1 protects cells from serum withdrawal-induced death (120). Another stress-activated kinase that phosphorylates NHE1 is the Nck-interacting kinase (NIK). Full phosphorylation and activation of NHE1 by NIK required binding to NHE1 at residues 538-638, and phosphorylation of NHE1 occurred at a site distal to NIK

binding (121). Furthermore, Ca^{2+}/calmodulin-dependent kinase II (CaMKII) has three consensus sequence sites in the cytoplasmic tail of NHE1 and was shown to phosphorylate NHE1 *in vitro* (122).

Studies *in vitro* have shown that NHE1 is a novel substrate of protein kinase B, specifically phosphorylating NHE1 at Ser^{648} and to a lesser extent Ser^{703} and Ser^{796} in the cytosolic tail (123). Additionally, phosphorylation of NHE1 at Ser^{648} by protein kinase B (otherwise known as Akt) interferes with CaM binding to NHE1 and, therefore, interrupts the relief of NHE1 autoinhibition (as discussed in section 1.4.2) (123). In contrast, recent studies in fibroblasts have shown that Akt phosphorylation of NHE1 increases its activity and is required for activation of NHE1 in response to insulin and PDGF (124). Furthermore, fibroblasts expressing NHE1 with Ser648Ala mutation were unable to disassemble actin stress fibers and, thus, exhibited decreased cell proliferation (124).

Other protein kinases are known to regulate NHE1 activity but not by direct phosphorylation. *In vitro* assays examining phosphorylation of NHE1's cytoplasmic tail found that protein kinase A (PKA), C (PKC), and D did not phosphorylate the cytoplasmic tail of NHE1 (105,122,125,126). Table I summarizes the protein kinases involved in NHE1 regulation.

Lysophosphatidic acid (LPA) is a bioactive phospholipid that mediates signaling by the LPA receptor subtypes 1-4. LPA stimulates NHE1 via the Gα13 via Cdc42 and RhoA dependent pathways (116). As described above, this activates NHE1 via a RhoA, p160ROCK dependent pathway (118).

1.4.4 Dephosphorylation of NHE1

Since phosphorylation plays an essential role in the regulation of NHE1 activity, dephosphorylation must also play an important role in eventual removal of phosphate groups. Dephosphorylation occurs via a family of proteins called the Serine/Threonine protein phosphatases, which play a regulatory role in many physiological processes (reviewed in (127)). In the myocardium, protein phosphatases (PP), PP-1, PP-2A, PP-2B, and PP-2C are present (128,129). PP-1 is susceptible to inhibition by I-2, and PP-2A, PP-2B and PP-2C are dependent on Ca^{2+} and Mg^{2+}, although PP-2A retains activity in the absence of divalent cations. Both PP2A and PP2C are known to regulate the MAPK cascade via ERK1/2 and $p90^{RSK}$ (130,131).

There are few studies examining the role of phosphatases and NHE1. These studies mainly used pharmacological approaches to gain insights into which phosphatases regulate NHE1. These inhibitors include okadaic acid, a polyether derivative of a fatty acid, which has different inhibitory potencies against the various phosphatase isoforms. For example, it inhibits PP-2A about 10 to 100 times better than PP-1, and is an even weaker inhibitor of PP-2B (132). Okadaic acid has been shown to maintain NHE1 in a phosphorylated state and thereby increase NHE1 activity in rat thymocytes, human bladder carcinoma cell line MGH-U1, Chinese hamster lung fibroblasts, and platelets (106,133,134). Furthermore, okadaic acid treatment results in increased activated ERK1/2 and this stimulates NHE1 activity in red blood cells; this effect on NHE1 is reversed by the MEK inhibitor, PD98059 (135). Studies examining phosphatases that

directly regulate NHE1 include ßNHE in trout, which was regulated by PP-1 (136). Furthermore, NHE1 binds to the adaptor protein 14-3-3 via phosphorylated Ser^{703} and this prevents dephosphorylation *in vitro* (104). Studies with 14-3-3 display another mechanism whereby NHE1 is regulated, by protecting NHE1 against dephosphorylation. PP-1 plays a major role in NHE1 regulation both *in vitro* and *in vivo*, where it binds to NHE1 and regulates NHE1 activity (137). It was determined that NHE1 dephosphorylation occurred primarily by PP-1 and to a lesser extent by PP-2A (137). PP-2A has recently been shown to have a role in adenosine A1 receptor mediated regulation of NHE1 activity in adult rat ventricular cardiomyocytes. However, Snabaitis *et al*. tested only amino acids 625-747 in the cytoplasmic tail of NHE1, excluding potentially important sites of NHE1 phosphorylation including Ser^{766}, Ser^{770}, Ser^{771}, Thr^{779} and Ser^{785} (138).

NHE1 has recently been shown to bind to the Src-homology 2 domain-containing protein tyrosine phosphatase (SHP2). Overexpression of SHP2 in a mouse bone marrow-derived pro-B cell line resulted in both increased steady state pH_i and NHE1 activity (139). Recent studies have shown that inhibition of SHP2 in vascular smooth muscle and endothelial cells results in decreased NHE1 activity (140). It is notable that there are no tyrosine residues known to be phosphorylated on NHE1. However, these phosphatases may function to dephosphorylate regulatory proteins of NHE1.

Table I: Proteins, factors and kinases interacting with the cytosolic region of NHE1. The type and location of the interactions are indicated plus other related information. A more detailed discussion of this information is in section 1.4.

Protein -Factor	Binding Location	Function/Other Information	Reference
Calmodulin	636-656	High affinity site, stimulatory	(85)
Calmodulin	657-700	Low affinity site	(85)
Carbonic Anhydrase II	790-802	Phosphorylation regulated, stimulatory	(100)
CHP1	518-537	Solution structure determined, essential cofactor	(95)
CHP2	518-537	Crystal structure	(141)
CHP3, Tescalcin	530-535, Second more distal binding site also reported	Promotes maturation and protein half life	(58,99)
Daxx	567-637	Activates	(142)
Ezrin, radixin, moesin (ERM)	552-560	Affects focal adhesions, stress fiber formation, cell shape, apoptosis	(12,44)
Hsp70	C-terminus	May affect protein folding	(139,143)
PIP2	506-576 (two sites)	Mediates inhibitory effect of ATP depletion	(90)
14-3-3	C terminus, phospho-Ser^{703}	Scaffolding protein, stress	(104)
Bin I, Amphiphysin I, BMX, Fas, CIDE-B, Maspin, CAS, DR4	Identified by antibody array analysis	Functional role not yet characterized	(139)

Protein Kinase	Phosphorylation/ Dephosphorylation Site	Other	Reference
CaM Kinase II	C-terminus	Phosphorylation shown *in vitro*	(122)
ERK1-2	Ser^{770}, Ser^{771}	Activated by sustained acidosis	(114)
$p90^{RSK}$	Ser^{703}	Serum stimulated	(82)
p38 MAPK	Thr^{718}, Ser^{723}, Ser^{726}, Ser^{729} (one or more)	Activation by trophic factor withdrawal in proβ cells	(43)
Nck interacting kinase	Binds 563-638 and phosphorylates distally	Activates	(121)
Protein kinase D	May act indirectly	Inhibitory	(126)
p160ROCK	C-terminus	Mediates RhoA activation	(118)
Protein kinase B	Ser^{648}	Phosphorylation shown *in vitro* and *in vivo*	(123,124)

Phosphatase	Dephosphorylation Site	Other	Reference
Protein Phosphatase 1	C-terminus	Inhibitory	(137)
Protein Phosphatase 2A	Unknown, co-localizes and dephosphorylates NHE1	Counteracts α_1-adenosine receptor mediated activation	(138)
SHP-2 tyrosine phosphatase	C-terminus	Stimulatory	(139)

1.5 NHE1 in the myocardium

NHE1 is the predominant plasma membrane isoform in the myocardium (6,7), where it is concentrated along the intercalated discs and transverse tubule system (8). The distribution of other NHE isoforms in the heart has only been analyzed as small parts of other studies. NHE2 was very faintly detected in northern blots in rat myocardium and northern blot analysis and reverse transcriptase PCR detected none, or very faint traces of NHE3 in both human and rat heart tissue (18,144-146). Both NHE4 and 5 were undetectable in the rat myocardium by northern blot analysis; however, there was a very small amount of cross-reactive material with the NHE4 antibody in one study (21,144,147).

1.5.1 Physiological role of NHE1 in the myocardium

NHE1 is responsible for 50-60% of proton efflux and, thus, plays an essential role in the maintenance of pH_i homeostasis and cardiomyocyte contractility (148). The remaining percentage of total acid efflux is mostly mediated by the HCO_3^- based transporters; however, bicarbonate transporters are more active at alkaline pH's (148-150). Thus, at physiological and acidic pH's (<6.5) NHE1 is the major mechanism of acid extrusion. Many studies that investigate the regulation of NHE1 activity are performed with medium lacking HCO_3^- so as to selectively study only the NHE-mediated acid extrusion pathway.

Myocardial NHE1 has a very steep relationship between pH_i and its activity and the lower the pH_i the more active NHE1 is (151). In myocardial cells NHE1 displays a Hill coefficient of 3, indicating that more than one H^+ ion is

binding to NHE1 (151). Section 1.3.4 discusses the identification and role of a proton sensor in the membrane domain of NHE1.

Interestingly, stimulation of NHE1 by hormones (such as endothelin-1), α_1-adrenergic stimulation and phosphorylation of its cytoplasmic tail shifts the pH dependence of NHE1 toward a more alkaline range, making NHE1 more active over a larger range of intracellular pH (109,152). Studies from our laboratory and others have shown that both NHE1 expression and activity are increased in both the fetal and neonatal myocardium (153,154). Furthermore, recent studies from our laboratory have shown that NHE1 facilitates cardiomyocyte embryonic stem cell differentiation (40). Although NHE1 plays a major role in normal cardiac function, the vast majority of studies have examined its role in various pathological states.

1.5.2 NHE1 and pathological roles in the myocardium

The Na^+/H^+ exchanger isoform 1 contributes to several pathological conditions in the myocardium. The most studied role of NHE1 in heart disease is in ischemia reperfusion (I/R) injury. NHE1 activity and mRNA levels are increased with ischemia and reperfusion (76,155). The specific role that NHE1 has in I/R injury can be described by the "coupled exchanger theory" (156,157). During ischemia, anaerobic glycolysis occurs resulting in the production of protons, and causing intracellular acidosis (by approximately 1 pH unit). NHE1 is activated by low pH_i leading to an increase in intracellular Na^+ ions and alkalinization of the cytoplasm. This excess intracellular Na^+ results in the

reversal of Na^+/Ca^{2+} exchanger activity, resulting in increased intracellular Ca^{2+}. The buildup of Ca^{2+} results in the activation of a variety of pathways in the myocardium, which ultimately leads to cardiac cell death, thus damaging the heart. The importance of NHE1 in I/R injury is supported by studies where genetic ablation of NHE1 in mice protects the heart from I/R injury (158). Activation of NHE1 regulatory pathways has also been suggested to be important in NHE1-mediated damage to the heart and it has been shown that ischemia and reperfusion may activate NHE1 regulatory kinases (83). Furthermore, during ischemia NHE1 is activated by ischemic metabolites such as hydrogen peroxide (H_2O_2) (159). Studies of ischemia in non-myocardial cells (neuronal cells) also found that *in vitro* simulated ischemia increased NHE1 activity in an ERK1/2 dependent manner (160).

Numerous studies have examined the effects of NHE1 inhibition in myocardial ischemia reperfusion injury. NHE1 inhibitors, including amiloride, cariporide, and their derivatives have been shown to be cardioprotective in animal models in many studies (161-170). Moreover, NHE1 inhibition has been shown to be useful as a tool in cardioprotection that is superior to ischemic preconditioning (166,171,172). Although, NHE1 inhibitors have been developed for use in clinical trials, the results of such trials (Phase I, II and III) have not yielded significant benefits for clinical application (13,73,173,174).

The first clinical study, termed GUARDIAN (Guard During Ischemia Against Necrosis), a phase II/phase III study with more than 11,590 patients assessed cariporide in patients with acute coronary syndromes, demonstrated no

overall significant benefit (173). The ESCAMI (Evaluation of the Safety and Cardioprotective effects of Eniporide in Acute Myocardial Infarction), a phase II trial where eniporide was assessed in patients undergoing thrombolysis or angioplasty for acute myocardial infarction (MI), also yielded no beneficial effects (174). However, it is notable that a lack of beneficial effect could be a result of lack of optimal dosing (GUARDIAN) and administration during reperfusion only (ESCAMI) (13). The more recent trial, EXPEDITION (Na^+/H^+ Exchange Inhibition to Prevent Coronary Events in Acute Cardiac Conditions), examined the effects of cariporide on mortality and incidence of MI in high-risk patients undergoing coronary artery bypass graft surgery. The results showed a decrease in MI with treatment of cariporide, however, there were unexpected side effects, which increased mortality due to cerebrovascular events (13,73). Based on these clinical findings, more research is required to better understand the regulation of NHE1 in pathophysiological conditions.

In addition to the role of NHE1 in I/R injury, NHE1 has been implicated in the development of cardiac hypertrophy (reviewed in (175)). Cardiac hypertrophy can be described as an enlargement of the heart due to cell growth rather than an increase in number of cardiomyocytes. This is an early maladaptive response to heart failure or increased afterload (reviewed in (152,176)). Hypertrophic stimuli, such as angiotensin II, norepinephrine, aldosterone, endothelin-1, α_1-adrenergic receptor agonist and mechanical stimulation also activate NHE1 (177-180). NHE1 is activated by MAP kinases and protein kinase C dependent pathways, and these pathways are also important in hypertrophic and remodeling processes (83). Mice

over-expressing a human hyperactive NHE1, present with significant cardiac hypertrophy, contractile dysfunction and heart failure (181). The mechanism of heart failure in the transgenic mice was shown to involve activation of CaMKII, which caused increased phospholamban phosphorylation and increases in sarcoplasmic reticulum Ca^{2+} loading and cytosolic Ca^{2+}. This led to Ca^{2+} dependent hypertrophic stimuli and acceleration of cell death (181). Additionally, transgenic mice displayed increased levels of activated ERK1/2 and p38MAPK (181). Studies from our laboratory have shown that transgenic mice overexpressing NHE1 have enhanced cardiac hypertrophy in the presence of phenylephrine (α_1-adrenergic receptor agonist) (182,183). The mechanism by which this occurs may be via the MAPK cascade. Phenylephrine is known to activate the MAPK cascade specifically increasing activation of the kinases ERK1/2 and $p90^{RSK}$ (184), which have both been shown to phosphorylate and stimulate the Na^+/H^+ exchanger (82,110,114,185). Inhibition of NHE1 has been shown to prevent cardiac hypertrophy both *in vivo* and *in vitro* (179,181,186-188). The clinical benefits of NHE1 inhibition and hypertrophy have yet to be examined.

NHE1 activity is also elevated in human and animal models of hypertension (189-193). Spontaneously hypertensive rats display increased NHE1 activity and phosphorylation, and the 90 kDa NHE1 kinase shows increased activity (192,194). Furthermore, this 90 kDa NHE1 kinase was PKC independent and partially dependent on the MAPK pathway.

The lack of success of NHE1 inhibitors in clinical trials warrants closer examination of NHE1 stimulation. Increased NHE1 activity causes detrimental effects in various forms of heart disease, and thus potential inhibition of NHE1 stimulation is an attractive target for prevention of ischemia reperfusion injury and hypertrophy.

1.6 Regulation of NHE1 in the myocardium

A variety of growth factors and hormones regulate the Na^+/H^+ exchanger isoform 1. Many have been shown to activate NHE1 in a multitude of tissues, such as thrombin, serum, epidermal growth factor, insulin, angiotensin II, and lysophosphatidic acid (105,112,192,195-197). These various extracellular agonists mediate their effects through several classes of cell surface receptors. NHE1 activity is regulated by these factors mainly through modifications of NHE1's cytosolic domain. These modifications include protein-protein interactions and phosphorylation. They act to control NHE1 transport by altering the affinity of the transport domain for intracellular protons (reviewed in (3)).

1.6.1 NHE1 regulation in the myocardium by receptors

There are key autocrine, paracrine and hormonal factors in the myocardium important to cardiac pathology. Many of these pathways act through receptors such as the G protein coupled receptors (GPCR). GPCR systems are linked to heterotrimeric G proteins of the G_q subtype, and mediate an increase in

NHE1 activity. Serum has been shown to activate NHE1, however, serum contains a myriad of agonists (109).

One example of GPCR mediated stimulation of NHE1 is by catecholamines via the α_1-adrenergic receptor (AR), requiring ERK and PKC activity (195,198). Additionally, neurohormonal stimulation of NHE1 occurs via altered pH_i sensitivity (199). Endothelin-1 (ET-1), a 21 amino acid vasoactive peptide, increases resting pH_i and also stimulates NHE1 activity in the myocardium (200,201). This effect acts via an ERK-dependent pathway (109). Angiotensin II (Ang II), a peptide hormone, is another potent activator of NHE1 activity in the myocardium (202). Stimulation of NHE1 by Ang II occurs through the AT_1 subtype of angiotensin receptors and is antagonized by AT_2 receptor activation (203). In rat vascular smooth muscle cells, Ang II activates NHE1 via a $p90^{RSK}$-dependent pathway (192,197). However, other studies in rat vascular smooth muscle cells showed that Ang II signaling activates NHE1 by p38MAPK and not ERK1/2 (119).

Thrombin and the synthetic thrombin receptor activating peptide, SFLLRN, increase NHE1 activity (204). Thrombin acts by means of a PKC-dependent pathway, however, it is noteworthy that PKC does not directly phosphorylate NHE1 (204). Furthermore, interaction of thrombin with its receptor, protease activated receptor-1 (PAR-1), initiates a cascade of signaling events including the MAPK cascade activating kinases ERK1/2 and $p90^{RSK}$ resulting in NHE1 phosphorylation and activation (106,133).

GPCRs can also mediate inhibition of NHE activity through the G protein subtypes G_s and G_i. These pathways include the β_1-adrenergic receptors, angiotensin AT_2 receptors and adenosine A_1 receptors (203,205,206).

Finally, NHE1 is also stimulated by receptors that respond to growth factors such as epidermal growth factor (EGF) and serum (containing many growth factors) (109,207). Activation of NHE1, by both EGF and serum, have been shown to act via the ERK1/2 and $p90^{RSK}$ kinases. The hormone insulin also activates NHE1 by activating the MAPK cascade and PKC. Specifically in erythrocytes, insulin has been shown to induce IP_3 production which activates PKC, which has been shown to indirectly activate NHE1 (112,208).

1.6.2 NHE1 and protein kinase regulation in the myocardium

The effects of hormonal regulation of NHE1 are mediated by protein-protein interactions and by phosphorylation of the regulatory NHE1 cytosolic domain. These have been primarily studied in non-myocardial tissues (section 1.4.3-1.4.4). In non-myocardial cells, NHE1 is phosphorylated by a number of kinases such as ERK1/2, $p90^{RSK}$, p38MAPK, p160ROCK, NIK, CaMKII and PKB (see above 1.4.3). However, in the myocardium NHE1 is likely phosphorylated by only a subset of these protein kinases including ERK1/2, $p90^{RSK}$ and protein kinase B (109,123).

The first evidence that myocardial kinases were involved in the regulation of NHE1 came from the Fliegel laboratory, where immunoprecipitated ERK1/2 and $p90^{RSK}$ were shown to phosphorylate NHE1 directly in the rat myocardium

(109). Both endothelin-1 (ET-1) and serum enhanced this phosphorylation, stimulated exchanger activity and resulted in an alkaline shift of steady-state pH_i. The use of PD98059 (inhibitor of the upstream activator of ERK1/2, MAPK kinase (MEK)) abolished the effect observed by ET-1 and serum on NHE1 stimulation. Recently, studies of transgenic mice overexpressing a highly active NHE1 displayed increased levels of activated phosphorylated ERK1/2 (181).

The MAPK cascade, which is activated by H_2O_2 and ischemia/reperfusion, plays an important role in the activation of NHE1 leading to myocardial damage (83,111,209,210). H_2O_2 is also a metabolite of ischemia and, thus, contributes to the increase in NHE1 activity during ischemia and reperfusion injury as well as detrimental effects, such as contractile malfunction (111,207,210). Another mechanism of H_2O_2 mediated NHE1 activation has been described in vascular smooth muscle and endothelial cells and this occurs via SHP2 inhibition (140).

Studies of the downstream kinase of ERK1/2, $p90^{RSK}$ have shown that the expression of dominant negative $p90^{RSK}$ led to a reduced effect of I/R injury, decreased cardiac apoptosis and partially preserved cardiac function (211). Additionally, the novel specific $p90^{RSK}$ inhibitor, fmk, when used to pre-treat adult rat cardiomyocytes, was able to inhibit phenylephrine induced increases in NHE1 phosphorylation and activity (185).

Sustained intracellular acidosis (SIA) has been shown to rapidly stimulate NHE1 activity and phosphorylation via an ERK-dependent pathway in neonatal and adult ventricular cardiomyocytes and non-myocardial COS-7 cells (212). The sustained acidosis treatment of cells was accomplished by subjecting cells to an

ammonium chloride induced acid load, cells were then washed and maintained in Na^+-free buffer, resulting in prolonged decreased pH*i*. SIA activated ERK1/2 and $p90^{RSK}$ in parallel to the increase in NHE1 activity. Furthermore, in adult ventricular cardiomyocytes, SIA activates ERK1/2 through activation of the Ras/Raf/MEK pathway (184).

Recent studies demonstrated that, *in vitro*, NHE1 is phosphorylated at Ser648 by protein kinase B (123). Studies in adult rat ventricular cardiomyocytes revealed that overexpression of PKB resulted in increased NHE1 phosphorylation and decreased NHE1 activity (123). Therefore, unlike the protein kinases discussed above, PKB phosphorylation of NHE1 is inhibitory in the myocardium.

In-gel kinase assays have revealed that protein kinases other than ERK1/2 and $p90^{RSK}$ and of molecular weight 40, 44 and 55 kDa also phosphorylate NHE1 in the myocardium, but are not yet identified (83). Also CaM kinase II and myosin light chain kinase are important in osmotic regulation of NHE1 in myocardial cells (213). Activation of CaM kinase II was recently shown to be very important in the mechanism whereby overexpression of NHE1 leads to cardiac hypertrophy and remodeling (181). Kinases, including p38MAPK, JNK, and MEK were not shown to directly phosphorylate NHE1 in the myocardium.

The precise amino acids that are phosphorylated on the cytoplasmic tail of NHE1 in the myocardium and the specific myocardial kinases, which phosphorylate these sites, remain largely unknown.

1.6.3 NHE1 stimulation by sustained intracellular acidosis

As described above in section 1.6.2 sustained intracellular acidosis (SIA) has been shown to be a stimulatory mechanism of NHE1 in the myocardium. Treatment of neonatal or adult rat ventricular cardiomyocytes for 3 minutes with SIA increased NHE1 activity. Sustained intracellular acidosis was achieved by incubating myocardial cells in a Na^+-free buffer after ammonium chloride washout for an extended time. This resulted in prolonged cellular acidification. However, in doing so there would also be depletion of extracellular Na^+ and it was possible that there would also be a depletion of intracellular Na^+. Studies in sheep have shown that reduction of intracellular sodium ($[Na^+]i$) leads to increased NHE1 activity (214). To rule out the possibility that the stimulation of NHE1 was due to depletion of $[Na^+]i$, NHE1 activity was examined in cells in which SIA treatment was maintained by the NHE1 inhibitor, cariporide (with normal concentrations of extracellular sodium) (212). The results demonstrated that the effects of SIA maintained by NHE1 inhibition was the same as that maintained by removal of extracellular Na^+. This suggested that the mechanism of activation of NHE1 by SIA was due solely to the acidification of the cell. Haworth *et al.* then examined the role of protein kinases in NHE1 stimulation by sustained intracellular acidosis. Stimulation by sustained acidosis also activated the kinases, ERK1/2 and $p90^{RSK}$, and inhibition of the MAPK cascade (with U0126 or PD98059), resulted in the abolishment of NHE1 stimulation by SIA (212). Follow-up studies established that the activation of NHE1 by SIA occurs via the Ras/Raf/MEK pathway (184).

1.6.4 Osmotic activation of NHE1

Osmotic changes to the cell also activate the Na^+/H^+ exchanger (215). Considering that NHE1 functions to regulate intracellular pH, by extruding H^+ and importing Na^+, it is not surprising that this results in changes in cellular ion concentrations and greatly influences cell volume. In non-myocardial cells, responsiveness to osmotic challenge was mediated by residues of NHE1 between the membrane domain and amino acid 566, and was shown to involve phosphorylation independent activation of NHE1 (84,216).

1.7 Thesis objectives

To understand how NHE1 is regulated in heart disease, it is critical to understand and elucidate the mechanisms whereby this protein is activated. The objective of this doctoral research project was to examine the regulation of NHE1 in the myocardium. This was done by 1) identifying and characterizing the exact sites of phosphorylation on the C-terminus of NHE1 responding to physiological stimuli, and by 2) determining the protein kinases which regulate NHE1 directly in the myocardium. The **hypothesis** of this research proposal was that specific protein kinases phosphorylate one or more amino acids of the cytosolic tail of NHE1 that are critical to pH regulation of NHE1 in the myocardium. Not only did we examine the precise amino acids that are phosphorylated on NHE1, but we also characterized the regulation of NHE1 in the myocardium by treating myocardial cells with physiological stimuli, specifically sustained intracellular acidosis and hormonal (α_1-adrenergic) stimulation.

Chapter 2:

Materials and Methods

2.1 Adenovirus production: sub-cloning of DNA of interest into shuttle vector pAdTrack-CMV

To study the regulation of the NHE1 protein in isolated cardiomyocytes, we developed a series of adenoviral vectors that efficiently express the mammalian NHE1 protein in isolated cardiomyocytes. These adenoviral vectors were produced according to the AdEasy™ Vector System by Quantum (217). A schematic of the gene used for production of adenovirus is shown in Figure 2.1 and a simple outline of this procedure is shown in Figure 2.2.

Figure 2.1 The Na^+/H^+ exchanger gene construct used for adenoviral expression of the NHE1 protein in the myocardium. CMV, cytomegalovirus promoter; NHE1, Na^+/H^+ exchanger isoform 1; GFP, green fluorescent protein; HA, hemagglutinin tag; PA, polyadenylation signal; LTR, long terminal repeat, and mutations of Leu163Phe and Gly174Ser.

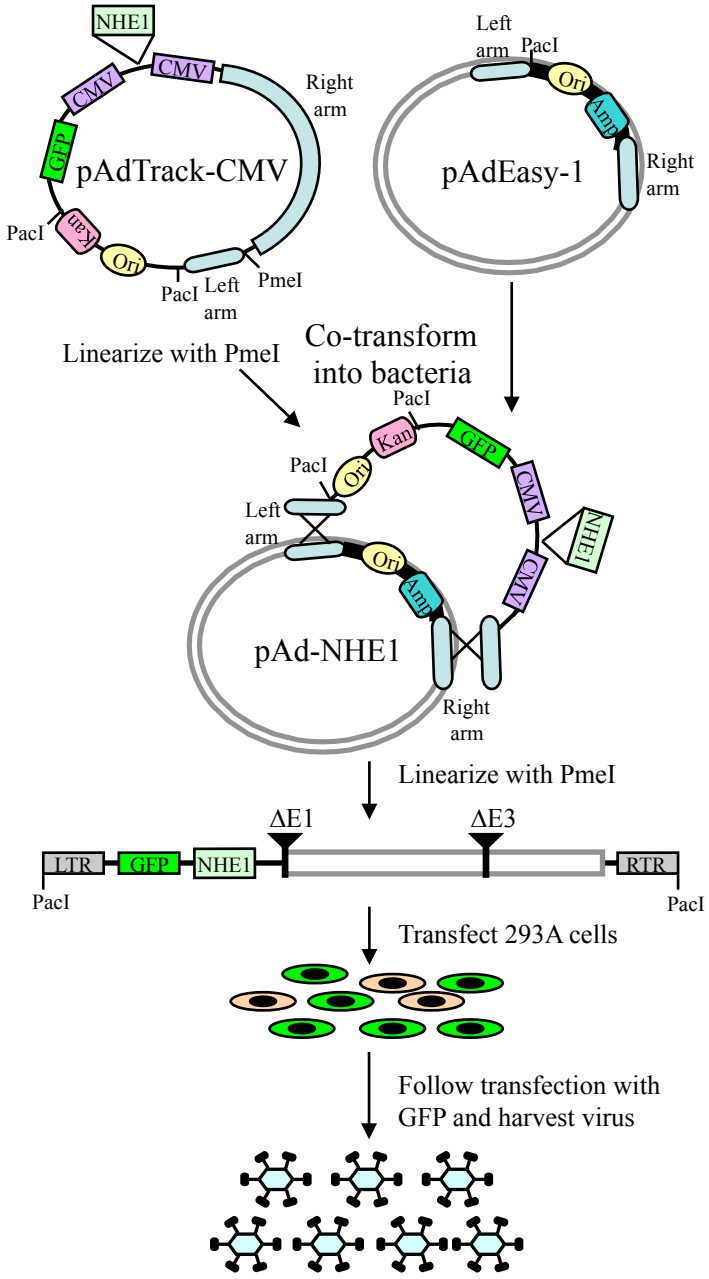

Figure 2.2 Overview of Adenoviral Vector Production. Cartoon representation of the basic steps of adenoviral vector production. (This figure was adopted from He *et al*., (217)).

2.1.1 PCR (production of NHE1 inhibitor resistant mutations and phosphorylation mutations)

Polymerase chain reaction (PCR) was used to introduce DNA mutations into NHE1. Initially the hemagluttin (HA) tagged Na^+/H^+ exchanger was mutated to make it resistant to inhibition by amiloride analogues. The double mutation Leu163Phe/Gly174Ser in NHE1 increases the resistance to inhibition to a level similar to NHE3 resistance (218). These were considered inhibitor resistant mutations (IRM) and were made in the NHE1 cDNA in the plasmid pYN4+. pYN4+ contains the human cDNA of NHE1 that is HA tagged. Expression is driven by the Rous Sarcoma Virus (RSV) promoter and the plasmid contains an ampicillin resistance cassette (Amp^R) for selection (52). The primers for this mutant were pYN4L163Ff GACGTCTTCTTCtTCTTCCTcCTcCCGCCCATCATCC, pYN4L163Fr GGATGATGGGCGGgAGgAGGAAGAaGAAGAAGACGTC and pYN4+Gly174Sf CATCCTGGATGCtagCTACTTCCTGCCAC, pYN4+Gly174Sr GTGGCAGGAAGTAGctaGCATCCAGGATG. Mutations (lower case) were also introduced to add restriction enzyme sites (Nhe1 and BseR1) used for screening plasmids.

To localize the amino acids important in regulation of the NHE1 protein in the myocardium several mutant NHE1 cDNA's were utilized. They were chosen based on earlier studies which demonstrated that four regions of the cytosolic domain of NHE1 were susceptible to ERK2 dependent phosphorylation (113). The mutants were as follows: Mutant #1, Ser693Ala; Mutant #2,

Thr718Ala/Ser723/726/729Ala; Mutant #3, Ser766/770/771Ala; and Mutant #4, Thr779Ala/Ser785Ala. We also mutated the individual amino acids Ser770Ala and Ser771Ala of NHE1 separately. Mutation of pYN4+ plasmids was as described earlier (114). Figure 2.3 depicts the cytoplasmic tail with phosphorylation group mutations (A) and single mutations (B).

For production of NHE1 cDNA with both the inhibitor resistant mutations and the phosphorylation site mutations, we performed restriction enzyme digests and ligations (see below). PCR was used to transfer the cDNA of NHE1 into a new template plasmid, pAdTrack-CMV. This was done for both the control cDNA NHE1 with IRM and the new combined cDNA NHE1 IRM and phosphorylation site mutations from pYN4+. The following primers were used: MackPynF CCGGGGTACCGCCACCATGGTTCTGCGGTCTGG and MackPynR GGAAGCTTAAGCTTCTACTGAGCAGCGTAATCTGGAAC that flanked the insert with Kpn I and Hind III restriction sites, respectively. pAdTrack-CMV drives expression via the cytomegalovirus (CMV) promoter, and contains a kanamycin resistance cassette (Kan^R) for selection.

All PCR reaction mixtures included: 1.5 mM $MgSO_4$, 250 µM dNTPs, 5 µl High Fidelity 10X PCR buffer, 10 µM forward primer, 10 µM reverse primer, 750 ng template DNA, 2.5 U of Platinum® Taq Polymerase High Fidelity (Invitrogen), and were brought to a final volume of 50 µl with ddH_20. PCR was performed using one cycle of 94˚C for 3 minutes, and 16 cycles of 94˚C for 1 minute, and 55˚C for 2 minutes, and 72˚C for 1.5 minutes. After PCR was complete all PCR products were digested with 1 Unit of the restriction enzyme

Dpn1 (New England Biolabs) for 1 hour at 37˚C to remove methylated template DNA. All PCR products were run on a 1% agarose gel to confirm the PCR product was correct, and of the proper size.

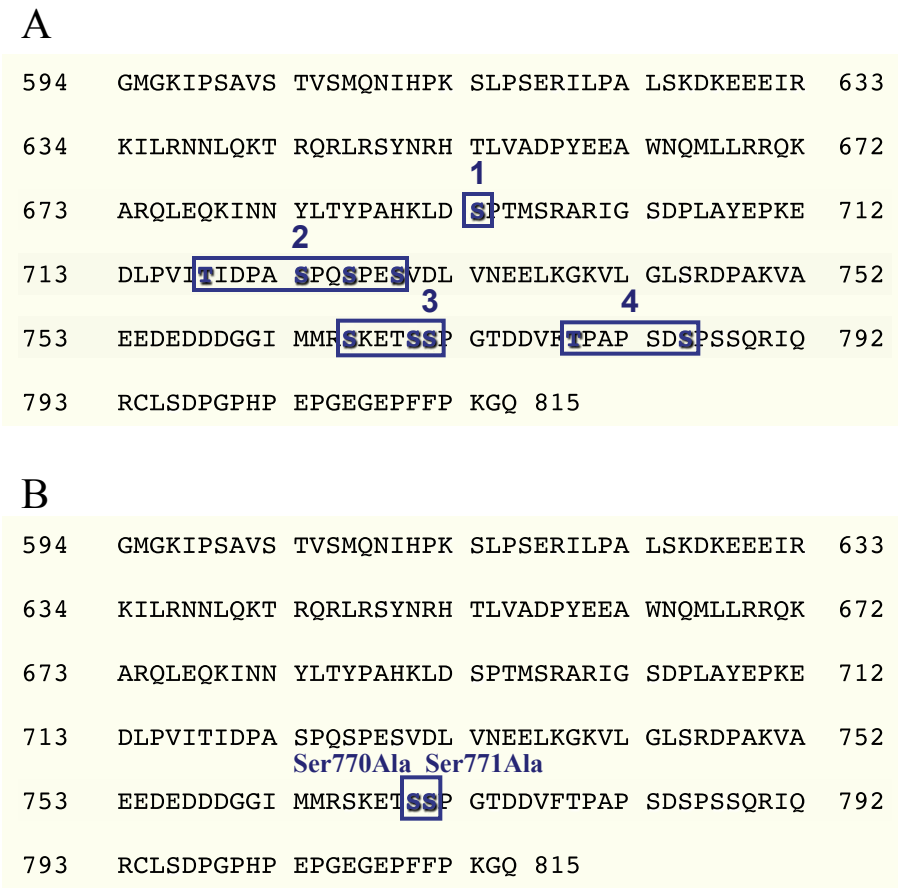

Figure 2.3 Phosphorylation/mutation sites in the cytosolic domain of NHE1. The sequence of the final 221 amino acids of the cytosolic domain of human NHE1. **(A)** Boxed and highlighted in blue are the phosphorylation mutants of NHE1: 1, Ser693Ala; 2, Thr718Ala & Ser723/726/729Ala; 3, Ser766/770/771Ala; and 4, Thr779Ala & Ser785Ala all in which serine and threonine residues have been mutated to alanine. **(B)** Boxed and highlighted in blue are the sites of the single phosphorylation site mutation, Ser770Ala and Ser771Ala.

2.1.2 Restriction enzyme digestion of bacterial DNA

To make the phosphorylation defective mutant NHE1 cDNA's resistant to inhibition, restriction enzyme digests were performed, which moved the resistance containing region into plasmids containing the various phosphorylation site mutations. NHE1 cDNA's with the phosphorylation site mutations and NHE1 cDNA with the inhibitor resistant mutations were digested with the enzymes Age1 and Spe1 (For Mutant #1, 3, 4, 5, 6) and ApaI and Xma1 (Mutant #2). The digests were resolved on a 1% low melting point agarose gel, and specific bands excised, purified and ligated (see below).

Additionally, restriction enzyme digests were performed to confirm that all PCR reactions introduced the proper mutations in the NHE1 cDNA, and ligations of cDNA were complete. The restriction enzyme digests were run on 1% agarose gels to confirm the presence or absence of restriction sites.

The 50 µl digestion reactions contained: 5 µl 10x buffer selected based on the manufacturer's specification. When digests were done with two restriction enzymes, the buffer that best met the conditions for both enzymes was used. The reaction was brought to volume with ddH_20, and 10 Units of enzyme, 1 µl of template DNA (purified plasmid DNA) were used at recommended temperatures and times.

2.1.3 DNA purification from 1% low melting point agarose gel

Selected DNA digests were run on a 1% low melting point agarose gel. They were briefly exposed to UV light to mark bands for excision. After bands

were removed gel slices were placed in Eppendorf tubes which were placed in liquid nitrogen to freeze the gel slices. Frozen gel slices were placed between clean Parafilm, gently squeezed between the Parafilm with fingers so that the gel slice began to melt and the liquid containing DNA was collected with a pipet and placed in a new Eppendorf tube. DNA was purified by adding phenol: chloroform: isoamyl alcohol (PCI) 25: 24: 1 solution. Eppendorf tubes were spun with PCI and DNA solution in a tabletop centrifuge at 14,000 rpm for 5 minutes at room temperature. Then the aqueous layer (top) was extracted and placed into a new Eppendorf tube and the PCI extraction was repeated once more. DNA was precipitated from the aqueous solution using a 10% volume of 3 M NaAcetate pH 5.2, and 2X volume of 100% ethanol. The mixtures were inverted and placed in the -20˚C freezer for 2 hours. Samples were spun in a tabletop centrifuge at 14,000 rpm for 20 minutes at room temperature. The 100% ethanol was gently poured off and the pellet was washed with 70% ethanol. The DNA pellet was resuspended in 10 µl ddH_20.

2.1.4 DNA ligation

Purified DNA fragments were ligated using the New England Biolabs T4 DNA ligase. The 20 µl ligation reaction mixture contained: 2 µl 10x T4 DNA Ligase Buffer, 20 Units of T4 DNA Ligase, a ratio of 9:1 of insert to vector DNA (9 µl insert DNA and 1 µl vector DNA). The ligase reactions were incubated at 16˚C for 16 hours.

2.1.5 Bacterial cell transformations

Electrocompetent *E. coli* strains XL1-Blue and BJ5183-AD-1 (Stratagene) were transformed by electroporation using 1.8 kV, 25 μF capacitance, 150 Ω resistance (Biorad Gene Pulser® II). Purified plasmid DNA or ligated DNA (approximately 1 μl) was used to transform bacterial cells. Once transformed, bacterial cells were transferred to 1 mL of cold LB (Luria-Bertani) medium and placed in a 37˚C shaker for 1 hour. Bacterial cells were then plated on LB agarose plates containing either 100 μg/mL of ampicillin (Amp) or kanamycin (Kan) and incubated at 37˚C for 18 hours.

2.1.6 Isolation of bacterial plasmid DNA

Transformed *E. coli* colonies were picked individually and cultured in 2 mL of LB media containing either 100 μg/mL Amp or Kan and incubated in a 37˚C shaker for 18 hours. Bacterial cells were harvested by centrifugation at 12,000 rpm for 2 minutes and plasmid DNA was isolated using the rapid miniprep protocol. Bacterial cell pellets were resuspended in 150 μl of Solution 1 (50 mM Glucose, 25 mM Tris pH 8.0, 10 mM EDTA), followed by addition of 150 μl of Solution 2 (0.2 M NaOH, 1% SDS). The solution was gently mixed by inverting the Eppendorf tubes five times and the mixture was left to stand at room temperature for 5 minutes. 150 μl of Solution 3 was then added (3 M K-Actetate, and 1.5% glacial acetic acid). The solution was mixed by inverting five times and then left to stand on ice for 10 minutes. The cell suspension was then centrifuged at 12,000 rpm for 5 minutes. The supernatant was transferred to a new Eppendorf

tube and plasmid DNA was extracted using PCI (as described above in 2.1.3). Eppendorf tubes with PCI and DNA solution were centrifuged in a tabletop centrifuge at 14,000 rpm for 5 minutes at room temperature. The aqueous layer (top) was then transferred to a new Eppendorf tube. DNA was precipitated from the aqueous solution using 10% volume of 3 M NaAcetate pH 5.2, and 2X volume of 100% ethanol. Eppendorfs were inverted and then placed in a -20˚C freezer for 2 hours. Samples were centrifuged in a tabletop centrifuge at 14,000 rpm for 20 minutes at room temperature. The 100% ethanol was gently poured off and the DNA pellet was washed with 70% ethanol. The DNA pellet was then resuspended in 50 µl TE buffer solution (10 mM Tris pH 8.0 and 1 mM EDTA pH 8.0) with 20 µg/mL RNase and then incubated at 37˚C or 30 minutes.

2.1.7 DNA sequencing

DNA sequencing was used to confirm the presence of proper DNA mutations. Sequencing was performed for all pYN4+ plasmid mutants as well as for all pAdTrack-CMV with cDNA's of NHE1 inserted. For sequencing, DNA was purified by PCI and resuspended in ddH_20. The University of Alberta, Department of Biochemistry DNA Core Services Ltd performed all sequencing.

2.2 Adenovirus production: generation of recombinant adenoviral plasmids by homologous recombination in *E. coli*

All constructs of pAdTrack-CMV with cDNA containing either NHE1-IRM or NHE1-IRM plus phosphorylation site mutations were cloned into the vector pAdEasy-1 (Figure 2.2). pAdEasy-1 contained the adenovirus genome with two genes (E1 and E3) deleted, as well as two recombinant arms, and an ampicillin resistance cassette (Amp^R) for selection. All pAdTrack-CMV-NHE1 constructs were linearized with the restriction enzyme, Pme1. The restriction enzyme digests were purified by running on 1% low melting point agarose and the DNA was extracted, purified and precipitated (as described in section 2.1.3).

Pme1 cut shuttle vector (300 ng) was used to transform *E. coli* strain BJ5183 (Stratagene) (as described in section 2.1.5). BJ1583 is an *E. coli* strain that is recombination proficient and carries the p-AdEasy-1 plasmid that encodes the Adenovirus-5 genome (E1/E3 deleted), and for selection the streptomycin (Str^R) and ampicillin (Amp^R) resistance cassettes (Figure 2.2). These cells supply the components necessary to execute a recombination event between the pAdEasy-1 vector and the pAdTrack-CMV shuttle vector containing the NHE1 gene (which we inserted). The result of this transformation is the generation of a recombinant adenovirus genome that contains the NHE1 gene. All recombinants were screened as described above. Isolated plasmid DNA was confirmed by digestion with restriction enzyme Pac1 and run on long 1% agarose gels. Non-

recombined pAdEasy-1 has only one Pac1 site (size of band ~23 kb), and properly recombined pAdEasy-1 and pAdTrack-CMV has two Pac 1 sites (size of bands, ~20 kb and 3 or 4.5 kb). The smaller band of 3 or 4.5 kb results from differences in recombination sites. All constructs made resulted in a 4.5 kb band. As the BJ1583 cell line contains recombinases, which could result in unwanted recombination events, the newly produced recombinant DNA was retransformed into XL-1 Blue *E. Coli*. This is a bacterial cell line with relatively low recombinase levels. This resulted in large-scale production, extraction, purification and precipitation of bacterial plasmid DNA (as described in section 2.1.6). As before, all recombinants were screened using restriction enzyme digests including enzymes Pac1, BamH1, EcoR1 and SnaB1. Figure 2.4 shows an agarose gel of recombinant NHE1-IRM plasmid digested with Pac1 and BamH1. All patterns were compared to control with no recombination, pAdEasy-1. Prior to transfection of recombinant plasmid in 293A cells, 8 μg of recombinant plasmid DNA was digested with Pac1 for 18 hrs at 37°C and then heat inactivated at 65 °C for 20 minutes.

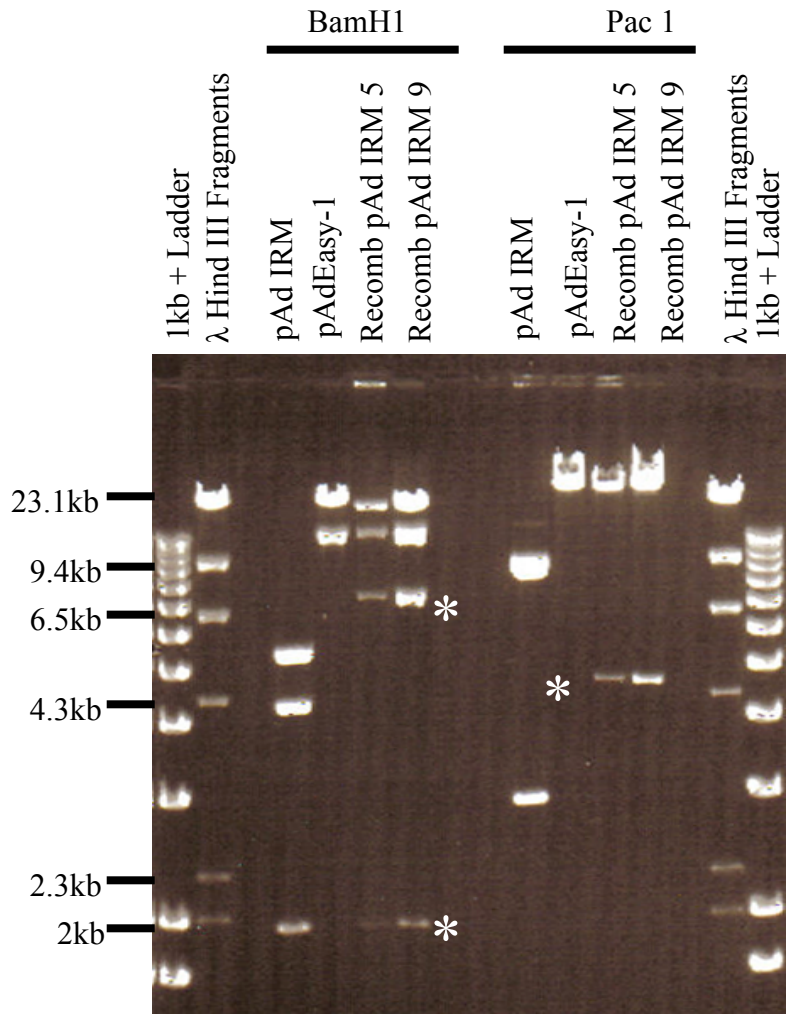

Figure 2.4 DNA agarose gel of putative positive plasmids containing NHE1-IRM recombinants. Lanes indicate the contents including the DNA from various constructs and the restriction enzyme used. * denotes a restriction fragment that indicates that the NHE1-IRM properly recombined.

2.3 Adenovirus production: production, purification, and titration of adenovirus

2.3.1 Production of adenovirus in mammalian cells

For production of adenovirus in mammalian cells we used the HEK293A cell line, a human embryonic kidney cell line that was transformed with sheared adenovirus type 5 DNA. These cells contain and express the early region 1 of the Adenovirus 5 virus (Stratagene) and thus they complete growth of E1 defective adenovirus mutants and vectors.

HEK293A cells were grown to 70% confluency in two T25 flasks (Falcon) with αMEM medium, 10% fetal bovine serum (FBS) and 10 µg/mL gentimicin, for transfection. For transfection two solutions were made: Solution A (8 µg of heat inactivated Pac 1 digested recombinant plasmid and 200 µl serum free Opti-MEM® I Reduced Serum Medium (Invitrogen)) and Solution B (40 µl Lipofectamine™ 2000 (Invitrogen) and 200 µl serum free Opti-MEM®). Both solutions were mixed gently. The solutions were then incubated separately for 30 minutes at room temperature and then were combined gently and incubated for 15 minutes at room temperature. The HEK293A cells in the T25 flasks were rinsed with 4 mL of serum free Opti-MEM, and then 2.5 mL of serum free Opti-MEM was added to each flask. The cells were then incubated at 37 °C and 5% CO_2 for 10 minutes. In the meantime, 1.6 mL of serum free Opti-MEM was added to the combined Solutions A and B and mixed gently. Then, 1 mL of the Solution A and

B combination in Opti-MEM was added to each T25 flask of 293A cells. Cells were incubated for 4 hours at 37 °C and 5% CO_2, after which the transfection medium was vacuumed off the cells and 6 mL of alpha minimum essential medium (αMEM) (Sigma), with 5% horse serum (HS) and 10 µg/mL of gentimicin was added. Cells were incubated at 37°C and 5% CO_2. Medium with horse serum as opposed to fetal bovine serum is used to slow cell growth to prolong the time cells can be infected, resulting in a stronger viral lysate. Transfection is monitored by green fluorescent protein (GFP) fluorescence using a microscope; GFP fluorescence only occurs in producing viruses, as GFP is one of the genes that is expressed in the recombinant plasmid. Viral production is monitored and takes approximately 7 days for viral lysis to occur. 2 mL of medium is exchanged from the T25 flask with fresh medium every 2-3 days. When cell lysis reaches ~70%, cells are harvested in 50 mL conical tubes and frozen in liquid nitrogen, thawed at 4°C and centrifuged for 5 minutes at 5,000 rpm at room temperature to remove cell debris. This viral lysate produced was tested for proper NHE1 expression. 150 µl of lysate was added to 60 mm dishes of confluent AP-1 cells and then NHE1 protein extracted using RIPA lysates and analyzed by Western blotingt (as described in sections 2.4.2 and 2.4.3). This lysate was then used for large-scale adenovirus production.

2.3.2 Large-scale production of adenovirus

HEK293A cells were seeded on 150 mm dishes to a confluency of 70% with 20 mL of fresh αMEM medium with 5% HS and 10 µg/mL gentimicin. Then

1 mL of viral lysate (prepared as described in section 2.3.1) was added to a 150 mm dish. Cells were then incubated at 37°C and 5% CO_2 for approximately 3 days until 80% cell lysis occurred, after which the cells and media were collected in a 50 mL conical tube, frozen in liquid nitrogen, thawed and centrifuged for 5 minutes at 5,000 rpm at 4°C. This new viral lysate of ~20 mL was then used for the larger scale infection of 24 150 mm dishes of cells at 90% confluency. New αMEM medium with 5% HS and 10 μg/mL gentimicin medium was added to each 150 mm dish (20 mL), as well as ~0.8 mL of viral lysate. Cells were then incubated at 37°C and 5% CO_2 from 2-5 days so that 80-100% cell lysis occurred. Once the cells were lysed, the cells and media were collected in 50 mL conical tubes, frozen in liquid nitrogen and stored at -80°C.

2.3.3 Adenovirus purification

Large-scale viral lysates were subjected to three freeze-thaw cycles. Lysates were stored in 50 mL conical tubes and thawed at 37°C (not letting lysates reach warm temperatures). They were then vortexed and refrozen in liquid nitrogen. Two 50 mL tubes of lysate were then stored at -80°C for future use. The remaining lysates were centrifuged for 15 minutes at 4,000 rpm at 4°C to remove cellular debris. The supernatants were transferred to a 500 mL sterile glass beaker. Powdered ammonium sulfate (242.3g per liter) was added slowly to the beaker with lysate and allowed to dissolve. The lysate-ammonium sulfate solution was stirred gently for 2 hours at room temperature. Once the solution had stirred, it was transferred to clean 50 mL conicals and centrifuged for 15 minutes at 1614 x

g at room temperature. The supernatant was discarded and the pellet was dissolved in ~1 mL of phosphate buffered saline (PBS) per conical tube. Dissolved pellets were then centrifuged for 15 minutes at 4,000 rpm at room temperature to remove debris. At this stage the adenovirus is in the dissolved pellet as a result of the ammonium sulfate precipitation of adenovirus from culture. This method was used as it increased the virus titer concentration (219).

Following the ammonium sulfate adenovirus precipitation, the adenovirus was purified using a cesium chloride (CsCl) gradient. To make the CsCl gradient, CsCl was made up in H_20 at three concentrations: 1.5 g/mL, 1.35 g/mL and 1.25 g/mL. To ensure the proper weight/volume of CsCl, 1 mL aliquots of each solution were weighed several times. The final solutions were filter sterilized through a 0.2 μm filter.

A discontinuous CsCl gradient was made by gently layering 1 mL of 1.5 g/mL of CsCl solution then 6 mL of the 1.35 g/mL CsCl solution and finally 6 mL of the 1.25 g/mL CsCl solution. Note that the CsCl solution was added drop by drop very gently to create the gradient. The supernatant from the ammonium sulfate precipitation was then gently transferred to the top of the gradient. Ultracentrifugation tubes were then balanced and centrifuged at 150,000 x g for 22 hours at 10°C in a Ti60 rotor (Beckman L-80 Ultracentrifuge).

Using a sterile Pasteur pipette, the thin grey adenovirus bands from the CsCl gradient were removed and placed in a sterile 50 mL conical. The adenovirus bands appeared approximately ¾ down from the top of the ultracentrifugation tubes.

Dialysis was used to remove the CsCl. A 2 L dialysis solution of 1X PBS and 10% glycerol was prepared and filter sterilized. One liter of dialysis solution was placed in a sterile 1.5 L glass beaker. The Pierce Slide-A-Lyzer dialysis cassette (10,000 MWCO, 3-15 mL capacity, Pierce) was made wet with dialysis solution for 1 min. Using a 10 mL syringe with an 18G needle, the adenovirus solution was drawn up and the sample was injected in port 1. Residual air was then removed from port 2. The adenovirus was dialyzed for 5 hours at 4°C, with gentle stirring. After the 5 hours of dialysis, the dialysis buffer was changed and the sample was dialyzed overnight with gentle stirring at 4°C.

When dialysis was complete, the adenovirus was removed from the dialysis cassette using an 18G needle on a 10 mL syringe. Initially, a little air was injected into port 3 and then adenovirus solution was withdrawn. Port 4 was used to make sure all adenovirus had been collected. The collected adenovirus was centrifuged after dialysis for 5 minutes at 1620 x g at 4°C to remove any debris. It was then aliquoted into sterile microcentrifuge tubes, ~1 mL per tube, labeled and stored at 4°C for use.

2.3.4 Adenovirus titration

To determine the concentration of active adenoviruses we calculated viral titers using a plaque assay. HEK293A cells were plated to about 50-60% confluency in 35 mm dishes. Appropriate dilutions of adenovirus were made from 10^3 to 10^{11} in a final volume of 1 mL of αMEM, 5% HS and 10 μg/mL gentimicin. One control dish was without adenovirus. Medium was removed from

the cells and the diluted adenovirus was added to each appropriately labeled dish. The 35 mm dishes with adenovirus were then incubated at 37°C and 5% CO_2 for 4 hours. While the cells and adenovirus were incubating, the agar overlay solution was prepared. A previously prepared sterile solution of 1% agar in ddH_20 (Difco) was heated, and incubated at 42°C. 3XαMEM medium with bicarbonate at pH 7.2 was filter sterilized, and was heated to 37°C. In the 40°C water bath, the agar overlay solution was made (1X αMEM with bicarbonate, 5% HS, and 0.5% agar). The adenovirus was removed from the 35 mm dishes and 2 mL of agar overlay solution was added drop by drop to each dish and allowed to solidify at room temperature. Dishes were then maintained at 37°C and 5% CO_2. Every 4-5 days, 1.5 mL of fresh agar overlay solution (as described above) was added on top of existing agar. On day 4 post-infection, each dish was examined for fluorescent cells under the microscope (generally for the higher dilutions 10^8-10^{11}), after which the formation of visible plaques was counted every day for 2 weeks. From the number of fluorescent cells and the dilution of the adenovirus, a fluorescent titer was calculated. From the total number of plaques per dish a plaque titer was calculated. All titers were performed in duplicate with two different sets of diluted adenovirus. Table II lists the various adenoviruses made and their titers.

Adenovirus	Titer (particle forming units (PFU)/mL)
Ad-GFP	2.65×10^{10}
Ad-NHE1-IRM	3.00×10^{9}
Ad-Ser693Ala-IRM (Mutant 1)	2.90×10^{9}
Ad-Thr718Ala, Ser723/726/729Ala-IRM (Mutant 2)	1.00×10^{9}
Ad-Ser766/770/771Ala-IRM (Mutant 3)	2.25×10^{9}
Ad-Thr779Ala and Ser785Ala-IRM (Mutant 4)	1.95×10^{9}
Ad-Ser770Ala-IRM	7.90×10^{8}
Ad-Ser771Ala-IRM	1.48×10^{9}
Ad-CHP1	2.70×10^{9}

Table II: Adenoviruses produced and their titers. Titer is in particle forming units per ml (PFU/ml). Indicated by each Adenovirus (Ad) is what the adenovirus contains. NHE1: Na^+/H^+ exchanger isoform 1. IRM, inhibitor resistant mutations of NHE1. Also indicated are the mutations in the cytosolic tail of NHE1. CHP1, calcineurin homologous protein isoform 1.

2.4 Characterization of plasmids for adenovirus production

2.4.1 Transient transfection of AP-1 cells

To ensure that all NHE1 constructs in pAdTrack-CMV expressed a functional NHE1, we performed transient transfections in AP-1 cells. AP-1 cells are Chinese hamster ovary cells that are deficient in plasma membrane NHE activity (2). Cells were plated in 60 mm dishes at 80% confluency in complete minimum essential medium (MEM) (Sigma) supplemented with 10% bovine growth serum (BGS). 18 hours prior to transfection, cells were washed with PBS and serum starved with complete MEM supplemented with 0.5% BGS. For each plate transfected, the following solutions were prepared: 20 µl Lipofectamine™ 2000 (Invitrogen) per 0.5 mL Opti-MEM® I Reduced Serum Medium (Invitrogen), and 8 µg plasmid DNA per 0.5 mL Opti-MEM® I Reduced Serum Medium (Invitrogen). The solutions were incubated at room temperature for 5 minutes, and then combined and mixed gently and incubated at room temperature for 20 minutes; this allows DNA-Lipofectamine™ 2000 complex formation. Then 1 mL of DNA-Lipofectamine™ 2000 solution was added to each dish, and incubated at 37˚C and 5% CO_2 for 6 hours and then 0.5 mL of BGS was added to each dish. Dishes were incubated overnight at 37°C and 5% CO_2.

2.4.2 Preparation of NHE1 from cultured cells

NHE1 was extracted from AP-1 cells, 293A cells, and isolated neonatal cardiomyocytes using RIPA lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1

mM EGTA, 1% (v/v) NP-40, 0.1% (v/v) Triton X-100, 0.25% (w/v) SDS and protease inhibitors: 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM benzamidine, and 0.1% (v/v) protease inhibitor cocktail (220).

Confluent cells in 60 mm dishes were washed with cold PBS and then placed on ice where 100 µl of RIPA Lysis buffer was added to each dish. The cells with lysis buffer were incubated for 3 minutes on ice, after which cells were scraped using a disposable scraper for 2 minutes. The cell suspension was then transferred to an Eppendorf tube and centrifuged at 14,000 rpm for 5 minutes at 4˚C. After the centrifugation, the supernatant (containing solubilized NHE1) was transferred to new tubes, being careful not to disturb the pellet. The pellet containing cell debris was discarded. Lysates were frozen in liquid nitrogen and stored at -80˚C.

2.4.3 Western blot analysis of NHE1 expression

NHE1 was resolved on 10% acrylamide SDS-PAGE. Samples from RIPA lysate supernatants were mixed with 4X SDS-PAGE loading dye (30% glycerol, 3% 2-mercaptoethanol, 6% SDS, 0.13 M Tris at pH 6.8, and 0.133 mg/mL Bromophenol Blue) in a 3:1 ratio. The SDS-PAGE gel ran for 1 hour at 60V past the stacking gel (5% acrylamide) and then at 120V until the dye front had run off. Proteins from the SDS-PAGE gel were then transferred to a nitrocellulose membrane (Biorad) for 1 hour at 450 mA.

Nitrocellulose membranes were blocked by gently rocking with blocking buffer solution (LI-COR Biosciences, Lincoln, Nebraska, USA) for 1 hour.

Membranes were then rinsed in Tris-buffered saline (TBS). Primary antibodies used to detect NHE1 were either 1:2000 dilution of 12CA5 (monoclonal mouse anti-HA antibody) or 1:2000 dilution of Y11 (polyclonal rabbit anti-HA antibody, Santa Cruz) and were diluted in TBS + 0.1% Tween-20 overnight. MF 20 anti-myosin antibody was used to control for loading of samples at a 1:2000 dilution (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA). After incubation with primary antibodies, the membrane was washed with TBS + 0.1% Tween-20 4 times for 5 minutes each at room temperature. The membrane was then incubated with fluorescent labeled secondary antibody 1:10, 000 dilution of goat anti-mouse (GAM) IRDye 800CW or goat anti-rabbit (GAR) IRDye 680 (LI-COR Biosciences, Lincoln, Nebraska, USA) in TBS + 0.1% Tween-20 for 1 hour in a dark box at room temperature. The membrane was then washed 4 times for 5 minutes each in TBS + 0.1% Tween-20. After rinsing, the membrane was stored in TBS. To visualize immuno-reactive proteins, we used the Licor Odyssey Infrared Imager. Quantifications were done using the Odyssey system (LI-COR Biosciences, Lincoln, Nebraska, USA).

2.5. Characterization of NHE1 mutants in neonatal rat cardiomyocytes

2.5.1 Preparation of isolated rat neonatal cardiomyocytes

Primary cultures of cardiomyocytes were prepared from neonatal Sprague Dawley rat heart ventricles as described previously (155). Briefly, hearts were removed from 5-6 day old rats under aseptic conditions, atria were removed and ventricles minced to a small size. The tissue was digested with three treatments of 0.1% collagenase (Worthington) and 0.05% trypsin (Sigma) solution, 20 mL, 15 mL and 7.5 mL, each cycle lasting for 20 minutes at 37°C with gentle stirring. Digested tissue was neutralized with a solution containing fetal bovine serum and kept on ice. Each myocyte solution was filtered with a 70 μm cell strainer (Falcon) to get rid of debris. Mycoyte cell solution was then centrifuged for 8 minutes at 2,000 rpm at room temperature. Supernatant was removed and discarded and the remaining pellet containing cardiomyocytes was resuspended in a solution containing Dulbecco's modified Eagle's medium (DMEM/F12), 0.25 mg/mL Fetuin (Sigma), 5 μg/ml transferrin, 5 μg/ml insulin, 5 ng/ml selenium, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.72 mM calcium chloride, 0.11 mM L-ascorbic acid, 10 mg/ml bovine serum albumin (BSA), 0.1 mM minimum essential medium (MEM) non-essential amino acids, 10% MEM vitamin, pH 7.4.

To cause selective removal of non-myocardial cells, a differential attachment procedure was used. Dissociated cells (from above) were incubated in T-75 flasks at 37°C in a humidified atmosphere (95%O_2 and 5% CO_2) for a 20

minute period. During this time, non-cardiomyocytes (fibroblasts, endothelial cells, and smooth muscle cells) attach and the majority of cardiomyocytes remain in suspension. Subsequently, isolated primary cardiomyocytes were removed and plated onto glass coverslips for physiologic studies, or onto Primaria^{TM} (Falcon) culture dishes or flasks. Myocytes were maintained for 3-4 days in medium containing Dulbecco's modified Eagle's medium (DMEM/F12) supplemented with 10% fetal 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. Previous studies examined cardiomyocytes in culture for up to 2 weeks, and analysis by light microscopy and immunostaining revealed cultures to be essentially free of non-myocardial cells. Furthermore, the cardiomyocytes contained myocyte like morphology and characteristic cross striations (155).

2.5.2 Cardiomyocyte adenovirus infection

Prior to adenoviral infection, the number of cardiomyocytes was estimated. During each myocyte preparation a single 35 mm dish was plated with 1 mL of the cardiomyocyte cell suspension, which was used for cell counting. Cells were washed and 1mL of trypsin (0.25% Trypsin EDTA, Gibco) was added for 10 min (cardiomyocytes are more resiliant to trypsin than other cells, so they need to be treated longer). Cells were then diluted with 5 mL of myocyte medium

(described in section 2.5.1) and tritrated. Using a hemocytometer, cells were counted, and concentration per mL of cells determined. All adenoviral calculations were performed as follows: #cells/mL x mL cells/dish results in #cells/dish and then multiplied by multiplicity of infection (MOI) (number of PFU per cell). Number of cells/dish x MOI results in PFU required per dish. This number of PFU/dish is divided by the viral titer of PFU/mL and results in the volume in mL of virus stock needed for infection. Virus stock of appropriate volume was added to cells with a fresh medium change (either 10% or 0.5% FBS depending on treatment desired). Cells were then incubated at 37°C and 5% CO_2 for times as required.

2.5.3 RIPA lysates of NHE1 protein and Western blot analysis

RIPA lysates of NHE1 protein were performed as described in section 2.2. Prior to Western blotting, total protein concentrations of the RIPA lysates were calculated using the BioRad DC Protein Assay kit. Western blotting was performed as previously described in section 2.4.3, with 50-100 μg of total protein loaded per lane. Western blot analysis also included the use of a commercial antibody against the NHE1 cytoplasmic tail where appropriate (MAB349 from Chemicon, Billerica, MA).

2.5.4 Intracellular pH measurement

To examine NHE1 activity in isolated cardiomyocytes, cells were infected with Ad-NHE1-IRM or various phosphorylation site mutants of Ad-NHE1-IRM.

Infection of cardiomyocytes for NHE1 activity assays was performed as described in section 2.5.2. Cells were routinely infected with the specified adenovirus at a MOI of 20 for 24 hours prior to intracellular pH (pH*i*) measurement. Intracellular pH of cells on coverslips was measured using a PTI Deltascan spectrofluorometer. The activity of NHE1 was measured following an acid load (52).

Isolated cardiomyocytes, uninfected or infected with the specified adenoviruses, were incubated prior to the activity assay for 30 minutes with αMEM supplemented with 0.5% FBS and 2 μg/mL of the pH-sensitive dye 2',7-bis(2-carboxyethyl)-5(6) carboxyfluorescein-acetoxymethyl ester (BCECF-AM; Molecular Probes Inc., Eugene, OR, USA). BCECF-AM is cell permeable and non-fluorescent; however, once inside the cell, BCECF-AM is cleaved by cellular esterases to BCECF, which makes the dye impermeable and fluorescent (221). Using the dye BCECF, the PTI fluorometer excitation wavelengths were 425 nm and 503 nm and the emission wavelength was 524 nm. The ratio of BCECF emissions at 524 nm gives the measurement of pH*i*, which is independent of dye concentration.

After the cells were loaded with BCECF, activity assays were performed in solutions pre-warmed to 37°C. BCECF-loaded cells on coverslips were placed in a cuvette holder, with 2.5 mL of Na^+ normal buffer (135 mM NaCl, 5.0 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, 5.5 mM Glucose, 10 mM Hepes, solution pH 7.4) at 37°C for 1 minute, allowing the pH*i* to stabilize. Ammonium chloride (NH_4Cl) was then added to a concentration of 50 mM (50 μl of 2.5 M NH_4Cl stock solution) for 3 minutes at 37°C. Upon the addition of NH_4Cl there is an

initial alkalinization, followed by equilibration; this step primes the cell for an acid load. The coverslip was then removed from the cuvette and placed in a cuvette containing 2.5 mL Na^+-Free buffer (135 mM N-methyl-D-glucamine, 5.0 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, 5.5 mM glucose, 10 mM Hepes, solution pH 7.4) at 37°C. The coverslip was rinsed 5 times in the Na^+-Free buffer (~30 seconds) and then removed and placed in a cuvette with Na^+-normal buffer and allowed to recover for 3 minutes. Following the recovery from an acid load, the cells were equilibrated in a three-step pH calibration, using Na^+-free calibration buffer (135 mM N-Methyl Glucamine, 135 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, 5.5 mM glucose, 10 mM Hepes, and 5 μM nigercirin, at pH's of 6, 7 and 8). Cells were incubated in each calibration buffer for 2 minutes until equilibrium was established. The calibration measurements were used to convert fluorescence output into pH (as previously described (52)). NHE1 activity was then measured as the rate of recovery after an acid load, which was calculated from the first 20 seconds after return of Na^+ and expressed as change in pH/sec (ΔpH/sec) or alternatively pH/min (ΔpH/min).

2.5.5 Inhibition of NHE1 by EMD 87580

We performed two pulse activity assays to determine the concentration of the NHE1 specific inhibitor EMD87580 that was sufficient to inhibit endogenous NHE1 in isolated cardiomyocytes, but did not inhibit exogenous NHE1 with the Leu163Phe/Gly174Ser mutations (inhibitor resistant mutations, IRM) (222). Cardiomyocytes were either uninfected or infected with adenovirus NHE1-IRM

for 24 hours in 10% FBS medium and incubated at 37°C and 5% CO_2 overnight. EMD87580 was diluted in PBS at concentrations: 0 μM, 0.3 μM, 1 μM, 3 μM, 10 μM, 30 μM, 100 μM, and 300 μM. The two pulse acidification assay for EMD87580 treated cells was performed as follows:

EMD87580 Inhibition:

1. Incubated cells in Na^+ normal buffer at 37°C for 1 minute.
2. Added 50 mM NH_4Cl and incubated at 37°C for 3 minutes.
3. Removed NH_4Cl-containing buffer and acidified with Na^+-free buffer (~30 seconds).
4. Replaced Na^+-free buffer with Na^+ normal buffer and incubated at 37°C for 3 minutes. The first NHE activity was recorded from recovery in this step.
5. Added appropriate concentration of EMD 87580 (0 - 300 μM) for 3 minutes at 37°C.
6. Added 50 mM NH_4Cl and incubated at 37°C for 3 minutes (EMD 87580, 0 – 300 μM).
7. Removed NH_4Cl-containing buffer and acidified with Na^+-free buffer and incubated at 37°C for 3 minutes (EMD 87580, 0 – 300 μM).
8. Replaced Na^+-free buffer with Na^+ normal buffer and incubated at 37°C for 3 minutes (EMD 87580, 0 – 300 μM). Second recovery was calculated in this step.
9. Three-point calibration in calibration buffers of pH 6, 7, and 8.

Calculations for the two pulse acidification assays were done to show the effect of EMD87580 on NHE1 activity. The activity of the Na^+/H^+ exchanger in the second pulse (Step 8) in the presence of EMD87580 (0 – 300 μM) was compared to the NHE1 activity in the first pulse in the absence of EMD87580 (Step 4) and expressed as a percentage.

2.5.6 Stimulation of NHE1 activity

2.5.6A Sustained intracellular acidosis

To test the effects of sustained intracellular acidosis (SIA) (212), cells were infected in low serum (0.5% FBS) medium (section 2.5.2) and incubated at 37°C and 5% CO_2 overnight and activity assays were performed with a two-pulse acidification assay. The SIA treatment was as described earlier and has been previously described by others and our laboratory (114,212). The two-pulse method was used to measure the effect of SIA treatment on NHE1 activity, while enabling normalization for the activity of various NHE1 mutants. Assays were done in either the presence or absence of 10 μM EMD87580. EMD87580 was dissolved in PBS and added during ammonium chloride treatment, in Na^+-free medium and during recovery in NaCl. We previously determined that 10 μM EMD87580 was sufficient to inhibit endogenous NHE1 in isolated cardiomyocytes, but does not inhibit exogenous NHE1 with the Leu163Phe/Gly174Ser mutations (222). The two-pulse activity assays were performed for control cells and stimulated cells as follows:

Control:

1. Incubated cells in Na^+ normal buffer at 37°C for 1 minute.
2. Added 50 mM NH_4Cl and incubated at 37°C for 3 minutes.
3. Removed NH_4Cl-containing buffer and acidified with Na^+-free buffer (~30 seconds).
4. Replaced Na^+-free buffer with Na^+ normal buffer and incubated at 37°C for 3 minutes.
5. Repeated steps 2-4.
6. Three-point calibration in calibration buffers of pH 6, 7, and 8.

Stimulated:

1. Incubated cells in Na^+ normal buffer at 37°C for 1 minute.
2. Added 50 mM NH_4Cl and incubated at 37°C for 3 minutes.
3. Removed NH_4Cl-containing buffer and acidified with Na^+-free buffer (~30 seconds).
4. Replaced Na^+-free buffer with Na^+ normal buffer and incubated at 37°C for 3 minutes.
5. Added 50 mM NH_4Cl and incubated at 37°C for 3 minutes.
6. Removed NH_4Cl-containing buffer and acidified with Na^+-free buffer and incubated at 37°C for 3 minutes.
7. Replaced Na^+-free buffer with Na^+ normal buffer and incubated at 37°C for 3 minutes.
8. Three-point calibration in calibration buffers of pH 6, 7, and 8.

For another set of experiments, sustained intracellular acidosis was induced in the presence of Na^+ (135 mM NaCl) but was maintained by the addition of 20 μM EMD87580. Calculations for the two-pulse assay were done to show the effect of sustained acidosis on NHE1 activity, in ΔpH/min. The difference between the two pulses (second pulse minus first pulse) was compared for control versus SIA.

2.5.6B Phenylephrine stimulation

To test the effects of phenylephrine (PE) stimulation (184), cells were infected in 10% FBS medium (section 2.5.2) and incubated at 37°C and 5% CO_2 overnight. Activity assays were performed using the single pulse acidification assay. Assays were done in either the presence or absence of 10 μM EMD87580. EMD87580 was dissolved in PBS and added during ammonium chloride treatment, in Na^+-free medium and during recovery in NaCl. We previously determined that 10μM EMD87580 was sufficient to inhibit endogenous NHE1 in isolated cardiomyocytes, but does not inhibit exogenous NHE1 with the Leu163Phe/Gly174Ser mutations (222). To test the effect of phenylephrine on NHE1 activity, cells were treated for 6 minutes with 100 μM freshly made phenylephrine added to Na^+ normal buffer. 100 μM phenylephrine was maintained throughout the single pulse activity assay (described in section 2.5.4) (184). Control assays in the absence of phenylephrine were set to 100% rate

of recovery, and the rate of recovery of the phenylephrine stimulated NHE1 was expressed relative to the control, as a percentage.

2.5.7 RIPA lysates of ERK1/2 and p90^{RSK}

To analyze protein levels of activated and basal ERK1/2 and p90^{RSK} under control or stimulated conditions, the following MAPK assay was performed. Cardiomyocytes were plated on 100 mm dishes (~4 x10^6 cells/dish) and infected with adenovirus expressing NHE1-IRM as described in sections 2.5.1 and 2.5.2. Cells were treated with either control or stimulated conditions (SIA or PE). SIA cells were incubated in Na^+ normal buffer, then 50 mM NH_4Cl was added for 3 minutes followed by 1, 3 or 6 minutes incubation in Na^+-free buffer. All steps were performed in a 37°C room. For PE stimulation, cells were incubated in Na^+ normal buffer for 6 minutes with 100 μM freshly made PE, followed by 3 minutes 50 mM NH_4Cl treatment, and 30 seconds Na^+-free buffer incubation. All steps were performed in a 37°C room. Controls were performed alongside stimulated conditions, either in the absence of 3 minutes Na^+-free buffer incubation (no SIA) or in the absence of 100 μM PE. For treatment with phorbol 12-myristate-13-acetate (PMA; Sigma), cells were treated with 50 ng/ml for 20 minutes.

Cell treatments were followed by addition of 1 mL of MAPK cell lysis buffer (50 mM Na-pyrophosphate, 50 mM NaF, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1 mM sodiumorthovanadate, 0.1% Triton X-100, 10 mM Hepes pH 7.4, 0.5 mM PMSF, 10 mg/mL leupeptin (Sigma)). Cells were then frozen on dry ice in a 100% ethanol bath for 5 minutes, and thawed on ice (~15 minutes). Cells

were then scraped for 2 minutes and transferred to pre-chilled 2 mL Eppendorf tubes. Eppendorf tubes were sonicated in an ice water bath for 20 seconds at constant duty with an output of 30 (Branson Sonifier), and then centrifuged for 30 minutes at 14,000 rpm at 4°C. Pellets were discarded and supernatants (containing MAPK pathway proteins) were then transferred to new Eppendorf tubes, frozen in liquid nitrogen and stored for future use at -80°C.

2.5.8 Western blotting of ERK1/2 and p90^{RSK} proteins

RIPA lysates of MAPK pathway proteins ERK1/2 and p90^{RSK} were subjected to 10% SDS-PAGE (100 µg of total protein from cell lysates) and transferred to nitrocellulose membrane as previously described in section 2.4.3.

Nitrocellulose membranes were blocked by gently rocking with blocking buffer solution (LI-COR Biosciences, Lincoln, Nebraska, USA) for 1 hour. Membranes were then rinsed in Tris-buffered saline (TBS). Then primary antibodies to detect phosphorylated-ERK1/2 and ERK1/2 (Cell Signaling Technologies) or phosphoryated-p90^{RSK} and p90^{RSK} (Cell Signaling Technologies, and Santa Cruz, respectively) were diluted 1:1000 in TBS + 0.1% Tween-20 overnight (see Table III for the MAPK pathway specific antibodies used). The membrane was then washed with TBS + 0.1% Tween-20 4 times for 5 minutes each at room temperature. The membrane was then incubated with the appropriate fluorescent labeled secondary antibodies, goat anti-mouse (GAM) IRDye 800CW, goat anti-rabbit (GAR) IRDye 680, and donkey anti-goat IRDye 800CW (LI-COR Biosciences, Lincoln, Nebraska, USA) diluted 1:10,000 in TBS + 0.1% Tween-20

for 1 hour in a dark box at room temperature (see Table III, MAPK pathway kinase specific antibodies). The membrane was then washed 4 times for 5 minutes each in TBS + 0.1% Tween-20. Then the membrane was rinsed and stored in TBS. To visualize immuno-reactive proteins, we used the Licor Odyssey Infrared Imager. Quantifications were done using the Odyssey system (LI-COR Biosciences, Lincoln, Nebraska, USA).

	Phosphorylated-ERK1/2	**ERK1/2**	**Phosphorylated-$p90^{RSK}$**	**$p90^{RSK}$1/2/3**
Primary Antibody	Phospho-ERK (Thr202/Tyr204) Mouse monoclonal (Cell Signaling Technologies)	ERK1/2 (p44/p42 MAPK) Rabbit polyclonal (Cell Signaling Technologies)	Phospho-$p90^{RSK}$ (Ser 380) Rabbit polyclonal (Cell Signaling Technologies)	$p90^{RSK}$1/2/3 Goat monoclonal (Santa Cruz)
Secondary Antibody	Goat anti-mouse IRDye 800CW (Li-COR BioSciences)	Goat anti-rabbit IRDye 680 (Li-COR Biosciences)	Goat anti-rabbit IRDye 680 (Li-COR Biosciences)	Donkey anti-goat IRDye 800CW (Li-COR BioSciences)

Table III: Antibodies for the analysis of MAPK pathway kinases. ERK1/2, extracellular regulated kinase isoform 1 and 2 and $p90^{RSK}$, p90 ribosomal S6 kinase. Western blotting was done using antibodies for both the activated kinases (phosphorylated kinase) and total kinase. The source of the antibodies is indicated below the specified antibody.

2.5.9 Inhibition of the MAPK pathway

To inhibit the MAPK pathway upstream of ERK1/2 and $p90^{RSK}$, we used the MEK1/2 kinase inhibitor U0126 (Sigma) (223,224). The concentration of U0126 used was between 3-10 μM diluted in dimethylsulfoxide (DMSO). These concentrations have been shown to specifically inhibit MAPKK within the MAPK cascade (225). For inhibition of the kinases ERK1/2 and $p90^{RSK}$ for experiments testing NHE1 activity, cells were incubated with 3 μM U0126 in Na^+ normal buffer for 10 minutes before carrying out the control or stimulated (SIA or PE) experiments, and 3 μM U0126 was kept constantly throughout the entire assay (212). Control experiments were done in the absence of U0126 and were in the presence of DMSO to control for any effects of this vehicle. For *in vivo* phosphorylation experiments, and examination of the levels of phosphorylated ERK and $p90^{RSK}$, 10 μM of U0126 was used. Cells were pre-incubated in Na^+ normal buffer for 10 minutes and 10 μM U0126 kept throughout treatments of control and stimulation (SIA or PE).

2.5.10 Buffering capacity

Buffering capacity was determined for cardiomyocytes infected with Ad-GFP, Ad-NHE1 IRM and uninfected cells. For the adenovirus infected cells, isolated neonatal cardiomyocytes were infected with an MOI of 20 for 24 hours and then buffering capacity activity assays were performed.

After the cells were loaded with BCECF (as described in section 2.5.4), activity assays were performed as follows. All solutions used were pre-warmed

and kept at 37°C. The BCECF-loaded cells (on coverslip) were placed in a cuvette holder, with 2.5 mL of Na^+ normal buffer (135 mM NaCl, 5.0 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, 5.5 mM glucose, 10 mM Hepes, solution pH 7.4) at 37°C for 1 minute allowing the pH*i* to stabilize. Ammonium chloride (NH_4Cl) was then added in a stepwise gradient at different concentrations beginning with 30 mM NH_4Cl. This was then switched to 20 mM, 15 mM, 10 mM, 5 mM, 1 mM, and 0 mM. Each NH_4Cl solution was allowed to equilibrate for 30 seconds at 37°C. Following stepwise NH_4Cl treatments cells were equilibrated in a three-step pH calibration, using Na^+-free calibration buffer (135 mM N-methyl-D-glucamine, 135 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, 5.5 mM Glucose, 10 mM Hepes, and 5 μM nigercirin, at pH's of 6, 7 and 8). Cells were incubated in the calibration buffers for 1 minute each until equilibrium was established. The calibration measurements were used to convert fluorescence output into pH.

Buffering capacity (*B* in mM/pH unit) was estimated as the amount of acid loaded divided by the observed change of cellular pH produced by this load (as described earlier (226)). BCECF-loaded cells were incubated with NH_4Cl-containing buffer (as described above). During this period, intracellular pH (pH*i*) increases due to influx of NH_3. A slight decrease can follow due to NH_4^+ uptake. Removal of extracellular NH_4Cl produces an intracellular acidification due to dissociation of intracellular NH_4^+ (NH_4^+i) and the rapid exit of intracellular NH_3 (NH_3i).

Buffering Capacity, $B = \Delta NH_4^+i / \Delta pHi$

$\Delta NH_4^+i = NH_4Cl_{\text{concentration outside}} \times 10^{(pKa-pHi))\,/\,[1\,+\,10\,(pKa-pHo)]}$

The equilibrium between NH_4^+, NH_3 and pH in the extracellular medium is determined by the Henderson-Hasselbach relation using the p*K*a for NH_4^+ of 9.21.

Values of *B* were determined at various pH*i* by varying the amounts of NH_4Cl and then plotted in a graph of buffering capacity, *B* versus pH.

2.6 NHE1 protein *in vivo* phosphorylation labeling and immunoprecipitation

2.6.1 [^{32}P]- labeling of NHE1

To examine the level of *in vivo* phosphorylated NHE1 protein in the myocardium, isolated neonatal rat cardiomyocytes were plated in 100 mm dishes to a confluency of ~100% (approximately $4x10^6$ cells/dish). On the second day in culture cardiomyocytes were infected with the appropriate adenoviruses (for example NHE1-IRM) at an MOI of 20 PFU/cell for 24 hours at 37°C and 5% CO_2. Infection was done in fresh medium (as previously described in section 2.5.2) with 10% FBS or 0.5% FBS depending on treatment. All experiments testing for NHE1 stimulation with sustained intracellular acidosis (SIA) were performed with 0.5% FBS medium; however, in experiments where NHE1 was stimulated with phenylephrine (PE), cells were infected in 10% FBS medium.

After 24 hours of adenoviral infection, cells were washed twice with phosphate-free Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 0.5% FBS. Cells were then incubated with 8 mL phosphate-free DMEM with 0.5% FBS and incubated at 37°C and 5% CO_2 for 30 minutes. Cells were then washed twice more with phosphate-free DMEM with 0.5% FBS.

Finally, 2 mL of fresh phosphate-free DMEM with 0.5% FBS was added to cells, followed by addition of $H_3{}^{32}PO_4$ (Perkin Elmer) to a final concentration of 100 μCi/mL (total 200 μCi per dish). Cells were then incubated at 37°C and 5% CO_2 for 3 hours.

After the 3 hours of [^{32}P]-loading of cardiomyocytes, cells were treated as follows. For experiments testing stimulation by SIA, control and stimulated, and control and stimulated with 10 μM U0126. For experiments testing stimulation by PE the treatments were: control and PE stimulated, and control and PE stimulated with 10 μM U0126.

2.6.2 Preparation of cell lysates of [^{32}P]-labeled NHE1

Cell lysates were prepared after appropriate cell treatments by addition of 1 mL ice-cold detergent-free RIPA Buffer (150 mM NaCl, 80 mM NaF, 50 mM Tris-HCL pH 8.0, 5 mM EDTA, 1 mM EGTA, 25 mM NaPyrophosphate, 1 mM NaOrthovanadate, 0.1% (v/v) protein inhibitor cocktail (220), 0.1 mM benzamidine, 0.1 mM PMSF, 0.2 mM ALLN, (calpain inhibitor; Calbiochem). Cells were then subjected to three freeze/thaw cycles, freezing on dry ice, and thawing on ice. Freeze/thaw cycles were followed by scraping cells for 2 minutes and transferring the cell lysates into ice cold polycarbonate centrifuge tubes. Cells were then sonicated for 30 seconds at 30 amilute in an ice/water bath and then centrifuged for 1 hour at 35,000 rpm at 4°C. The supernatant was then discarded, and the pellet resuspended in 1 mL of detergent-containing RIPA Buffer (same as detergent-free RIPA buffer with the addition of 1.0% (v/v) NP-40, 0.5% (w/v)

deoxycholate, and 0.1% (w/v) SDS). Resuspended lysates were centrifuged for 30 minutes at 10,000 rpm at 4°C.

2.6.3 Immunoprecipitation of [^{32}P]-labeled NHE1

Immunoprecipitation (IP) of exogenously expressed NHE1 was performed using cell lysates (described in 2.6.2). Cell lysates (~1 mL) were pre-absorbed with 1% BSA (w/v) and 2 mg/mL Protein A-Sepharose CL-4B beads (Sigma) for 30 minutes at 4°C rotating end on end. Beads were removed by centrifugation for 3 minutes at 7,000 rpm at 4°C. The pre-absorbed supernatant was then transferred to a clean Eppendorf tube and beads discarded. The cell lysates were then treated with 1.4 μg/mL of an anti-hemagluttin (HA) tag antibody (Y11, Santa Cruz), as all exogenous NHE1 protein expressed contains an HA tag at its C-terminus, and incubated for 2 hours at 4°C rotating end on end. During the above procedure, a preparation of 100 mg/mL of Protein A-Sepharose CL-4B beads was prepared in detergent containing RIPA Buffer and 1% (w/v) BSA, incubated at 4°C and rotated end on end (2-3 hours); this eliminated non-specific binding of beads by blocking with BSA. The beads were then centrifuged for 5 minutes at 7,000 rpm at 4°C. The cell lysate with anti-HA antibody was then added to the blocked Protein A-Sepharose CL-4B beads and gently mixed and incubated at 4°C for 18 hours rotating end on end.

Samples were then centrifuged for 3 minutes at 7,000 rpm at 4°C to separate protein-bound beads. Beads were subsequently washed three times with 500 μl of detergent-containing RIPA Buffer. The protein-bound to the beads was

then eluted from washed beads with 45 µl of 1X SDS-PAGE sample loading buffer (5% 2-mercaptoethanol, 7.5% glycerol, 1.5% SDS, 0.03 M Tris, and 0.033 mg/mL Bromophenol Blue) and incubated for 15 minutes at 37°C. Finally the solution with eluted beads was centrifuged for 3 minutes at 7,000 rpm at room temperature, and the supernatant containing eluted immunoprecipitated protein was transferred to a new Eppendorf tube. Immunoprecipitated protein was run on a 10% acrylamide SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was then placed for exposure with a phosphor-imaging screen and placed in a dark drawer for one to two weeks. The phosphor-imaging screen was scanned with the Typhoon 9400 Variable Mode Imager (GE Heatlhcare, Piscataway, NJ, USA); this resulted in visualizing radioactively [^{32}P]-labeled NHE1 protein. After membranes were scanned for radioactivity, the membranes were blotted with Anti-HA antibody (Y11, Santa Cruz) to detect total immunoprecipitated NHE1 (as described in section 2.4.3).

2.6.4 Analysis of [^{32}P]-labeled NHE1

As described above in section 2.6.3, radioactively labeled immunoprecipitated NHE1 was detected using phosphor-imaging screens. Total immunoprecipitated protein was determined by Western blotting with an anti-HA antibody. Densitometry was performed using Image J software to analyze phosphorylated NHE1 ([^{32}P]-labeled NHE1) and total NHE1 protein immunoprecipitated. All analysis of phosphorylated NHE1 protein was corrected for protein loading differences. Comparison of control versus stimulated

treatments was determined by comparing relative levels of phosphorylated NHE1 between the two treatments.

2.7 CHP1 – Adenovirus production and characterization

2.7.1 CHP1 adenovirus production

Adenovirus expressing CHP1 was produced and characterized as described in section 2.1-3. PCR was used to extract CHP1 with a C-terminal V5 tag from the vector pDEST40-CHP with the following primers: pAdCHP-V5 forward 5'-CCGGGGTACCACCATGGGTTCTCGGGCCTCCAC-3' and pAdCHP-V5 reverse 5'-GCCCAAGCTTCTACGTAGAATCGAGACCGAGGAG-3' (94). Underlined regions show introduction of Kpn1 and HindIII restriction sites respectively. The resulting product was then ligated into pAdTrack-CMV, recombined with pAd-Easy-1, and made into an adenoviral vector in HEK293A cells as described in section 2.1-3.

2.7.2 CHP1 overexpression in cardiomyocytes

Cardiomyocytes were infected with adenovirus expressing CHP as previously described in section 2.5.2, with varying MOI's and times of infection. To examine expression of CHP1 in cardiomyocytes, RIPA lysates and Western blotting were performed as described in sections 2.5.2 and 2.5.3. However, 12%

SDS-PAGE was used as the molecular weight of CHP1 is around 22 kDa. The primary antibody used in Western blot analysis was J114 (rabbit polyclonal) at 1:1000 dilution. The J114 antibody was made against CHP1 (produced by our laboratory). The secondary antibody used was goat anti-rabbit IRDye 680 (LI-COR Biosciences).

2.7.3 CHP1 overexpression and NHE1 activity

The effect of overexpression of CHP1 on NHE1 activity was analyzed by measuring intracellular pH as described in section 2.5.4. Single pulse assays were performed for the following treatments: GFP, CHP1, NHE1, and CHP1/NHE1 infected cardiomyocytes (MOI 20 for 24 hrs).

2.7.4 CHP1 overexpression and cardiomyocyte buffering capacity

Cardiomyocytes were infected with an MOI of 20 for 24 hours with the following adenoviruses: Ad-GFP, Ad-NHE1-IRM, Ad-CHP and AdCHP1 and AdNHE1 together. Buffering capacity assays were performed as described in section 2.5.10.

2.7.5 Proton flux measurements

Proton flux is the measurement of the movement of protons out of the cell. Calculation of proton efflux is as follows: Buffering capacity, *B* (mM/pH units) x ΔpH/Δtime (min) and results in mM protons/Δtime (min). A graph is then plotted of proton efflux, JH^+ (Y axis) versus pH (X axis).

$JH^+ = d(B \cdot pH)/dt$

A volume correction was used to compare J_{H^+} plotting the intracellular pH (pH*i*) on the X axis against the mM NH_4Cl/pH Units on the Y axis. The linear regression is calculated and results in the equation of buffering capacity mM/pH units at a given pH.

2.8 Statistics

For measurements of NHE1 activity, results reported are the result of 10-12 experiments per treatment/condition. Western blot analysis of signal transduction proteins, activated and total ERK1/2 and activated and total $p90^{RSK}$ involved a minimum of three experiments per treatment/condition. For all experiments of *in vivo* NHE1 phosphorylation, densitometry results were calculated for a minimum of three experiments per treatment/condition. Statistics performed for all experiments used the Wilcoxon Mann-Whitney rank sum test and these statistics were performed for all data with the computing program KaleidaGraph 3.6.

Chapter 3:

Expression and characterization of an inhibitor resistant Na^+/H^+ exchanger in the mammalian myocardium

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3.1 Introduction

NHE1 is a highly regulated membrane protein that is responsible for the removal of excess intracellular protons in the myocardium. NHE1 removes one intracellular proton for one extracellular sodium. The cytosolic domain of NHE1 plays a crucial role in its regulation as the major site of protein interactions and phosphorylation (section 1.4). As discussed in section 1.4.3, phosphorylation of NHE1's cytosolic tail results in activation of the exchanger. However, it is notable that several studies examining the regulation of NHE1 were carried out in non-myocardial cells. Of particular interest is understanding the regulation of NHE1 in the myocardium as several studies have shown that NHE1 contributes to several pathological conditions in the heart (section 1.5.2). For example, increased activity of the exchanger has been shown to have detrimental effects on the myocardium in both ischemia/reperfusion injury and hypertrophy (see sections 1.5.2 and 1.6) (227,228). Furthermore, NHE1 mRNA levels are increased when subjected to chronic acidosis (229).

During myocardial I/R, intracellular pH decreases due to metabolic demands, which leads to the production of protons. In the myocardium NHE1 is the major mechanism of acid extrusion at physiological and acidic pH (<6.5) (148). Thus, this increased proton production leads to increased NHE1 activity, which causes elevated intracellular [Na^+]. Elevated intracellular [Na^+] leads to reverse mode Na^+/Ca^{2+} exchanger activity leading to calcium overload. Ca^{2+} overload results in activation of various pathways in the myocardium and ultimately leads to contractile dysfunction, myocyte death and cardiac

arrythymias (157,230,231).

The Na^+/H^+ exchanger's role in various forms of heart disease, and how expression and regulation of the protein varies in pathological settings (83), makes it of great interest to study the effects of elevated levels of NHE1 protein in the myocardium. Additionally, myocardial cells display differences in NHE1 activity kinetics and, therefore, it is important to study the exchanger in myocardial cells (151).

To examine the regulation of myocardial NHE1, it was crucial to find a method whereby exogenous NHE1 is introduced into the mammalian myocardium. Our laboratory has recently examined two expression systems for studying exogenous NHE1 in the mammalian myocardium. An animal model whereby NHE1 with a hemagluttinin (HA) tag was cloned behind an α-myosin heavy chain promoter and transgenic mice were made with either wild type or hyperactive NHE1 protein. Use of the α-myosin heavy chain promoter resulted in specific expression of NHE1 in the myocardium, and there was no expression in other intact tissues. Additionally, the exogenous NHE1 in transgenic one month old mice was present in isolated adult cardiomyocytes. However, expression from the promoter declined rapidly and very little exogenous NHE1 was detectable on the fourth day after cardiomyocyte isolation in culture (222). Therefore, this animal model would not be suitable for studies in isolated cultured cardiomyocytes.

Another approach that would enable overexpression of NHE1 in isolated cardiomyocytes was needed. Cultured cardiomyocytes are quite refractory to lipid

or calcium based transfection systems and, therefore, we examined the adenoviral vector expression system and its ability to express an active NHE1 that contained NHE1 inhibitor resistant mutations. Since cardiomyocytes contain endogenous plasma membrane NHE1, the inhibitor resistant mutations of the exogenous NHE1 (in the adenoviral vector) allowed us to inhibit activity of the endogenous NHE1 protein present in all cardiomyocytes while the exogenous NHE1 protein remained active (Figure 3.1). In the present study, we constructed an adenoviral vector containing our gene of interest and used it to infect cultured rat neonatal cardiomyocytes (Figure 2.1).

With the aid of the CIHR Adenoviral core facility directed by Dr. J. Dyck at the University of Alberta, we made the first adenovirus expressing NHE1. An HA tagged mutant NHE1 (inhibitor resistant mutations Leu163Phe and Gly174Ser) was transferred into an adenoviral expression system and used to infect cultured neonatal rat ventricular cardiomyocytes (NRVM) (61,62). This system resulted in robust expression and activity of exogenous NHE1, which we were able to distinguish from endogenous NHE1 by its resistance to NHE1 inhibitors (amiloride analogs). These results suggest that for long term studies on isolated cardiomyocytes use of an adenoviral expression system is superior to studies using NHE1 transgenic mice (222).

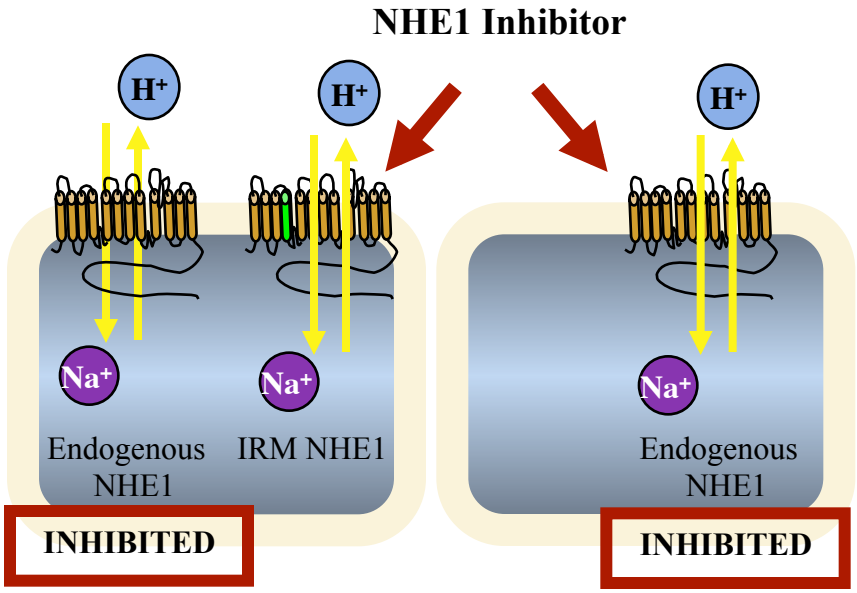

Figure 3.1 Cartoon of exogenous and endogenous NHE1 in myocardial cells. Cartoon representation of myocardial cells. The cell to the left contains both endogenous and exogenous NHE1 and the cell to the right contains only endogenous NHE1. Upon treatment with an NHE1 inhibitor (EMD87580), the endogenous NHE1 in both cells is inhibited but the exogenous NHE1 with inhibitor resistant mutations (IRM) remains active. This cartoon represents the premise of how we can examine exogenous NHE1 activity in myocardial cells.

3.2 Results

3.2.1 Production of adenovirus expressing inhibitor resistant NHE1

Production of recombinant adenoviruses is a technique that involves transferring the cDNA of interest (NHE1 mutants) into the shuttle vector pAdTrack-CMV, then recombining this construct with the vector pAdEasy-1 that contains the majority of the adenoviral genome with the exception of two essential genes, E1 and E3. Once the recombinant plasmid, which contains our mutant NHE1 and the adenoviral genome, is constructed and screened, it is used for the production of virus in the human embryonic kidney (HEK293A) cell line (Figure 2.2). HEK293A cells contain the E1 and E3 genes necessary for viral assembly enabling us to grow the virus in a large quantity for purification. Furthermore, when the viruses produced from this technique are used in cardiomyocytes they will successfully infect cells, but will be unable to reproduce since the viruses lack essential viral proteins for viral packaging (217).

The NHE1 protein expressed contained Leu163Phe/Gly174Ser mutations that made them resistant to inhibition, but did not affect NHE1 activity. This allowed us to inhibit activity of the endogenous NHE1 protein present in cardiomyocytes while the exogenous NHE1 protein remained active. Inhibitor resistant mutant NHE1 protein was initially cloned into the pAdTrack-CMV vector to begin adenoviral construction. For the construct, we confirmed that the clone was being expressed in this vector by transfecting AP-1 cells and checking for NHE activity (data not shown). AP-1 cells are a Chinese hamster ovary cell

line that are deficient in all isoforms of plasma membrane NHE (2). In addition Western blotting was used to confirm that the protein was being expressed (data not shown). Moreover the pAdTrack-CMV construct was sequenced for the entire NHE1 gene to confirm the proper gene and proper mutations. After completion of the recombinant adenovirus construction, we checked for expression and activity of the exogenous NHE1 protein in isolated cardiomyocytes.

3.2.2 Expression of inhibitor resistant NHE1

To determine the multiplicity of infection (MOI, number of infectious particles per cell) of adenovirus that should be used for infection of isolated cardiomyocytes, we performed a time course experiment of varying MOIs (10, 20, 30, and 40) and varying times of infection (18, 24, 36, 48, 72, and 96 hrs) (222). NHE1 expression was present as early as 24 hours after infection, and an MOI of 20 provided high levels of NHE1 protein. Figure 3.2A shows a Western blot examining the time course of expression of the NHE1 protein after infection of adenovirus with an inhibitor resistant mutant (IRM) of NHE1 at an MOI of 20. Exogenous NHE1 expression was present as early as 24 hours after infection and continued 96 hours post infection. Both the glycosylated and the deglycosylated form of the NHE1 protein are visible: protein bands at 110 kDa and 80 kDa, respectively, as described previously (50). Figure 3.2B are samples of Figure 3.2A probed with antibody to myosin heavy chain (MF-20) to assess protein loading. Protein concentrations declined slightly with time of infection, likely accounting for the slight decline in signal strength with time. Fluorescence microscopy

images of the GFP reporter from isolated cardiomyocytes infected with adenovirus expressing inhibitor resistant NHE1 at 24, 48, 72 and 96 hours post adenoviral infection are shown in Figure 3.2C.

We determined that infection of cardiomyocytes with adenovirus at an MOI of 20 for 24 hours expressing inhibitor resistant mutant NHE1 (NHE1-IRM), resulted in good production of NHE1 protein that was detected by Western blot analysis (Figure 3.3) (222). The lane AP-1 refers to a positive control made by transfection of AP1 cells with a HA-tagged NHE1. Lanes 1 and 2 are cell extracts from isolated cardiomyocytes infected with the Ad-NHE1-IRM adenovirus. Both the glycosylated and the deglycosylated form of the NHE1 protein are visible. Lane 3 shows a cell extract from cells infected with a control adenovirus not containing the HA-tagged NHE1 protein (Ad-GFP). Lanes 1-3 were reprobed with an antibody to the myosin heavy chain to assess equal protein loading in the cardiomyocyte cell extracts as shown in Figure 3.3.

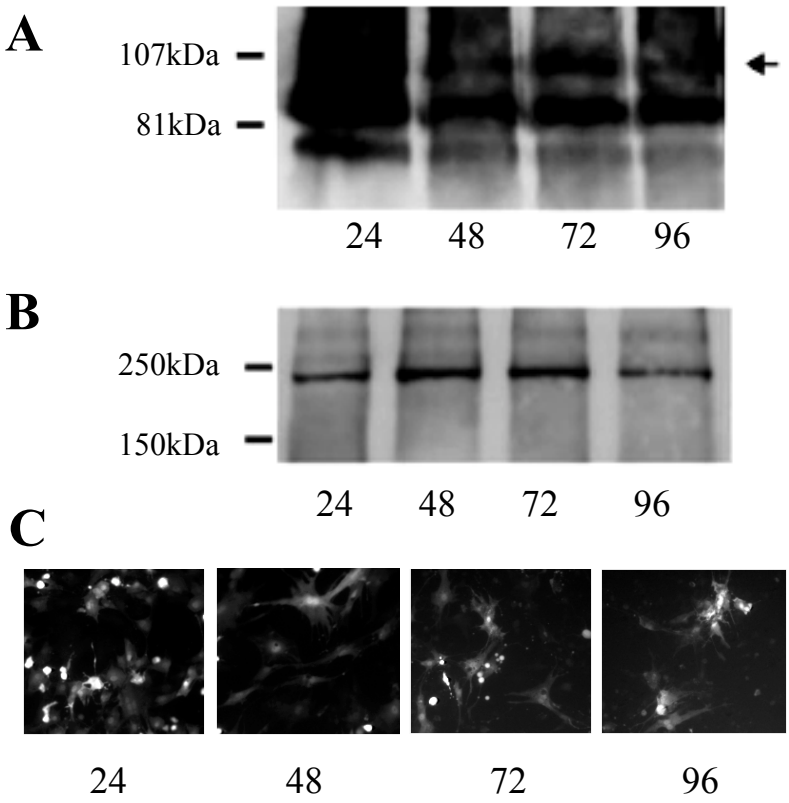

Figure 3.2 Time course of expression of NHE1 protein in isolated cardiomyocytes infected with adenovirus expressing inhibitor resistant NHE1. Sixty mm dishes of isolated neonatal cardiac myocytes were infected with the adenovirus containing the inhibitor resistant NHE1 with a multiplicity of infection of 20 for 24, 48, 72 and 96 hours. **(A)** Time course of expression was examined by Western blotting against the anti-HA tag. Arrow indicates the mobility of the fully glycosylated NHE1 isoform. **(B)** Samples of Fig. 3.2A probed with antibody to myosin (MF-20). **(C)** Fluorescence Microscopy pictures of the GFP reporter from isolated cardiomyocytes infected with adenovirus expressing inhibitor resistant NHE1. Cells were examined 24, 48, 72 and 96 hours after viral infection.

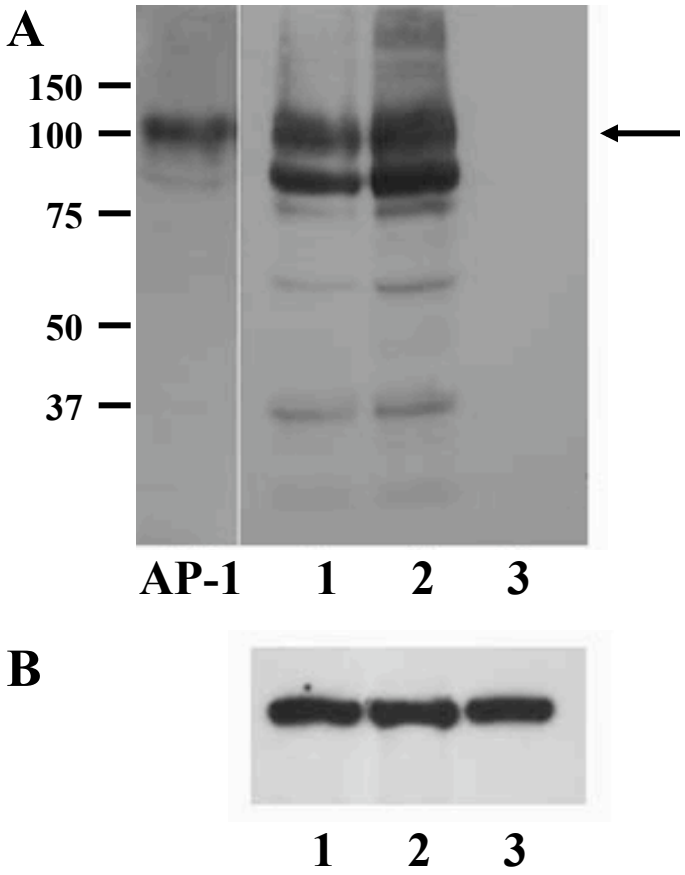

Figure 3.3 Characterization of expression of the Na^+/H^+ exchanger in isolated cardiomyocytes infected with adenovirus expressing NHE1. Isolated cardiomyocytes were made as described in section 2.5.1. Equal numbers of cells were infected with Adenovirus containing the HA-tagged inhibitor resistant NHE1 protein (AdNHE1-IRM). **(A)** Western blots of cell extracts were blotted with anti-HA antibody. AP-1 is a positive control made from AP-1 cells transfected with HA -tagged NHE1 protein. Lanes 1 and 2 were extracts from cardiomyocytes infected with AdNHE1-IRM. Lane 3 was from cells infected with a control adenovirus which had no NHE1. Arrow indicates the mobility of the fully glycosylated NHE1 isoform. **(B)** Western blots of lanes 1–3 were re-probed with antibody to myosin heavy chain.

3.2.3 Characterization of the inhibitor resistant NHE1

The inhibitor resistant mutations were designed to distinguish between endogenous and exogenous NHE1. Studies in CHO cells showed that mutations of residues Leu163Phe and Gly174Ser in NHE1 transmembrane segment IV resulted in NHE1 inhibitor IC_{50} concentrations of 20 μM compared to wild type NHE1 of 0.18 μM (100-fold difference) (62). The mutant NHE1 (Leu163Phe/Gly174Ser) protein functions normally with only a very slight decrease in Na^+ affinity. In earlier studies, we have used NHE1 inhibitors to characterize NHE1 activity (109,114,213). As the efficacy of the NHE1 inhibitors might be different in adenovirus (Ad) NHE1-IRM infected cardiomyocytes, we measured the activity of NHE1 in varying concentrations (0.3 – 300 μM) of the NHE1 inhibitor EMD87580 (cariporide derivative) using two-pulse activity assays (as described in the materials and methods section 2.5.5). The activity of the exchanger in the second pulse in the presence of the NHE1 inhibitor EMD87580 was compared to the activity of NHE1 in the first pulse in the absence of the NHE1 inhibitor EMD87580.

Our results showed that EMD87580 inhibited the myocardial endogenous NHE1 with an IC_{50} of 0.3 μM, and in cardiomyocytes infected with inhibitor resistant mutant of NHE1 the IC_{50} was 30 μM (Figure 3.4) (222). Thus, the inhibitor resistant mutations Leu163Phe and Gly174Ser resulted in a 100-fold difference in the IC_{50} of NHE1 in isolated cardiomyocytes. This demonstrated that in isolated cardiomyocytes we could use 10 μM of EMD87580 to completely

inhibit the endogenous NHE1 while retaining exogenous NHE1 activity (Figure 3.1). For future studies it is notable that EMD87580 is only applied during assays and thus does not pose any long-term effects of administration. Thus, we showed that EMD87580 resistant NHE1 activity was detected while endogenous NHE1 was inhibited, confirming that NHE1 activity was the result of adenoviral expression (222).

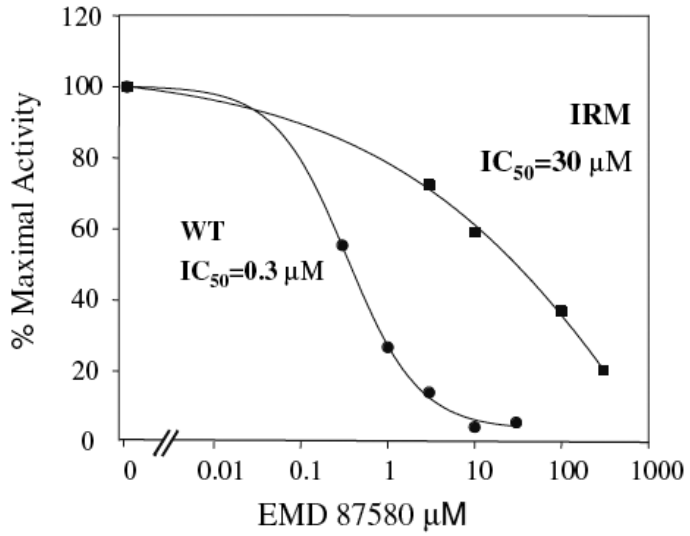

Figure 3.4 Characterization of the inhibitor resistant Na^+/H^+ exchanger activity in isolated cardiomyocytes. Isolated neonatal rat cardiomyocytes were uninfected or infected with adenovirus expressing inhibitor resistant NHE1 (Ad-NHE1-IRM) at an MOI of 20 for 24 hours. The cells were then subjected to a two-pulse Na^+/H^+ exchanger activity assay and the activity of the exchanger in the second pulse in the presence of the NHE1 inhibitor EMD87580 was compared to that of the first pulse in the absence of the inhibitor EMD 87580. Circles, uninfected cardiomyocytes and squares, cells infected with Ad-NHE1-IRM. WT, wild type Na^+/H^+ exchanger activity in uninfected cardiomyocytes; IRM, inhibitor resistant mutant activity of infected cardiomyocytes. IC_{50}, inhibitory concentration of 50% of NHE1 activity.

3.2.4 Activity of inhibitor resistant NHE1

To characterize the inhibitor resistant NHE1 protein in primary cultures of cardiomyocytes, we assayed the activity of the Na^+/H^+ exchanger in the presence or absence of the NHE1 inhibitor, EMD87580 (as described in materials and methods in section 2.5.5). The results are shown in Figure 3.5. In either mock infected cardiomyocytes, or cardiomyocytes infected with adenovirus without the NHE1 gene (Ad-GFP), the activity of the Na^+/H^+ exchanger was reduced to negligible levels upon the addition of 10 μM EMD87580. In cardiomyocytes infected with adenovirus containing the inhibitor resistant NHE1 (NHE1-IRM), Na^+/H^+ exchanger activity was retained after treatment with 10 μM EMD87580. Since the Na^+/H^+ exchanger activity initially measured was a combination of the endogenous NHE1 protein and the protein introduced by the adenovirus, the decline in activity due to 10 μM EMD87580, is likely due to decreased activity of the endogenous Na^+/H^+ exchanger. The results showed that in cells infected with AdNHE1-IRM, more than 50% of the Na^+/H^+ exchanger activity was due to introduction of the exogenous gene.

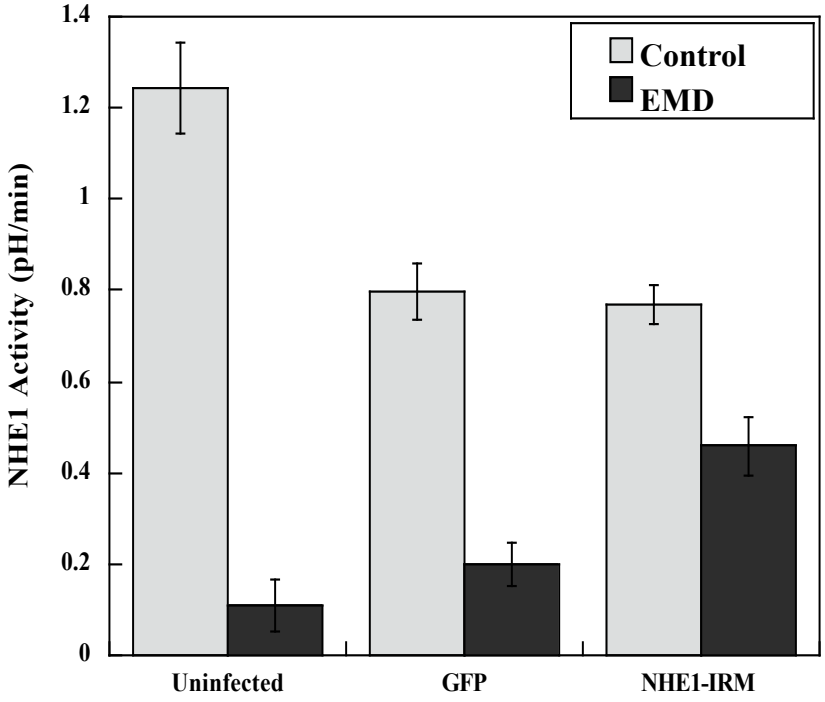

Figure 3.5 Na^+/H^+ exchanger activity in isolated cardiomyocytes infected with adenovirus containing NHE1 protein. Isolated cardiomyocytes were infected with NHE1 containing adenovirus as described in Figure 3.3. Cells were then assayed for NHE1 activity using a two-pulse activity assay, with the second pulse in the presence of 10 μM EMD87580. The activity of the second pulse (shaded dark grey) relative to the first pulse is displayed for each bar. Uninfected cardiomyocytes; Ad-GFP, cardiomyocytes infected with adenovirus without an NHE1 insert; Ad-NHE1-IRM, infected with adenovirus containing NHE1 insert with inhibitor resistant mutations; EMD, assayed in the presence of 10 μM EMD87580. Values are the mean of 12 experiments +/- standard error.

3.3 Discussion

The NHE1 isoform of the Na^+/H^+ exchanger is responsible for the removal of one intracellular proton (H^+) in exchange for one extracellular sodium ion (Na^+). NHE1 has been implicated in the damage that occurs to the myocardium during ischemia and reperfusion and plays an important role in cardiac hypertrophy (227,228). Myocardial NHE1 expression and activity have been shown to increase in diseased states (232). It was, therefore, of interest to develop animal models that mimic the increase in NHE1 levels and activity that occur in the pathophysiological state. In the present study, we examined the adenoviral vector expression system for increasing NHE1 expression and activity in the mammalian myocardium.

We developed a system for overexpression of the Na^+/H^+ exchanger in isolated cardiomyocytes. An adenovirus containing an inhibitor resistant NHE1 demonstrated that infection of isolated cardiomyocytes resulted in stable expression of the exogenous NHE1 gene in cultured isolated cardiomyocytes. Exogenous NHE1 was detected up to 96 hours post infection in isolated cardiomyocytes. Furthermore, fluorescence microscopy demonstrated that infection of the cells was almost complete (95-99%) by the adenovirus and the cardiomyocytes maintained their general morphology and contractility in culture after infection.

Cardiomyocytes possess their own plasma membrane NHE1 isoform of the Na^+/H^+ exchanger, so to distinguish activity and effects of an exogenous NHE1 protein, we made a mutant Na^+/H^+ exchanger that was resistant to NHE1

inhibitors. The Leu163Phe/Gly174Ser mutation caused an increase in the IC_{50} for NHE1 inhibition of over 100-fold. A detailed discussion of how these mutations inhibit NHE1 can be found in section 1.3.4. This enabled a large range whereby the use of 10 μM EMD87580 could completely inhibit the endogenous NHE1 in the isolated cardiomyocytes while enabling activity of the exogenous inhibitor resistant NHE1. Studies examining endogenous myocardial NHE1 activity in either uninfected or infected with adenovirus with the GFP reporter only (Ad-GFP) demonstrated that it was possible to eliminate endogenous NHE1 activity by inclusion of 10 μM of the NHE1 inhibitor EMD87580. However, in adenovirus infected cardiomyocytes expressing an inhibitor resistant NHE1, approximately 50-60% of total NHE1 activity was a result of the exogenous NHE1 activity. This system provided us with a useful tool to introduce NHE1 in myocardial cells and assess exogenous NHE1 activity.

In conclusion, the adenoviral expression system allows the study of NHE1 overexpression in primary cultures of isolated cardiomyocytes. By the inclusion of inhibitor resistant mutations, we were able to produce a system to introduce NHE1 in myocardial cells but also be able to differentiate the NHE1 which was exogenous from that which was endogenous. Thus, this method could be used to examine other NHE1 mutants and their regulation in isolated cardiomyocytes.

Chapter 4:

Sustained acidosis activates myocardial NHE1 by phosphorylation of Ser^{770} and Ser^{771}

A version of this chapter also appears in the

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4.1 Introduction

There are two major domains in the Na^+/H^+ exchanger, the N-terminal domain and the C-terminal domain. The N-terminal membrane domain contains approximately 500 amino acids, which are involved in ion affinity, transport and NHE inhibitor sensitivity. Specifically, the amino acids Leu^{163} and Gly^{174} in transmembrane IV are essential for NHE1 inhibitor sensitivity and when these residues are mutated to Phe and Ser, respectively, NHE is resistant to inhibition (61,62). The N-terminal domain is followed by the C-terminal cytosolic domain (~315 amino acids), which regulates ion exchange activity and is the site of phosphorylation and protein and cofactor interactions (Figure 1.1). The membrane proximal region of the C-terminal tail is more compact and the distal region more flexible (57), thereby potentially enabling structural changes due to protein binding or phosphorylation.

In fibroblasts, the cytoplasmic tail of NHE1 is phosphorylated in response to growth factors, thrombin, phorbol esters, and serum. This stimulation favors NHE1 activity at a more alkaline pH (105). Furthermore, thrombin, epidermal growth factor, and phosphatase PP-1 and PP-2A inhibitors increase phosphorylation of NHE1 at a common set of sites (106). Deletion analysis of the cytoplasmic tail of NHE1 further revealed that *in vivo* phosphorylation sites map to the last 178 amino acids of the NHE1 cytoplasmic tail, and deletion of this region resulted in a 50% decrease in growth factor-induced NHE1 stimulation (107). Many different protein kinases have been shown to phosphorylate and regulate NHE as described in section 1.4.

Our laboratory and others have shown that the mitogen activated protein kinase (MAPK) cascade, in particular ERK1/2, is critical in mediating NHE1 activation (109-112). ERK was also found to bind to NHE1 (unpublished data). $p90^{RSK}$, a kinase downstream of ERK1/2, has also been shown to directly phosphorylate NHE1 at Ser^{703} and stimulate its activity (82). Taken together, these results indicate that the MAPK signaling pathway is key to NHE1 regulation and activation. *In vitro* studies in our laboratory have shown that putative phosphorylation sites of ERK2 on the cytoplasmic tail of NHE1 include Ser^{693}, Ser^{766}, Ser^{770}, Thr^{779} and Ser^{785} (113). Experiments in CHO cells revealed that the amino acids Ser^{766}, Ser^{770} and Ser^{771} are important in the regulation of NHE1 activity and this was shown to occur in an ERK-dependent manner (114). In another report, we showed that amino acids Thr^{718}, Ser^{723}, Ser^{726} and Ser^{729} are phosphorylated *in vitro* by a p38 MAPK dependent pathway (43). Further studies in CHO cells also revealed that Ser^{726} and Ser^{729} mediate activation of NHE1 by apoptosis (120).

Understanding the regulation of NHE1 in the myocardium is important, as regulation of NHE1 varies amongst different cells (233,234). Furthermore, myocardial NHE1 has different kinetic characteristics than in other tissues (151) and is activated acutely by sustained intracellular acidosis (212). The mechanism of regulation of NHE1 by sustained intracellular acidosis may be of particular importance in activating the protein in heart failure. We have shown that ischemia and reperfusion activate the NHE1 protein and NHE1 directed protein kinases in the intact myocardium (31,83). In addition, decreased pH*i* is a characteristic

feature of ischemic heart disease. A more detailed discussion of the role of NHE1 in ischemia/reperfusion injury can be found in section 1.5.2.

Sustained intracellular acidosis (SIA) has been shown to rapidly stimulate NHE1 activity and phosphorylation via an ERK-dependent pathway in neonatal and adult ventricular cardiomyocytes and non-myocardial cells (212). SIA activated ERK1/2 and $p90^{RSK}$ in parallel to the increase in NHE1 activity. Furthermore, in adult ventricular cardiomyocytes, SIA activates ERK1/2 through proximal activation of the classical Ras/Raf/MEK pathway (184).

A large body of work from our laboratory and others has demonstrated that the mechanisms whereby phosphorylation regulates NHE1 are critical in understanding how NHE1 activity is stimulated. This is of great importance as stimulation of NHE1 is paramount in the maintenance of intracellular pH and damage to the myocardium. Therefore, in the present study, we examined phosphorylation site mutant NHE1 proteins and their ability to be stimulated by sustained intracellular acidosis. In the present study, examination of mutant NHE1 activity and the level of *in vivo* phosphorylated proteins revealed that amino acids Ser^{770} and Ser^{771} in the cytoplasmic tail of NHE1 are critical in stimulation of NHE1 by sustained acidosis and that this pathway is ERK-dependent. Our results are the first to define and elucidate this mechanism of regulation of the Na^+/H^+ exchanger in the mammalian myocardium.

4.2 Results

4.2.1 Expression of NHE1 phosphorylation site mutants

To study the regulation of the NHE1 protein in isolated cardiomyocytes, we developed adenoviral vectors that expressed the mammalian NHE1 protein. A series of mutations were made in the cytoplasmic regulatory region that contained putative phosphorylation sites for regulatory protein kinases. Our earlier studies in non-myocardial cells examined four groups of phosphorylation mutants of NHE1, Ser693Ala; Thr718Ala, Ser723/726/729Ala; Ser766/770/771Ala; & Thr779Ala, Ser785Ala (Figure 2.3) (114). The phosphorylation site mutants were performed in groups because in other transport proteins, such as the cystic fibrosis transmembrane conductance regulator (CFTR), groups of phosphorylatable amino acids have been shown to work together in an overlapping manner (235). Experiments in CHO cells have shown that phosphorylation of amino acids Ser^{766}, Ser^{770} and Ser^{771} are important in the regulation of NHE1 activity (114). However, regulation of NHE1 is tissue specific and myocardial NHE1 has different kinetic properties compared with other tissues (236).

All of the NHE1 phosphorylation site mutants grouped #1, Ser693Ala; #2, Thr718Ala and Ser723/726/729Ala; #3, Ser766/770/771Ala; and #4, Thr779Ala and Ser785Ala, were made with the inhibitor resistant mutations Leu163Phe/Gly174Ser. This enabled us to inhibit the activity of the endogenous NHE1 protein present in cardiomyocytes while exogenous NHE1 remained active

(222). These cDNA constructs were then used to construct the respective adenoviruses.

For production of adenoviral vectors, mutant NHE1 proteins were initially cloned into the pAdTrack-CMV vector to begin adenoviral construction. For all such constructs, we confirmed that the clone was being expressed and active in this vector by transfecting AP-1 cells and checking for NHE activity and expression (data not shown). After completion of the recombinant adenovirus construction, we tested for expression and activity of the exogenous NHE1 protein in isolated cardiomyocytes. Figure 4.1A shows typical results whereby exogenous NHE1 protein was expressed in isolated cardiomyocytes as detected by an antibody against the HA tag. Both a glycosylated and a deglycosylated or partially deglycosylated form of the NHE1 protein are visible as described earlier (50). Cells infected with adenovirus expressing the control adenovirus with GFP alone displayed no detectable NHE1 protein while cells infected with adenovirus containing the wild type or mutant NHE1s #1-4 all expressed the NHE1 protein. An antibody against myosin heavy chain was used to confirm that isolated cardiomyocyte proteins were present and transferred properly for immunoblotting (Figure 4.1A, lower panel). Additionally, Western blots of the cell extracts were probed for endogenous NHE1 protein using a commercial antibody against the NHE1 cytoplasmic tail (Figure 4.1B). The full length NHE1 protein was detected in the uninfected cells (Mock) and in cells infected with NHE1 containing adenovirus. Exogenous NHE1 protein was slightly larger in apparent molecular weight, likely due to the HA tag. Furthermore, degradation products of NHE1

were also present in all the lanes. The level of NHE1 protein was estimated to be 3-fold higher when exogenous NHE1 was introduced, however, quantification was compromised by the presence of endogenous NHE1 in all the adenovirus infected samples.

These results also suggested that the phosphorylation site mutations did not have significant effects on NHE1 protein maturation. As described above, for all mutants both the fully glycosylated NHE1 ~110 kDa and the partially glycosylated NHE1 ~85 kDa were present at similar levels to wild type NHE1 (Figure 4.1A). Previous work in our laboratory demonstrated that protein mistargeting results in predominantly the 85 kDa partially glycosylated NHE1 protein (52). However, the results from the Western blot analysis show that NHE1 mutants #1-4 are expressed at similar levels to wild type and have significant fully glycosylated NHE1, suggesting proper membrane targeting. Previous studies in CHO cells have shown that the same group of phosphorylation site mutant NHE1 proteins (1-4) were properly targeted to the plasma membrane (222).

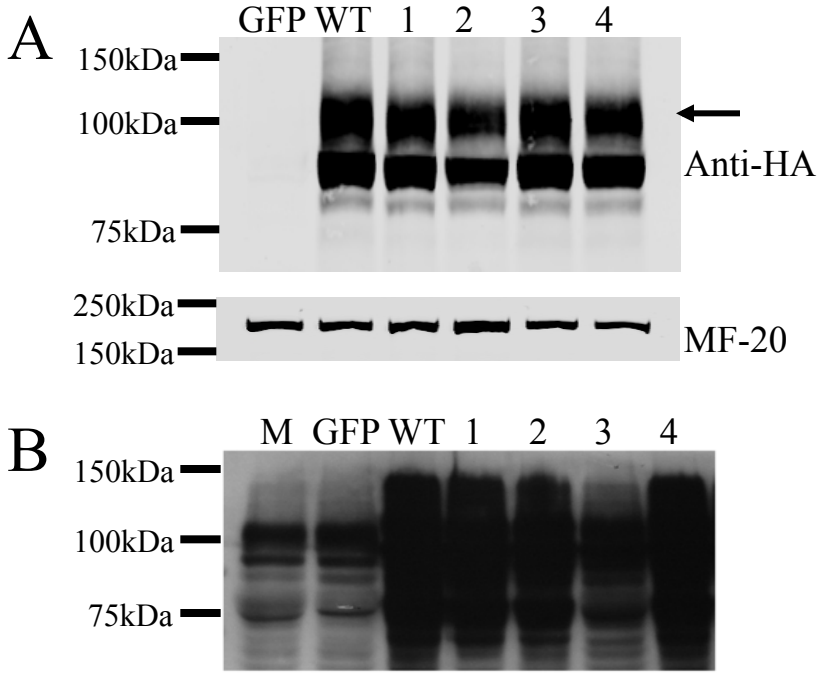


Figure 4.1 Western blot analysis of NHE1 expression in isolated cardiomyocytes infected with adenovirus expressing wild type or mutant NHE1 protein. Isolated cardiomyocytes were made from neonatal rat hearts as described in section 2.5.3. Cells were infected with the appropriate adenoviruses. GFP indicates cell extract from isolated cardiomyocytes infected with adenovirus expressing GFP protein alone. WT, and 1-4 indicate cell extracts from isolated cardiomyocytes infected with adenovirus containing wild type NHE1 (with inhibitor resistant mutations) and with phosphorylation site mutations #1-4, respectively. M, mock uninfected cells. **(A)** Upper panel, Western blot of cell extracts blotted with anti-HA antibody. Lower panel illustrates the samples immunoblotted for myosin heavy chain (MF-20). Arrow denotes fully glycosylated NHE1. **(B)** Samples were immunoblotted for total NHE1 (endogenous and exogenous) using an antibody against the C-terminus of the protein. Part B was performed by Dr. Pratap Karki.

4.2.2 Basal activity of NHE1 phosphorylation site mutants

Isolated cardiomyocytes were infected with adenovirus with or without the NHE1 cDNA insert. Over 99% of the cells appeared to be infected as shown by fluorescence of the GFP marker protein, which is contained in all adenoviral vectors made (not shown). Fluorescence from the GFP marker protein was only a small fraction of that of BCECF and did not interfere with the measurement of intracellular pH (pH*i*). Infection of isolated cardiomyocytes with adenoviral vectors did not alter the resting pH*i*.

To confirm that we had expressed various functional inhibitor resistant mutants in isolated cardiomyocytes, we measured NHE1 activity in isolated cardiomyocytes infected with NHE1 containing adenovirus at an MOI of 20 for 24 hours. The activity was measured in cells using a two-pulse assay with the second pulse in the presence of 10 μM EMD87580. We previously determined that this concentration inhibited the endogenous NHE1 protein while the NHE1 mutant with the Leu163Phe/Gly174Ser mutations were unaffected (222). We found that 10 μM EMD87580 resulted in approximately a 90% decrease in Na^+/H^+ exchanger activity in uninfected (Mock infected) or GFP infected cells, but approximately 45% decrease in activity in cells infected with inhibitor resistant NHE1 and the phosphorylation site mutants with the inhibitor resistant mutations (Table IV). In the presence of 10 μM EMD87580, activity of the NHE1 protein was reduced by approximately 45%. The decline in activity due to 10 μM EMD87580 is likely due to decreased activity of the endogenous Na^+/H^+ exchanger. Most of the 55% of the remaining activity was likely due to activity of

the inhibitor resistant NHE1 protein. The absolute rate of recovery of cells infected with the wild type inhibitor resistant mutant was 0.0128 +/- 0.007 ΔpH/sec and there was no significant decrease in the rate of recovery of mutants #1-4. In the absence of 10 μM EMD87580, the NHE1 activity after the second ammonium chloride pulse was equivalent to the first. There was also no difference in the buffering capacity of cells infected with various types of adenovirus relative to uninfected cells (Figure 4.2).

	Total NHE1 activity	NHE1 activity with 10 μM EMD87580
Mock infected	0.0207	0.0018 (8.69%)
GFP	0.0133	0.0030 (22.55%)
Inhibitor resistant NHE1	0.0128	0.0076 (59.37%)
1	0.0123	0.0052 (42.28%)
2	0.0148	0.0084 (56.76%)
3	0.0139	0.0065 (46.76%)
4	0.0163	0.0071 (43.56%)

Table IV: Activity of NHE1 mutants in the absence or presence of 10 μM EMD87580. The NHE1 activity is dpH/dt (sec^{-1}). Mock, are uninfected cells; GFP, adenovirus infected cells with GFP reporter; IRM, adenovirus infected with inhibitor resistant NHE1; Numbers 1-4 indicate adenovirus infected cells with NHE1 inhibitor resistant and phosphorylation site mutants 1-4. 1: Ser693Ala-IRM, 2: Thr718Ala and Ser723/726/729Ala-IRM, 3: Ser766/770/771Ala-IRM and 4: Thr779Ala and Ser785Ala-IRM. Total NHE1 activity indicates both endogenous and exogenous NHE1 assessed in the absence of EMD87580. NHE1 activity with 10 μM EMD87580 indicates the exogenous inhibitor resistant NHE1 assessed in the presence of 10 μM EMD87580. $p<0.05$ for all samples tested between 10 μM EMD87580 and Control.

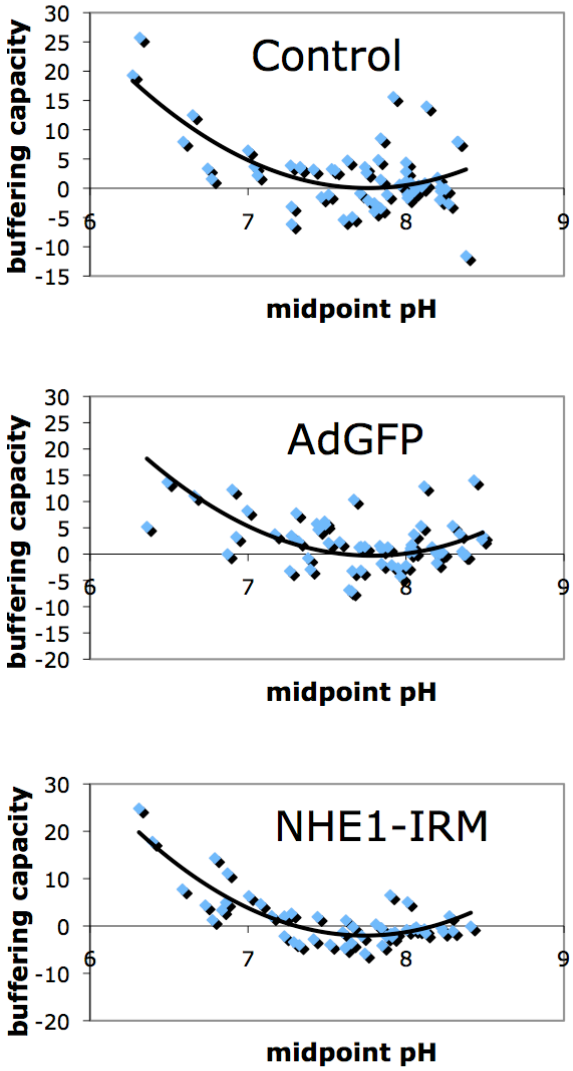


Figure 4.2 Comparison of intracellular buffering capacity of isolated cardiomyocytes infected with adenovirus. Upper panel, uninfected cells; middle panel, cells infected with GFP expressing adenovirus and lower panel, cells infected with inhibitor resistant NHE1 (NHE1-IRM) containing adenovirus. This experiment was performed by Dr. Pratap Karki.

4.2.3 Stimulation of NHE1 mutants by sustained acidosis

We characterized the effect of sustained intracellular acidosis on activity of various NHE1 proteins in isolated cardiomyocytes (Figures 4.3 and 4.4). As described in materials and methods section 2.5.6A, NHE1 stimulation by sustained acidosis was tested using a two-pulse assay. Briefly, NHE1 activity was assessed as the rate of pH*i* recovery (ROR) following an acute acid load on the first pulse, then cells were subjected to a second acid pulse where the acid loading was prolonged for 3 minutes before the second recovery was allowed (Figure 4.3). By comparing the rate of recovery of the second pulse to the first, we observed treatment related differences in NHE1 activity. For controls, two-pulse assays were performed where both pulses were acute acid loads. This enabled us to correct for potential assay differences between the first and second pulse. We found that infection of isolated cardiomyocytes did not alter the resting intracellular pH. There were no differences in the initial degree of acidification induced by ammonium chloride between NHE1 infected cells and controls (Ad-GFP infected cells) in either of the two pulses of the activity assays. The values between the second pulse with SIA to those in the control two-pulse assay (without SIA) were compared and there were no differences in the initial pH of recovery between the two pulses for both the control and SIA assays. An exclusion criterion of more than a difference of initial pH of 0.25 pH units between the first and second pulse was used for all activity assays. Furthermore, the increased period of incubation in Na^+-free medium in the SIA experiments changed the duration but not the extent of the intracellular acidosis. For example,

the minimum pH obtained with the first pulse in the GFP infected cells was 6.05 ± 0.07 while that of the second pulse with SIA treatment was 6.03 ± 0.06, and similar results were obtained with other groups of adenoviral infected cardiomyocytes. Both uninfected (Mock infected) and GFP infected isolated cardiomyocytes were assayed in the absence of the NHE1 inhibitor, EMD87580.

The rate of recovery in uninfected and GFP infected controls increased approximately 50-75 % by sustained acidosis (Figure 4.4). This effect also occurred when SIA was induced using EMD87580 in the presence of normal sodium (data not shown), which is consistent with previous studies (212). This indicated that the increased activity was not due to Na^+ depletion. When cardiomyocytes were infected with inhibitor resistant NHE1 protein in the presence of EMD87580, they behaved similar to controls. These results confirmed that the exogenous NHE1 was functional and responded similarly to endogenous NHE1 in isolated neonatal rat cardiomyocytes. The rate of recovery was elevated by about 60% with NHE1 phosphorylation site mutants 1, 2 and 4 when treated with sustained intracellular acidosis. The second pulse was not significantly different from that obtained with the wild type NHE1 inhibitor resistant protein. There was a slight decrease in the rate of recovery of mutant 4, though this was not significantly different from control values. In contrast, when cells were infected with the NHE1 phosphorylation site mutant protein 3, with mutations of amino acids Ser766/770/771Ala, there was significantly less activation by SIA in comparison to the activation achieved with wild type inhibitor resistant NHE1.

Figure 4.3 shows an example of the dual activity assay trace for both NHE1-IRM and the NHE mutant #3, with the SIA treatment.

These results confirm that SIA had a stimulatory effect on both endogenous and exogenous NHE1 activity. Furthermore, phosphorylation site mutants #1, 2, and 4 are not important in the regulation of NHE1 by sustained acidosis. However, NHE1 phosphorylation site mutant #3 with the amino acids Ser 766/770/771 mutated to Ala abolished the ability of NHE1 to be stimulated by sustained intracellular acidosis and therefore plays an important role in the regulation of NHE1 by phosphorylation.

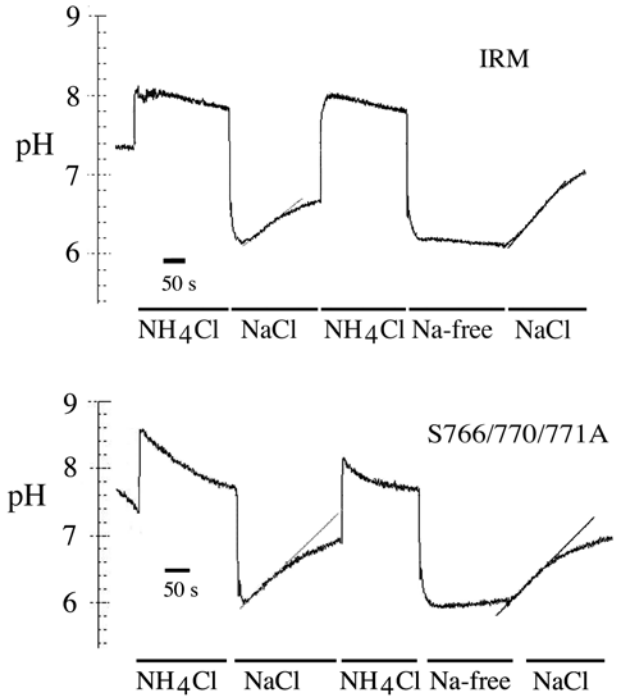


Figure 4.3 Examples of traces of dual pulse assays. Top panel, wild type inhibitor resistant NHE1 (NHE1-IRM). Lower panel, example of a dual pulse assay of NHE1 with mutations of S766/770/771A (mutant #3). Lines drawn at a tangent to the rate of recovery are for illustrative purposes only. Periods of NH_4Cl, NaCl and Na^+-free solution are indicated below the traces. A brief period of Na^+-free incubation after the initial NH_4Cl pulse is not illustrated.

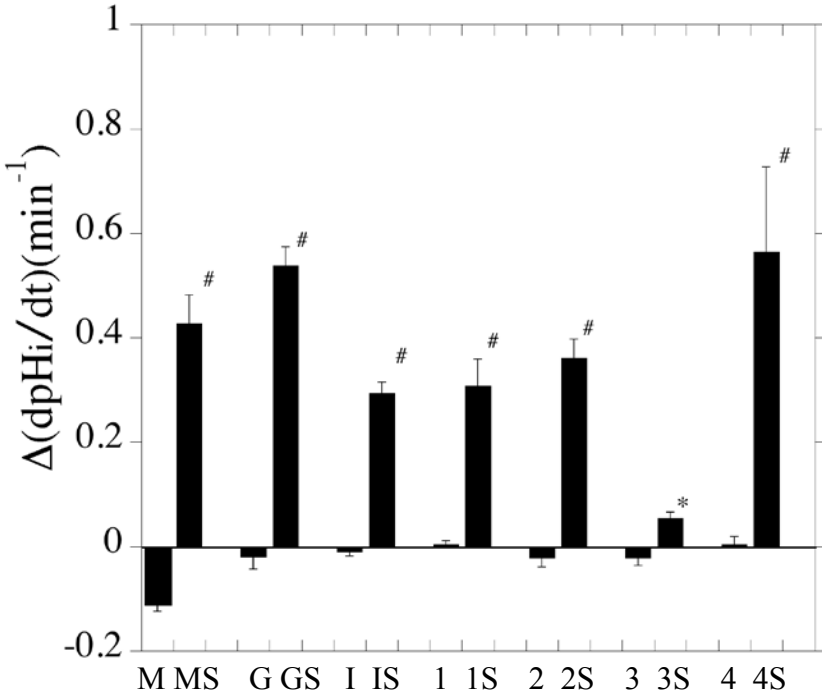


Figure 4.4 Effect of sustained intracellular acidosis on NHE1 activity in isolated cardiomyocytes. Isolated cardiomyocytes were uninfected, or infected with various adenovirus types at an MOI of 20 for 24 hours. M, uninfected cells; G, infected with virus without NHE1; I, infected with wild type inhibitor resistant mutant (IRM) NHE1 adenovirus; 1-4 NHE1, infected with IRM NHE1 adenovirus with mutations 1-4. The cells were then subjected to two-pulse activity assays and the activity of the exchanger in the second pulse was compared to that of the first pulse. The second activity assay pulse was done with a 3 minute period of sustained intracellular acidosis (SIA) immediately prior to recovery. Controls did not have an SIA in the second pulse. For uninfected and GFP infected cells, activity was in the absence of EMD87580, in the other cells activity was measured in the presence of 10 μM EMD87580. A summary of the difference between the second pulse and the first pulse is shown. # indicates significantly different from the first pulse at $p< 0.05$, * indicates significantly different relative to IRM NHE1 without mutations of phosphorylatable residues $p< 0.001$. Values are mean +/- SE of at least 10 experiments. The suffix S indicates that in the second pulse that group was treated with SIA, and when absent indicates that the second pulse did not have SIA.

4.2.4 Stimulation of ERK1/2 and p90^{RSK} by sustained acidosis

Previous reports have shown that SIA activates NHE1 in parallel to activation of the protein kinases ERK1/2 and p90$^{RSK\ (212)}$. Furthermore, in adult ventricular cardiomyocytes, SIA activates ERK1/2 through proximal activation of the classical Ras/Raf/MEK pathway (184). Therefore, we examined the effect of sustained acidosis on the activation of the protein kinases ERK1/2 and p90^{RSK}.

Isolated cardiomyocytes were infected for 24 hours with the adenovirus expressing inhibitor resistant NHE1 protein (MOI 20). Cardiomyocytes were then untreated, or treated with 3 minutes of sustained intracellular acidosis stimulation. The cardiomyocytes were lysed and cell lysates were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Western blot analysis was performed with the appropriate antibodies as described in Table III.

Kinase activation for both ERK1/2 and p90^{RSK} was assessed using antibodies specific to the phosphorylated form of the protein (Figure 4.5). The relative levels of phosphorylated ERK1/2 and p90^{RSK} were compared to total protein levels of ERK1/2 and p90^{RSK} using Image J Densitometry software.

ERK1/2 displayed significant activation by sustained intracellular acidosis (Figure 4.5A and C). Upper panels of the immunoblots were probed with the anti-phospho kinase antibodies and lower panels were probed with the anti-kinase antibodies, which were used to normalize the results. The MAPKK inhibitor, U0126, was used to block the activation of ERK1/2 and p90^{RSK} (223,224). Studies have shown that treatment with 10 μM of U0126 suppresses activation of the MAPKK cascade without inhibiting other protein kinases (225). Treatment of

cells with 10 μM MAPKK inhibitor U0126 for 10 minutes prior to acidosis treatment abolished activation of ERK1/2 in myocardial cells (Figure 4.5).

ERK1/2 activation was significantly stimulated by 3 minutes of SIA. However, we wanted to examine the ERK1/2 and $p90^{RSK}$ activation at varying times of sustained acidosis: 1, 3, and 6 minutes. This enabled us to assess the time for both ERK and $p90^{RSK}$ activation as $p90^{RSK}$ is downstream of ERK in the MAPK cascade and may require more time to be activated by sustained acidosis. Experiments were performed as above in Figure 4.5, with 1, 3, and 6 minutes of sustained acidosis. Figure 4.6 illustrates that ERK1/2 was rapidly activated within 1 minute of sustained acidosis stimulation. These results are consistent with previous studies, which examined ERK1/2 activation with sustained acidosis in neonatal rat ventricular cardiomyocytes and adult rat ventricular cardiomyocytes (184,212). Analysis of activation of $p90^{RSK}$ for 1, 3, and 6 minutes revealed that there was no significant increase in the phosphorylation levels of $p90^{RSK}$ isoforms 1 and 2 with the SIA stimulation over the 6 minutes acidosis period examined (Figure 4.7). These results are not consistent with previous studies, which showed an increase in phosphorylated $p90^{RSK}$ with 3 and 6 minutes of sustained acidosis (212). Furthermore, we examined the effect of the MAPKK inhibitor U0126 on the basal level of phospho-$p90^{RSK}$ (Figure 4.8). U0126 did not decrease the level of $p90^{RSK}$ and thus indicated that $p90^{RSK}$ was not already stimulated. Treatment with phorbol ester (PMA) caused a significant increase in the level of phosphorylated $p90^{RSK}$, and confirmed that we were able to stimulate phosphorylation of $p90^{RSK}$ in our system.

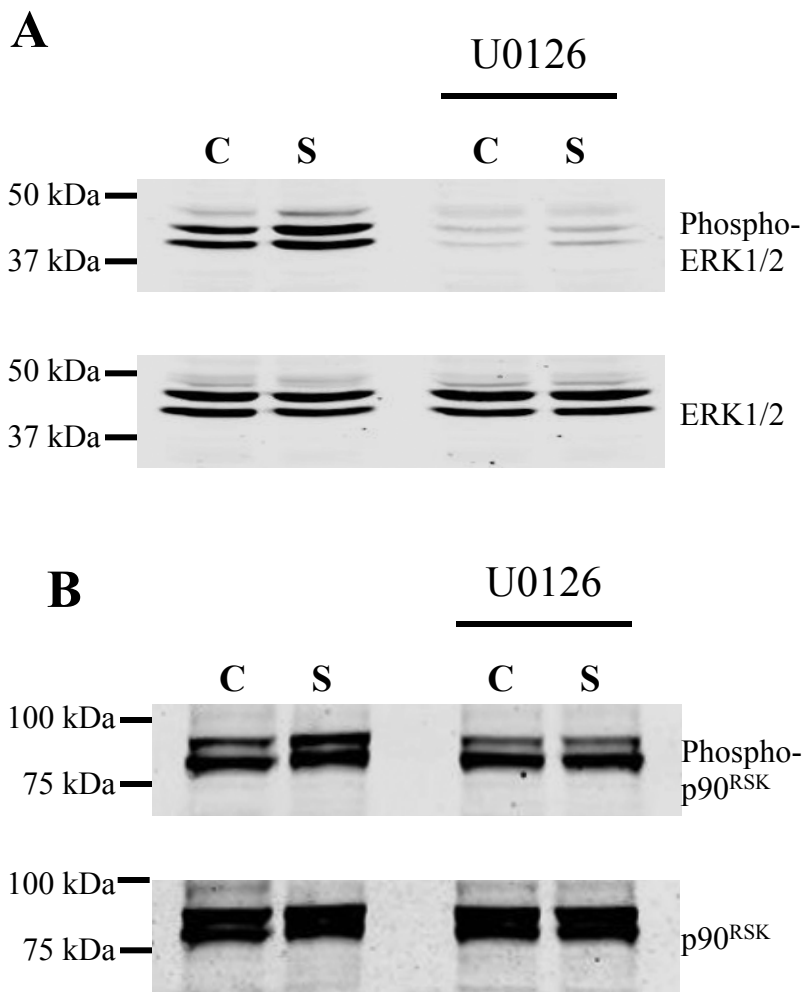


Figure 4.5 Stimulation of ERK1/2 and p90^{RSK} by sustained acidosis. (see below for figure legend)

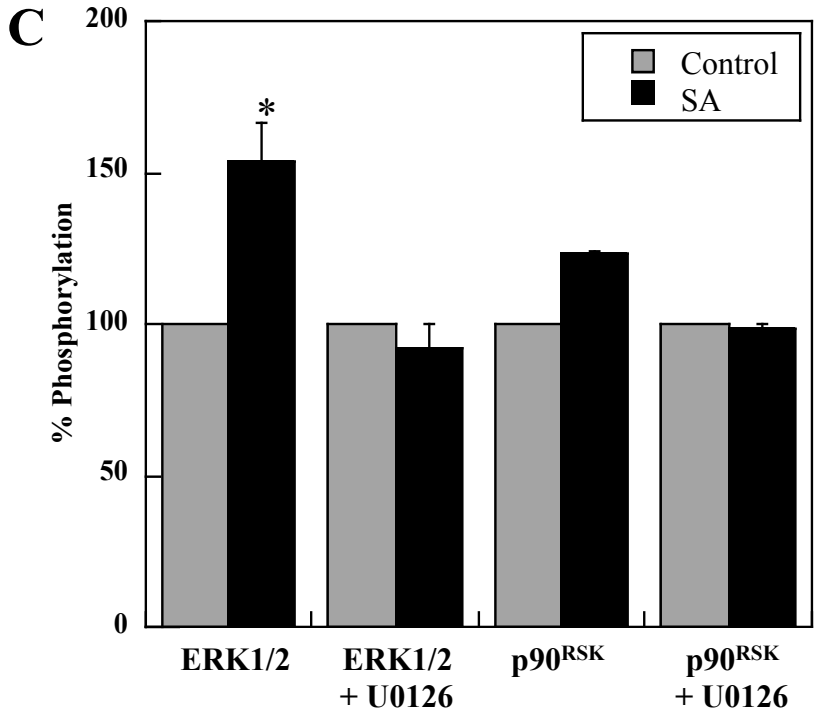


Figure 4.5 Stimulation of ERK1/2 and p90^{RSK} by sustained acidosis. Isolated cardiac myocytes were subjected to sustained acidosis as described in section 2.5.7. Levels of ERK and p90^{RSK} were determined. **(A)** Western blot with anti -phospho ERK1/2 (upper panel) and ERK1/2 (lower panel) antibodies. Where indicated, cells were treated in the presence of 10 μM U0126. "C", indicates control cells while "S" indicates cells were subjected to sustained intracellular acidosis for 3 minutes. **(B)** Western blot with p90^{RSK} (lower panel) and anti-phospho p90^{RSK} (upper panel) antibodies. **(C)** Summary of the effects of SIA on ERK and p90^{RSK} activation. Controls were set to 100% and sustained acidosis (SA) was normalized to controls. Values are the mean +/- SE of at least three experiments. * indicates significantly elevated over the level of control at $p<0.05$.

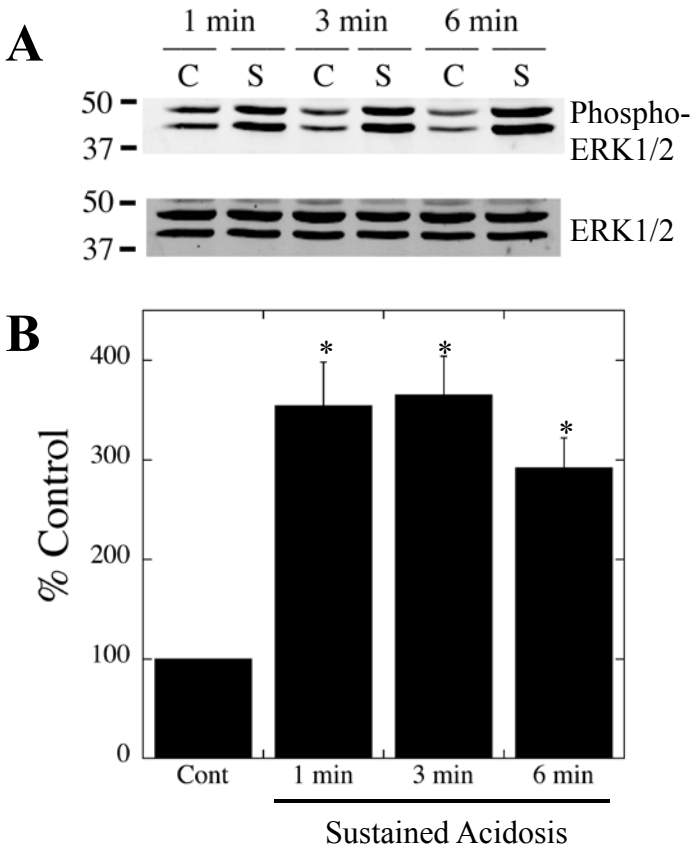


Figure 4.6 Time course of stimulation of ERK1/2 by sustained acidosis. Isolated cardiomyocytes were subjected to sustained intracellular acidosis as described in section 2.5.7. **(A)** Western blot with ERK1/2 (lower panel) and anti-phospho ERK1/2 (upper panel) after 1, 3 and 6 minutes of SIA stimulation as indicated. "C", indicates control cells while "S", indicates cells were subjected to SIA. **(B)** Summary of the effects of SIA on ERK activation. Control was set to 100% and sustained acidosis was normalized to control levels. Values are the mean +/- SE of at least three experiments. * indicates significantly elevated over the level of control at $p<0.05$. This experiment was performed by Dr. Pratap Karki.

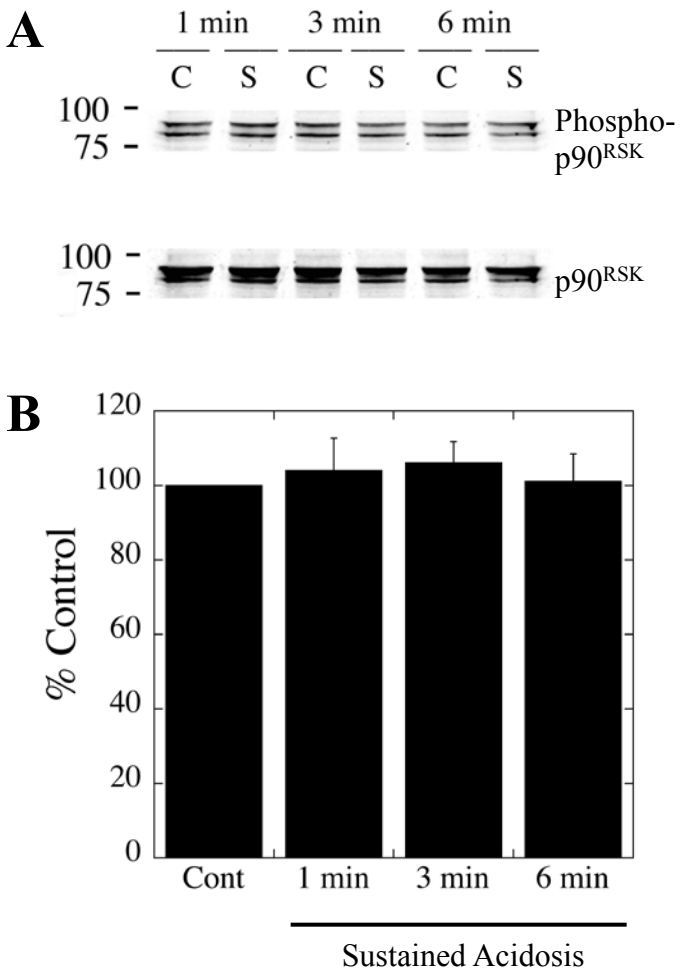


Figure 4.7 Time course of stimulation of p90^{RSK} by sustained acidosis. Isolated cardiomyocytes were subjected to sustained intracellular acidosis (SIA) as described in section 2.5.7. **(A)** Western blot with anti-p90^{RSK} (lower panel) and anti-phospho p90^{RSK} (upper panel) after 1, 3 and 6 minutes of SIA stimulation as indicated. "C", indicates control cells while "S" indicates cells were subjected to SIA. **(B)** Summary of the effects of SIA on p90^{RSK} activation. Control was set to 100% and sustained acidosis was normalized to control levels. Values are the mean +/- SE of at least three experiments. This experiment was performed by Dr. Pratap Karki.

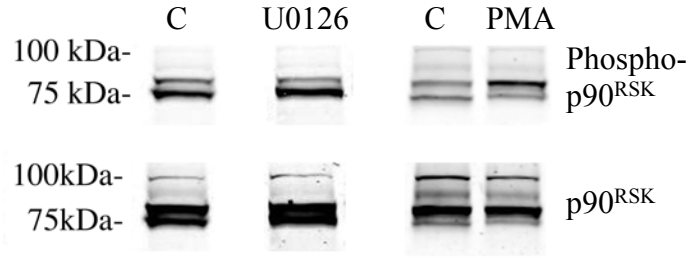


Figure 4.8 Analysis of p90^{RSK} with U0126 and phorbol ester treatment. Isolated cardiac myocytes were untreated, treated with U0126, or phorbol ester (PMA). **(A)** Western blot with p90^{RSK} (lower panel) and anti-phospho p90^{RSK} (upper panel). "C", control cells that were untreated. "U0126", indicates cells were treated for 10 minutes with 10 μM U0126. "PMA", cells were treated for 20 minutes with 10 μM phorbol ester. Results are typical of three experiments. This experiment was performed by Dr. Pratap Karki.

4.2.5 Inhibition of ERK1/2 and p90^{RSK} and NHE1 activity

Previous reports have shown that stimulation of NHE1 by sustained acidosis occurs through an ERK-dependent pathway (83,212) and we had observed increases in activated ERK1/2 with sustained acidosis (Figure 4.5 and 4.6). Therefore, we examined the effect of inhibiting the kinases ERK1/2 and p90^{RSK} on the ability of sustained intracellular acidosis to activate NHE1 activity.

To assess NHE1 activity using the MAPKK inhibitor U0126 (inhibiting the kinase upstream of ERK1/2 in the MAPK pathway, MEK), two-pulse activity assays were performed as described in sections 2.5.6 and 2.5.9 (223-225). Previous studies in our laboratory examined the effect of the vehicle on NHE1 activity, in a two-pulse assay. These studies found that the solvent DMSO did not affect NHE1 activity (114). Our control two-pulse assays were performed using 3 μM U0126 in DMSO for a 10 minute pre-pulse and then throughout two acute acid loads. Stimulatory two pulse assays involved the first pulse with an acute acid load but the second pulse with a prolonged acidosis of 3 minutes, still maintaining 3 μM U0126 in DMSO throughout the assay. The rate of pH*i* recovery (ROR) for the first and second pulse of each set of treatments was compared by examining the difference between the first and second pulse in Δ(dpH/dt) (min^{-1}).

The results in Figure 4.9 show that treatment of cardiomyocytes with the MAPKK inhibitor, U0126 prevented the activation of NHE1 by SIA in isolated cardiomyocytes. This inhibition of NHE1 stimulation was observed in all groups (uninfected, infected with control GFP, the inhibitor resistant mutant (IRM)

NHE1 and the IRM NHE1 with the mutations #1-4) and there was no significant increase in NHE1 activity upon treatment with sustained acidosis in the presence of 3 μM U0126 (Figure 4.9).

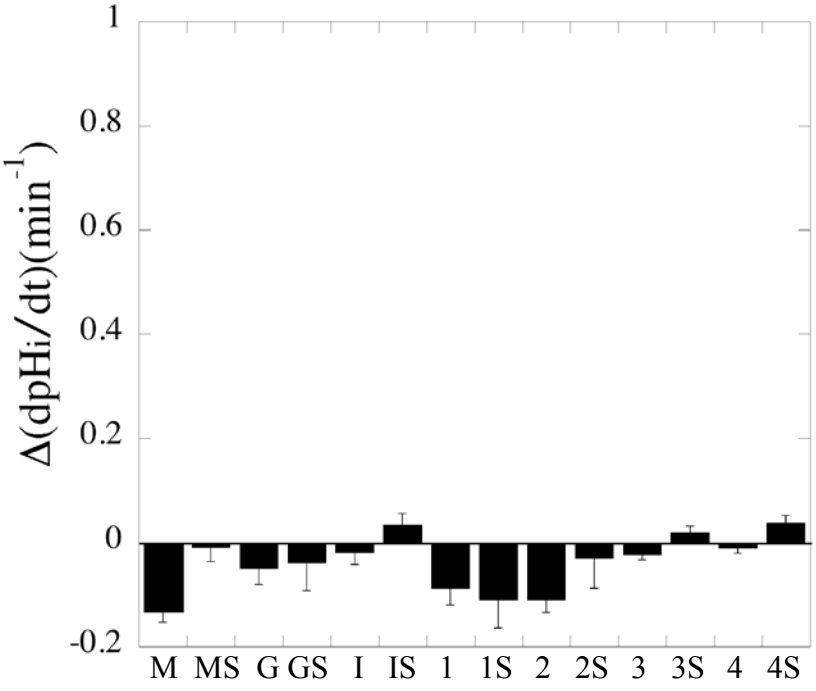


Figure 4.9 Effect of inhibition of ERK1/2 and p90^{RSK} on NHE1 stimulation by sustained acidosis. M, uninfected cells; G, infected with GFP containing virus without NHE1; I, infected with wild type inhibitor resistant (IRM) NHE1 adenovirus; 1-4 NHE1, infected with IRM NHE1 adenovirus with mutations 1-4 as indicated. For uninfected and GFP infected cells, activity was in the absence of EMD 87580 and for other cells activity was measured in the presence of 10 μM EMD 87580. Cells were uninfected, or infected with various adenoviruses at an MOI of 20 for 24 hours. The cells were then treated for 10 minutes with 3 μM U0126 (MAPKK inhibitor) and subjected to two-pulse Na^+/H^+ exchanger activity assays. U0126 was present for the entire assay period. The second activity assay pulse was done with a 3 minute period of sustained intracellular acidosis immediately prior to recovery. A summary of the difference between the second pulse and the first pulse is shown as Δ(dpH/dt) (min^{-1}). Values are the mean +/- SE of at least 10 experiments.

4.2.6 *In vivo* phosphorylation of NHE1 mutants

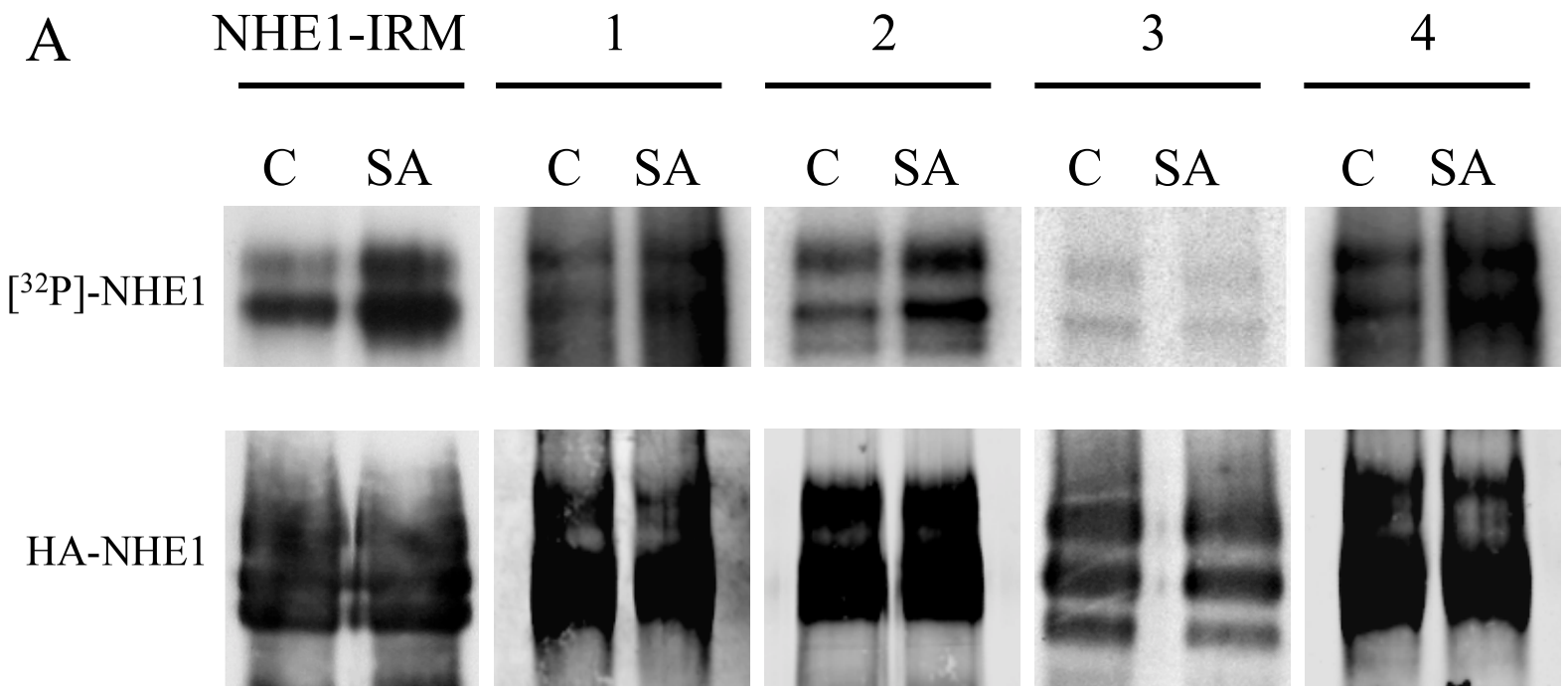
We examined the effect of intracellular acidosis and MAPK cascade inhibition on phosphorylation levels of the NHE1 in cardiomyocytes *in vivo*. Since previous results showed that the phosphorylation site mutant Ser776/770/771Ala of NHE1 abolished the ability of NHE1 activity to be stimulated by sustained acidosis, we then examined the differences in the actual level of phosphorylated NHE1 proteins. Cardiomyocytes were infected for 24 hours with the appropriate adenoviruses (NHE1-IRM and NHE1-IRM phosphorylation site mutants #1-4) at an MOI of 20. Cells were incubated in phosphate free buffer with radioactively labeled $H_3{}^{32}PO_4$. Cells were then treated with control or stimulatory conditions in the presence or absence of the MAPKK inhibitor, 10 μM U0126; this was followed by cell lysis and immunoprecipitation of the HA-tagged NHE1 protein with an anti-HA antibody. Immunoprecipitated protein was run on a 10% SDS-PAGE, transferred to a nitrocellulose membrane and exposed on a phosphor-imaging screen for one to two weeks. After exposure, Western blot analysis for total immunoprecipitated protein was performed using an anti-HA antibody. Table V shows the relative levels of phosphorylated NHE1 in WT and NHE1 mutant #3 under control or stimulated conditions. The absolute phosphorylation level of mutant #3 was 5% compared to wild type NHE1.

	Control	Sustained Acidosis
NHE1-IRM	100	140.18
Ser766/770/771Ala-IRM	3.39	3.87

Table V: Comparison of relative levels of phosphorylated NHE1 in both wild type inhibitor resistant mutant (IRM) NHE1 and phosphorylation mutant NHE1 Ser766/770/771Ala. Ratios of [^{32}P]-labeled NHE1 to total NHE1 immunoprecipitated were expressed as % of control NHE1-IRM.

SIA increased the level of phosphorylation of the NHE1 IRM protein in isolated cardiomyocytes. Figure 4.10A shows an example of an autoradiogram that demonstrates increased phosphorylation of the NHE1 protein in response to sustained acidosis. This occurred for the wild type NHE1 and for mutants 1, 2, and 4. There was no apparent increase in the level of phosphorylation in mutant 3 in response to sustained acidosis. The level of phosphorylation of mutant 3 was always much less than that of the wild type and of the other mutant proteins. Exposure times were increased greatly to facilitate accurate quantification of the levels of phosphorylation. Figure 4.10B is a summary of the experiments.

All experiments were paired and we examined the levels of NHE1 phosphorylation of wild type and mutant proteins comparing the phosphorylation levels of NHE1 protein with and without sustained acidosis. The results indicate that the phosphorylation level increased approximately 40-45% in response to sustained acidosis in wild type NHE1 and in mutants 1 and 2. There was a smaller increase in mutant 4, which was still significantly elevated over control values. Mutant 3, however, showed no increase in the level of phosphorylation in response to acidosis. In all cases we measured the amount of total immunoprecipitated protein and normalized the phosphorylated levels using these values (Figure 4.10B). Similar experiments were done in the presence of U0126 to determine whether the pathways involved in phosphorylation were indeed ERK-dependent. In the presence of 10 μM U0126 (Figure 4.11) there was no activation of the NHE1 protein. Thus, the increase in phosphorylated NHE1 upon sustained acidosis is ERK1/2 dependent.


A
NHE1-IRM
1
2
3
4
C
SA
C
SA
C
SA
C
SA
C
SA
[32P]-NHE1
HA-NHE1

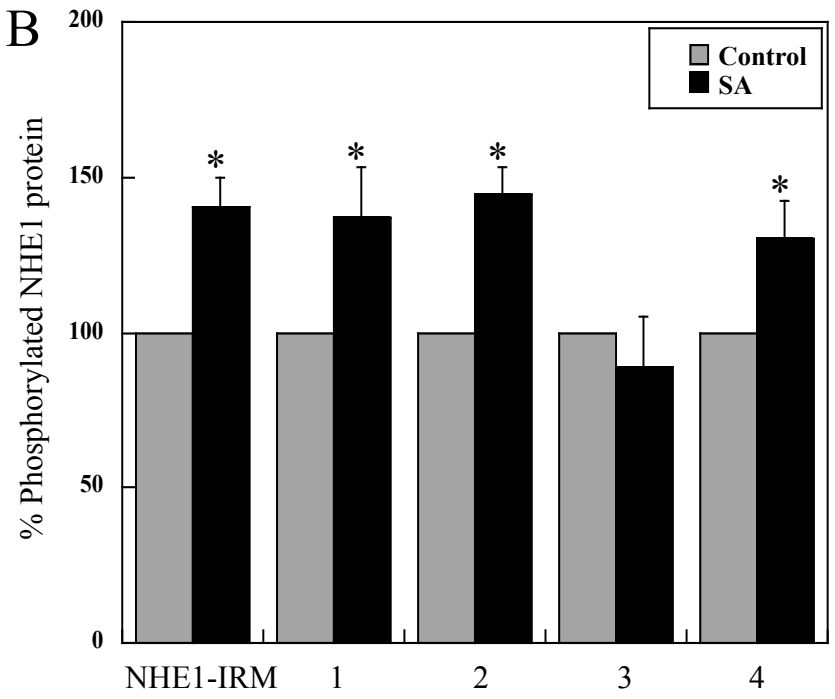
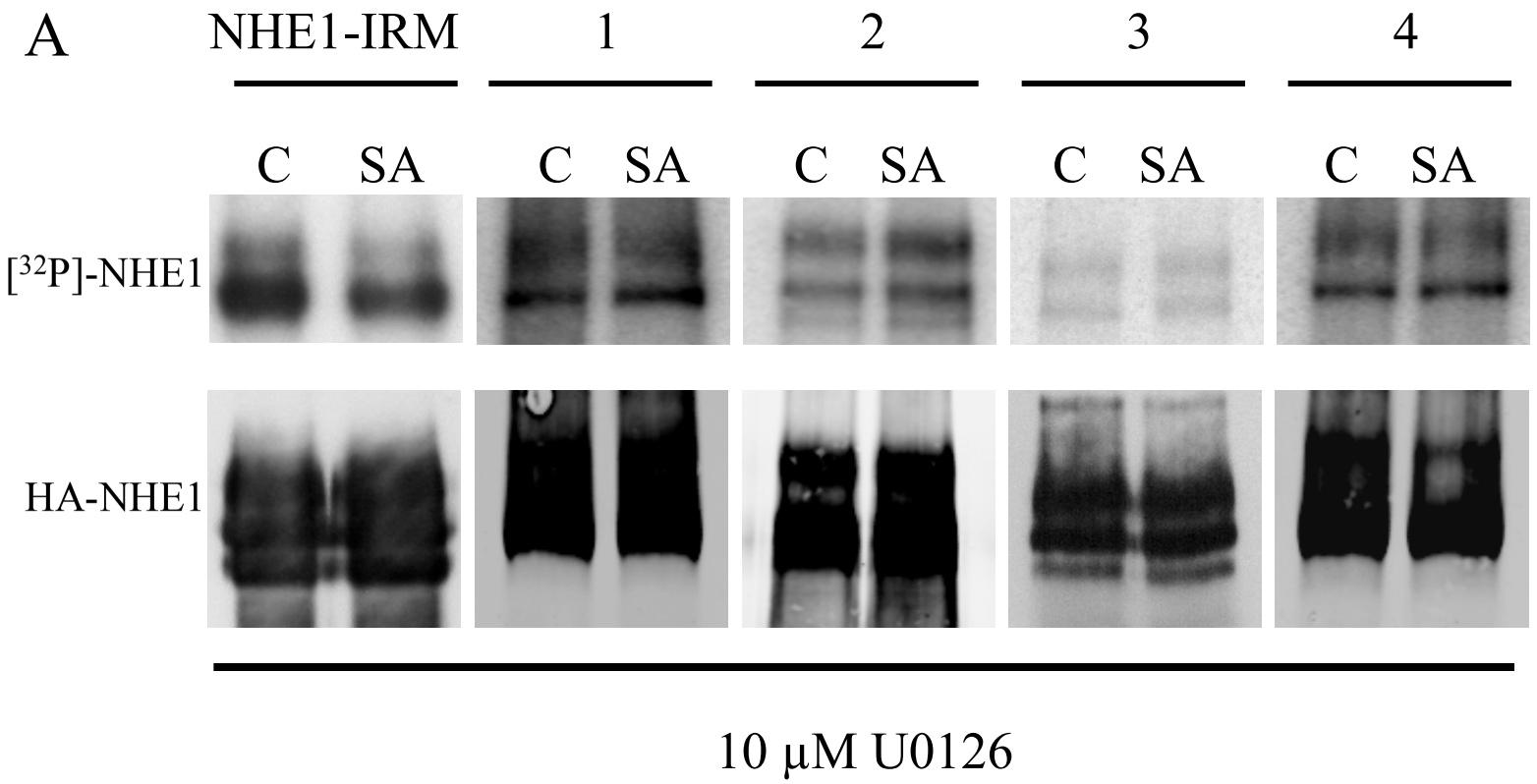


Figure 4.10 Effects of sustained intracellular acidosis on phosphorylation levels of NHE1 in isolated cardiomyocytes. **(A)** NHE1 protein was immunoprecipitated from cardiomyocytes incubated with [^{32}P], as described in 2.6. Upper part of each panel is the autoradiogram, illustrating phosphorylated NHE1 protein. Lower panel is the anti-HA western blot used to correct for total amount of immunoprecipitated protein. "C", immunoprecipitated NHE1 protein from control cells, "SA", immunoprecipitated NHE1 protein from cells stimulated for 3 minutes by sustained acidosis. NHE1-IRM, wild type NHE1 protein with inhibitor resistant mutations; 1-4 indicates phosphorylation site mutant NHE1 proteins 1 to 4. Exposure times for WT and mutants 1, 2 and 4 were equivalent. The exposure time for examination of [^{32}P] of mutant 3 was increased in order to make the bands visible. **(B)** Summary of phosphorylation levels of control versus stimulated by SA. Grey bars represent control C, black bars represent stimulated by SA. Values are the mean +/- SE of at least three experiments. * indicates significantly elevated over control $p<0.01$.


A
NHE1-IRM
1
2
3
4
C SA C SA C SA C SA C SA
[32P]-NHE1
HA-NHE1
10 μM U0126

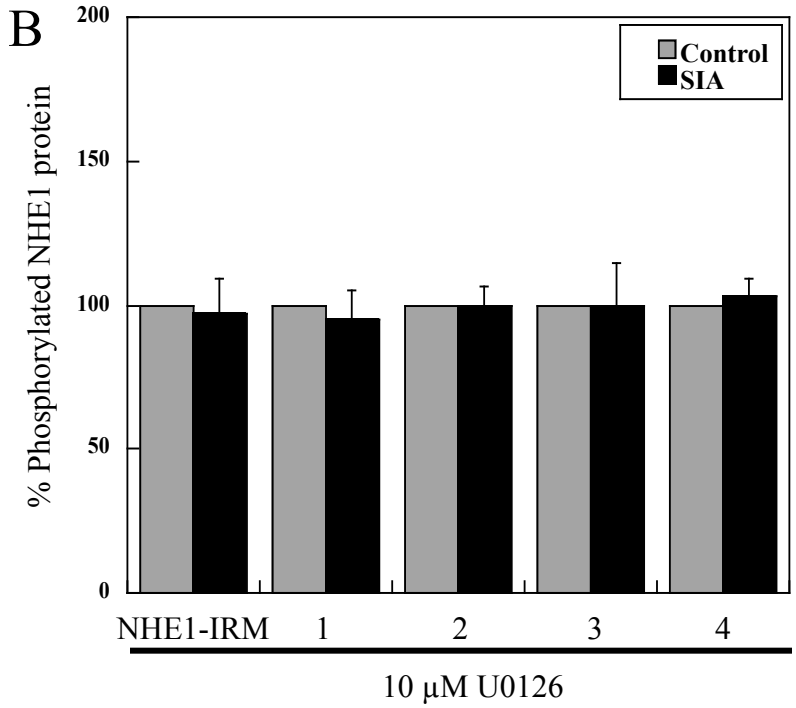


Figure 4.11 Phosphorylation of NHE1 when stimulated by sustained acidosis is ERK1/2 dependent. (A) NHE1 protein was immunoprecipitated from cardiomyocytes incubated with [^{32}P] in the presence of 10 uM U0126 as described in section 2.6. Upper part of each panel is the autoradiogram, illustrating phosphorylated NHE1 protein. Lower part illustrates anti-HA Western blot used to correct for the total amount of immunoprecipitated protein. "C", immunoprecipitated NHE1 protein from control cells, "SA", immunoprecipitated NHE1 protein from cells stimulated for 3 minutes by sustained acidosis. NHE1-IRM, wild type NHE1 protein with inhibitor resistant mutations; 1-4 indicates phosphorylation site mutant NHE1 proteins 1 to 4. Exposure times for WT and mutants 1, 2 and 4 were equivalent. The exposure time for examination of [^{32}P] of mutant 3 was increased several fold in order to make the bands visible. **(B)** Summary of phosphorylation levels of control versus sustained acidosis. Grey bars represent control, black bars represent stimulated by SIA. Values are the mean +/- SE of at least three experiments.

4.2.7 Characterization of NHE1 single phosphorylation mutants, Ser770Ala and Ser771Ala

The NHE1 mutant #3 with the mutations of Ser766/770/771Ala resulted in no increase in NHE1 activity in response to sustained acidosis, as well as low levels of basal phosphorylated NHE1 protein plus no increase in phosphorylated NHE1 upon sustained acidosis. Therefore, to identify the exact amino acid(s) involved in the regulation of activity of NHE1 in the myocardium we studied the effects of mutation of amino acids Ser^{770} and Ser^{771} to Ala. It was previously shown that the amino acid Ser^{766} was not involved in NHE1 regulation of activity by sustained intracellular acidosis in CHO cells (114). To examine the single phosphorylation site mutants of NHE1 in myocardial cells, adenoviruses were made containing the NHE1 protein with IRM and with single phosphorylation site mutations, Ser770Ala and Ser771Ala (Figure 2.3B). Effects on NHE1 activity and phosphorylation levels were analyzed by the same procedures as with other mutants.

Figure 4.12 displays the western blot analysis of cell lysates from cardiomyocytes infected for 24 hours with an MOI of 20 with either the NHE1-Ser770Ala-IRM or NHE1-Ser771Ala-IRM adenovirus. Western blot analysis revealed that both mutants expressed high levels of NHE1 protein, with both mature and immature NHE1 at similar levels to wild type. Furthermore, basal levels of NHE1 activity were similar to wild type, and when treated with 10 μM EMD87580 approximately 50% of NHE1 activity was detected indicating exogenous NHE1 (Table VI).

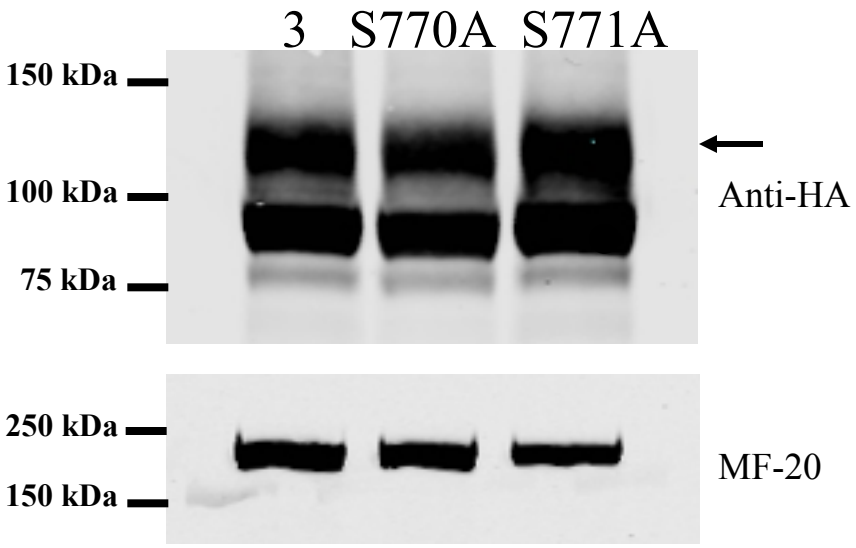


Figure 4.12 Western blot analysis of NHE1 protein mutant Ser770Ala and Ser771Ala in isolated cardiomyocytes. Isolated cardiomyocytes were prepared and infected at an MOIof 20 for 24 hours with adenovirus containing NHE1 inhibitor resistant mutant with single mutations of either Ser770Ala or Ser771Ala. The expression of the mutant NHE1 proteins was determined as described in section 2.5.3. Upper panel, Western blot of cell extracts blotted with anti-HA antibody. 3, S770A and S771A indicate cell extracts from isolated cardiomyocytes infected with phosphorylation site mutations, group #3 (Ser766/770/771Ala), Ser770Ala and Ser771Ala. Arrow denotes fully glycocyslated NHE1. Lower panel illustrates the same samples immunoblotted for myosin heavy chain (MF-20).

	Total NHE1 activity	NHE1 activity with 10 μM EMD87580
Ser770Ala	0.0156	0.0061 (39.10%)
Ser771Ala	0.0149	0.0060 (40.27%)

Table VI: Basal activity of NHE1 mutants Ser770Ala and Ser771Ala in the absence and presence of an NHE1 inhibitor. The NHE1 activity is in dpH/dt (sec^{-1}). Inhibitor resistant NHE1 with single phosphorylation site mutations Ser770Ala and Ser771Ala are indicated. NHE1 activity was assessed in the presence or absence of 10 μM EMD87580 as indicated. $p<0.05$ for all samples tested between total activity and EMD treated.

To examine the effect of these single NHE1 mutations on the ability of sustained acidosis to activate NHE1, activity was measured as previously described (section 2.5.4-6). Figure 4.13 is a summary of these results. As demonstrated earlier (Figure 4.4) wild type NHE1 and mutants #1, 2 and 4 were activated by sustained acidosis. However, NHE1 mutant #3 Ser766/770/771Ala was not activated by sustained acidosis. The Na^+/H^+ exchanger activity of the single phosphorylation site NHE1 mutants, Ser770Ala and Ser771Ala was not activated by sustained intracellular acidosis (Figure 4.13). We therefore examined the effect of sustained acidosis on phosphorylation levels of these mutant proteins. Figure 4.14 demonstrates that there was no increase in the phosphorylation of NHE1 mutant proteins Ser770Ala and Ser771Ala in response to sustained intracellular acidosis. This suggests that the amino acids Ser^{770} and Ser^{771} are phosphorylated by ERK1/2 and are essential for the activation of NHE1 by sustained acidosis in isolated cardiomyocytes.

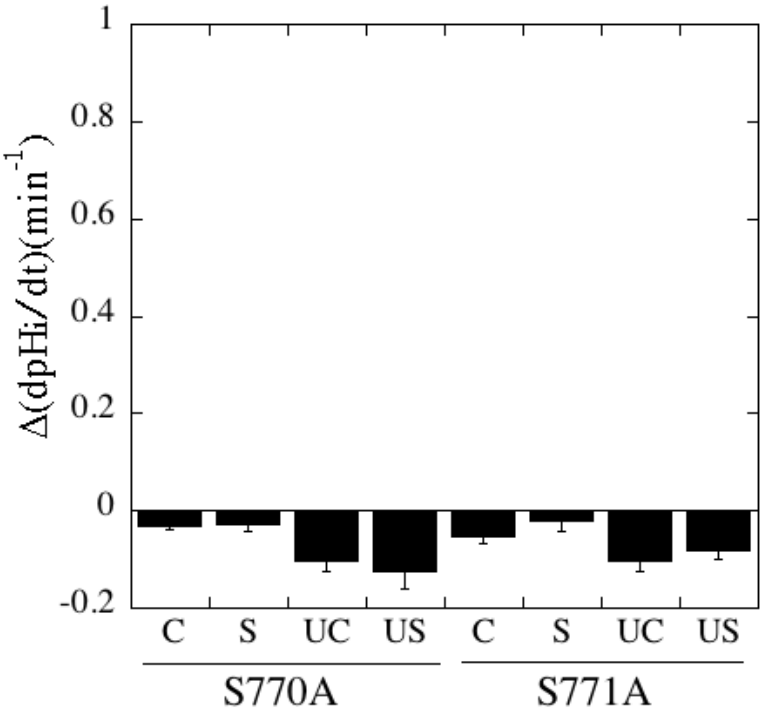
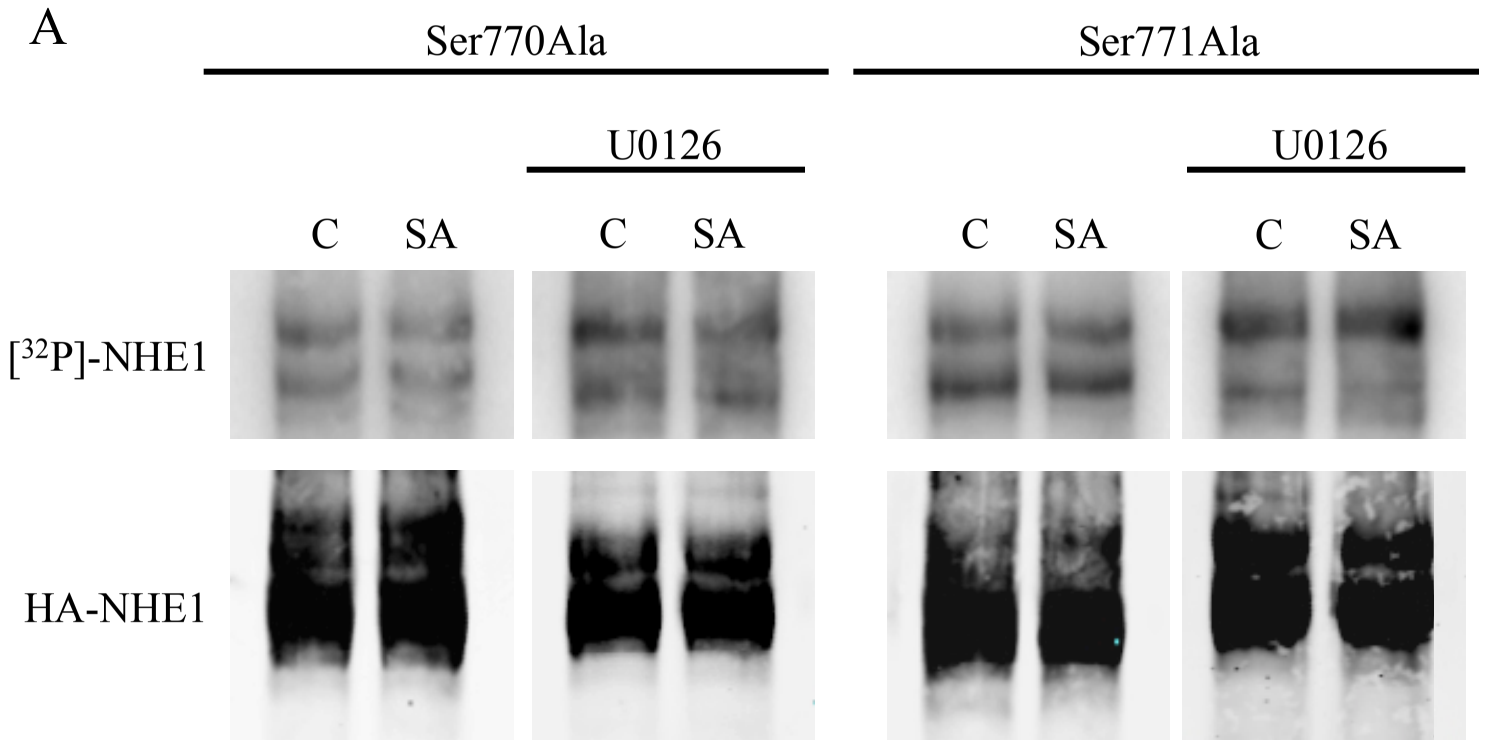


Figure 4.13 Effect of mutation of amino acids Ser^{770} and Ser^{771} on NHE1 activity in isolated cardiomyocytes. Isolated cardiomyocytes were prepared and infected at an MOI of 20 for 24 hours with adenovirus containing NHE1 inhibitor resistant mutant with single mutations of either Ser770Ala or Ser771Ala. The activity of the mutant NHE1 protein was determined as described in section 2.5.6. The bar graph displays the effect of sustained acidosis on activity of NHE1 mutants. Bars represent control "C", stimulated by sustained acidosis for 3 minutes "S", and in the presence of 3 μM U0126 "U". Values are the mean +/- SE of at least 12 experiments.


A
Ser770Ala
Ser771Ala
U0126
U0126
C
SA
C
SA
C
SA
C
SA
[32P]-NHE1
HA-NHE1

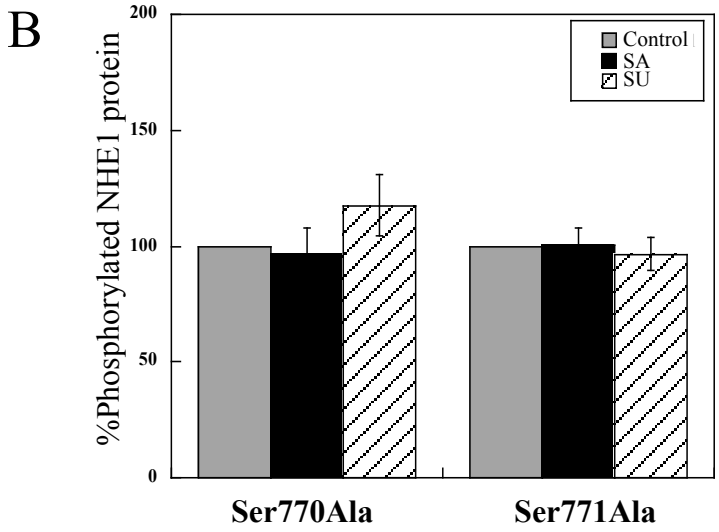


Figure 4.14 Effect of mutation of amino acids Ser^{770} and Ser^{771} on NHE1 activity in isolated cardiomyocytes. Isolated cardiomyocytes were infected at an MOI of 20 for 24 hrs with adenovirus containing NHE1 inhibitor resistant mutant with single mutations of either Ser770Ala or SerS771Ala. Phosphorylation of the mutant NHE1 proteins was determined as described in 2.6. **(A)** Examples of the effect of sustained acidosis (SA) on phosphorylation levels of NHE1 mutants Ser770Ala and Ser771Ala, in the presence or absence of 10 μM U0126. Upper part of each panel is the autoradiogram, which illustrates phosphorylated NHE1 protein. Lower part illustrates anti-HA Western blot. "C", immunopreciptated NHE1 protein from control cardiomyocytes; "SA", immunoprecipitated NHE1 protein from cardiomyocytes stimulated for 3 minutes by SA; Ser770Ala, and Ser771Ala, NHE1 proteins with Ser770Ala or Ser771Ala phosphorylation site mutations, respectively. **(B)** Summary of effects of SA on phosphorylation levels of Ser770Ala and Ser771Ala mutant NHE1 proteins in isolated cardiomyocytes. Control, immunopreciptated NHE1 protein from control cardiomyocytes, "SA", immunoprecipitated NHE1 protein from cardiomyocytes stimulated for 3 minutes by SA; "SU", immunoprecipitated NHE1 protein from cardiomyocytes stimulated by SA in the presence of 10 μM U0126. Values are the mean +/- SE of four experiments.

4.3 Discussion

The Na^+/H^+ exchanger isoform 1 (NHE1) plays critical roles in many cardiovascular disorders including ischemia reperfusion injury, hypertrophy and hypertension (described in more detail in section 1.5.2). Both increases in NHE1 expression and activity have been shown to contribute to its detrimental role in heart disease, such as ischemia reperfusion injury and hypertrophy. It is notable that although NHE1 expression is increased in various forms of cardiac pathologies, increased expression alone is not sufficient to enhance the susceptibility to ischemia and reperfusion injury (76,155,226). In fact, it has been recently suggested that regulation of NHE1 is crucial in enhancing susceptibility of the myocardium to ischemia/reperfusion injury (226). Earlier studies examining the regulation of NHE1 have shown that NHE1 is phosphorylated by the protein kinase ERK1/2 in response to ischemia/reperfusion and reactive oxygen species (83,210). Furthermore, Moor *et al*. showed that NHE1 displayed increased activity (2-10 fold) in three different models of ischemia and reperfusion both in intact myocardium and isolated cardiomyocytes (83). Taken together, these studies suggest that the MAPK cascade could be important in NHE1 regulation in cardiac disease. Therefore, our studies examined the precise mechanisms whereby NHE1 is regulated by phosphorylation in the myocardium.

Regulation of NHE1 is tissue specific and myocardial NHE1 has different kinetic properties compared with other tissues (233,234,236). Therefore, our laboratory attempted to develop two expression systems to study NHE1 overexpression in the myocardium (222) (chapter 3). Studies of the animal model

in which transgenic mice overexpressed NHE1 revealed that expression from the α-myosin promoter declined rapidly and very little exogenous NHE1 was detectable on the fourth day after cardiomyocyte isolation in culture (222). Therefore, this animal model was not suitable for studies in isolated cultured cardiomyocytes.

However, my studies showed we were able to use the adenoviral expression system to study NHE1 overexpression in primary cultures of isolated cardiomyocytes. By the inclusion of inhibitor resistant mutations (Leu163Phe/Gly174Ser), we were able to produce a system to introduce NHE1 in myocardial cells. We were able to differentiate the NHE1 that was exogenous from that which was endogenous with the addition of an NHE1 inhibitor. Thus, this method was used to examine NHE1 mutants and their regulation in isolated cardiomyocytes.

Previous studies have analyzed potential amino acids that mediate NHE1 activity by phosphorylation. *In vitro* studies from our laboratory in collaboration with Dr. Liang Liu (Department of Chemistry, University of Alberta) showed that residues Ser^{693}, Ser^{766}, Ser^{770}, Thr^{779} and Ser^{785} were phosphorylated by ERK2 by mass spectroscopy (113). Due to the close proximity of Ser^{771}, this residue was also included in my studies and in previous studies from our laboratory (114). Amino acid Ser^{703}, the target of $p90^{RSK}$, was not shown to be phosphorylated by ERK2 (113). Furthermore, the amino acids Thr^{718}, Ser^{723}, Ser^{726} and Ser^{729} were analyzed based on studies which showed that these residues were important in the activation of NHE1 following trophic factor withdrawal (43). These residues were

also shown to be phosphorylated *in vitro* by the p38MAPK, specifically amino acids Ser^{726} and Ser^{729} are important in the regulation of NHE1 in response to apoptotic stimuli (43,120).

Earlier studies from our laboratory in non-myocardial cells examined four groups of phosphorylation site mutants of NHE1: Ser693Ala; Thr718Ala, Ser723/726/729Ala; Ser766/770/771Ala; & Thr779Ala, Ser785Ala (114). The rationale for analyzing the phosphorylation sites in NHE1 as groups came from studies of the cystic fibrosis transmembrane conductance regulator (CFTR) protein. The CFTR protein is regulated by phosphorylation in its cytosolic tail, as is NHE1. Furthermore, it was shown that regulation of CFTR was dependent on the simultaneous phosphorylation of more than one residue (235). Also, by analyzing groups of phosphorylatable residues, we were able to make fewer adenoviral vectors and to efficiently study all these important amino acids in the myocardium. A summary of the mutations in the NHE1 protein is outlined in Table VII.

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

Table VII: Summary of mutations in the NHE1 protein. IRM, inhibitor resistant mutant NHE1 with mutations L163F/G174S. All mutations were made in the full length NHE isoform 1 of the Na^+/H^+ exchanger. All mutants expressed contained the IRM mutations in addition to the phosphorylation site mutations which are indicated in the table above.

Sustained intracellular acidosis (SIA) has been shown to rapidly stimulate NHE1 activity and phosphorylation via an ERK-dependent pathway in neonatal and adult ventricular cardiomyocytes and non-myocardial cells (212). SIA activated ERK1/2 and $p90^{RSK}$ in parallel to the increase in NHE1 activity. Furthermore, in adult ventricular cardiomyocytes, SIA activates ERK1/2 through proximal activation of the classical Ras/Raf/MEK pathway (184). We, therefore, examined the precise amino acids that regulate NHE1 stimulation by sustained acidosis, by characterizing phosphorylation site mutants of NHE1 in myocardial cells treated with sustained acidosis.

The present study revealed that myocardial NHE1 activity was stimulated by sustained acidosis and this occurred in an ERK-dependent manner. However, mutation of amino acids Ser^{770} and Ser^{771} to Ala in the cytosolic tail of NHE1 resulted in the inability of NHE1 to be stimulated by sustained acidosis. These results are consistent with previous studies of sustained acidosis in non-myocardial cells (114). Studies examining the level of phosphorylated NHE1 protein demonstrated that treatment of isolated neonatal rat ventricular cardiomyocytes with sustained acidosis resulted in a large increase in detectable phosphorylated NHE1 protein and this increase was ERK dependent. However, mutations of Ser^{770} and Ser^{771} to Ala greatly reduced (by 90%) the level of phosphorylated NHE1 and there was no increase in phosphorylation upon stimulation with sustained acidosis. Taken together our results indicate that the amino acids Ser^{770} and Ser^{771} were responsible for mediating NHE1 stimulation.

Mutation of either Ser^{770} or Ser^{771} to Ala decreased NHE1 activity and phosphorylation. When examining the single NHE1 mutants, one possibility is that only one of the two sites is phosphorylated. The other is that mutation of the one amino acid makes the neighboring residue unphosphorylatable by alteration of the consensus sequence. This could explain why we did not observe increased phosphorylation with the individual NHE1 mutants Ser770Ala and Ser771Ala. Furthermore, it is notable that Ser^{771} precedes Pro^{772} and this forms a standard ERK1/2 consensus sequence (Ser/Thr-Pro) (237). Thus, mutation of Ser^{770} may alter the consensus sequence thereby disrupting Ser^{771} phosphorylation. This would suggest that Ser^{771} is the critical phosphorylatable residue, however further investigations are needed to confirm this suggestion. In conclusion our results verify that amino acids Ser^{770} and Ser^{771} play an essential role in the stimulation of NHE1 by sustained acidosis in the rat myocardium.

Our studies also examined the role of the MAPK pathway, in particular ERK, which has been shown by our laboratory and others to regulate NHE1 (83,109,110,114,184,212). We found that treatment with sustained acidosis resulted in an increase in NHE1 activity and phosphorylation levels and that this could be abolished by treatment with the MAPKK inhibitor, U0126. A model of NHE1 regulation by sustained acidosis is shown in Figure 4.15.

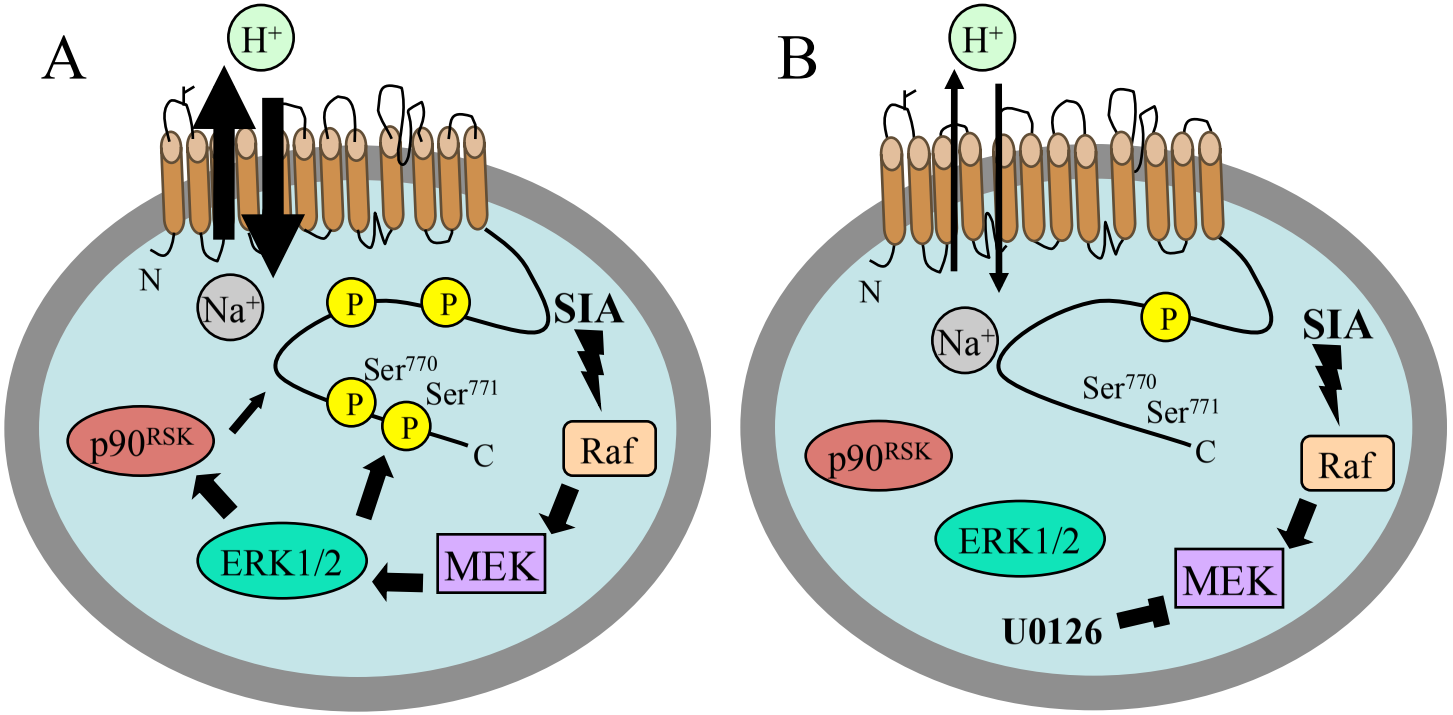


Figure 4.15 Model of NHE1 regulation by sustained intracellular acidosis. Residues phosphorylated are indicated Ser^{770} and Ser^{771}. **(A)** Model of sustained intracellular acidosis (SIA) activating NHE1. (**B)** Model of SIA and the effects in the presence of the MEK inhibitor, U0126. Raf, mitogen activated protein kinase (MAPK) kinase kinase; MEK, MAPK kinase; ERK1/2, extracellular regulated kinase isoform 1 and 2; $p90^{RSK}$, p90 ribosomal protein S6 kinase.

We next examined the effect of sustained acidosis on the activation of ERK1/2 and $p90^{RSK}$ in cardiomyocytes. Experiments were completed in the presence of protease inhibitors to prevent protein degradation as well as phosphatase inhibitors to prevent dephosphorylation of the kinases analyzed. Western blot analysis revealed that ERK1/2 was rapidly activated by sustained acidosis as early as 1 minute, while no $p90^{RSK}$ activation was detected up to 6 minutes of sustained acidosis. Our results with ERK1/2 activation are consistent with previous reports examining ERK1/2 activation with sustained acidosis; however, our results are inconsistent with what is observed in terms of $p90^{RSK}$ activation (114,184,212). Our results suggest that amino acids Ser^{770} and Ser^{771} are likely phosphorylated by ERK1/2 and this is essential for the stimulation of the Na^+/H^+ exchanger isoform 1 by sustained acidosis in the neonatal rat myocardium.

There have been various studies examining the role of Ser^{703} in NHE1 regulation (82,104,185). Studies have shown that Ser^{703} is phosphorylated by $p90^{RSK}$, and phosphorylated Ser^{703} binds to the adaptor protein 14-3-3 and this increases NHE1 activity (82,104). In the present study, we found that there was a rapid and large increase in the level of phosphorylated ERK1/2 by sustained acidosis. In contrast, we found only minor increases in phosphorylation of $p90^{RSK}$ by sustained acidosis. Previous studies from our laboratory have shown that ERK1/2 has a faster time course of activation in comparison to $p90^{RSK}$ (109). The same study by Moor *et al.* demonstrated that upon stimulation of NHE1 with serum, ERK1/2 was fully phosphorylated in 1 minute, while $p90^{RSK}$ required

several minutes for activation. This is consistent with our results, which demonstrated stimulation of cells by sustained acidosis for 1, 3 and 6 minutes resulted in a large and rapid activation of ERK1/2, while only minimal activation of $p90^{RSK}$ was observed. These results suggest that ERK1/2 is responsible for the rapid regulation of NHE1 at amino acids Ser^{770} and Ser^{771}. However, $p90^{RSK}$ might be responsible for longer-term regulation of NHE1 at Ser^{703}. Our results are also supported by the analysis of the level of phosphorylated NHE1 upon sustained acidosis treatment. We observed that there was a very large decrease in the majority of rapidly exchangeable phosphate on the NHE1 Ser766/770/771Ala, Ser770Ala and Ser771Ala mutants, thereby indicating that the majority of the rapidly exchangeable phosphate on NHE1 occurred at residues Ser^{770} and Ser^{771}.

At present, we are unable to determine whether the target of $p90^{RSK}$, Ser^{703} is phosphorylated or not in our study. Previous studies have shown that Ser^{703} is important in NHE1 regulation using both dominant negative $p90^{RSK}$ as well as specific $p90^{RSK}$ inhibitors (185,211). Further studies in our laboratory will examine NHE1 regulation by sustained acidosis in neonatal rat cardiomyocytes treated with a dominant negative $p90^{RSK}$ and will be able to address precisely the role of $p90^{RSK}$ and NHE1 stimulation by sustained acidosis. Preliminary results suggest that NHE1 is stimulated by sustained acidosis even in the absence of $p90^{RSK}$ activity, further corroborating our results.

We postulate that Ser^{703} is constitutively phosphorylated and therefore did not contribute to the rapid response of NHE1 when stimulated with sustained acidosis in our studies. Additionally, a difference between our studies and others

is that we used a shorter duration of NHE1 stimulation and all our studies were done in isolated neonatal rat ventricular cardiomyocytes, whereas the studies examining $p90^{RSK}$ used isolated adult rat ventricular cardiomyocytes. It was important to our study design to use neonatal cardiomyocytes, as opposed to adult, as they are stable in culture for longer periods of time (222). Adult cardiomyocytes have been shown to dedifferentiate in culture more rapidly than cultured neonatal cardiomyocytes (238-240).

Studying the regulation of neonatal NHE1 is of great interest and has important physiological implications. For example, the ERK pathway has been shown to be active in the neonatal myocardium and also plays an important role in myocardial development (241,242). Studies from our laboratory and others have shown that both NHE1 expression and activity are increased in the fetal and neonatal myocardium (153,154). Additionally, studies have shown that in the neonatal myocardium, ERK regulation of NHE1 is implicated in Ca^{2+} overload and damage to the myocardium (83,210). ERK expression is much higher in neonatal rat myocardium then adult rat myocardium (243). Taken together, this may explain the integral role that ERK has in the regulation of NHE1 by sustained intracellular acidosis in our study.

Various reports suggest that the neonatal myocardium is more susceptible to ischemic injury in comparison to the adult myocardium (244-246). Studies in immature rabbit hearts showed that preconditioning with an NHE1 inhibitor provided protection from ischemia/reperfusion injury in the immature myocardium (164). This is highly relevant as ischemia reperfusion injury has been

shown to be a clinical problem in children undergoing cardiovascular surgery (247). This could be due to the age related differences in pH*i* buffering of cardiomyocytes during ischemic injury (248). However, the mechanism whereby this occurs remains unknown.

In conclusion, we have examined the mechanism whereby sustained acidosis mediates NHE1 stimulation in isolated neonatal cardiomyocytes. We demonstrated for the first time that in the myocardium the amino acids Ser^{770} and Ser^{771} of NHE1 are essential for the stimulation of the Na^+/H^+ exchanger isoform 1 by sustained acidosis. This occurs in an ERK-dependent manner. We have, therefore, identified not only the precise amino acids, which are essential in the regulation of NHE1 by sustained acidosis but have also identified the protein kinase that is responsible for the phosphorylation of these amino acids.

Chapter 5:

Phenylephrine stimulates NHE1 by phosphorylation of Ser^{770} and Ser^{771}

A version of this chapter also appears in the

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5.1 Introduction

There are key hormonal factors in the myocardium important in cardiac pathology. Many of these hormones act through receptors such as the G protein coupled receptors (GPCR). A more detailed discussion of this occurs in section 1.6.1 and is reviewed in (249). GPCR systems are linked to heterotrimeric G proteins of the G_q subtype and mediate an increase in NHE1 activity.

One example of GPCR mediated stimulation of NHE1 is by catecholamines via the α_1-adrenergic receptor (AR), requiring both ERK and PKC activity (195,198). Additionally, stimulation of NHE1 occurs via altered pH_i sensitivity (199). Endothelin-1 (ET-1), a 21 amino acid vasoconstricting peptide, increases resting pH_i and has been shown to stimulate NHE1 activity in the myocardium (200,201). Previous studies from our laboratory demonstrated that the effect of ET-1 on NHE1 activity acts via an ERK-dependent pathway (109). Angiotensin II (Ang II), a peptide hormone, is another potent activator of NHE1 activity in the myocardium (202). Stimulation of NHE1 by Ang II occurs through the AT_1 subtype of angiotensin receptors and is antagonized by AT_2 receptor activation (203). Thrombin and the synthetic thrombin receptor activating peptide SFLLRN have also been shown to increase NHE1 activity (204).

Although there have been many studies of hormonal regulation of NHE1 in the myocardium, what may be the most characterized mechanism of NHE1 stimulation via the α_1-adrenergic receptors. Pure α_1-adrenergic selective agonists such as phenylephrine have been shown to increase the pH*i* sensitivity of NHE1 flux (195,198,199,250,251). The α_1-adrenergic receptor-mediated stimulation of

sarcolemmal NHE activity in rat ventricular cardiomyocytes required activation of ERK (but not the p38) of the MAPK cascade (195).

Since SIA has been shown to activate the Na^+/H^+ exchanger via an ERK-dependent pathway in the myocardium (Chapter 4), we wanted to examine whether hormone induced activation of the NHE1 protein in the myocardium is mediated by the same mechanism. Therefore, we examined the ability of NHE1 and all of the NHE1 phosphorylation site mutants (#1-4 and Ser770Ala and Ser771Ala) to be stimulated by phenylephrine. Phenylephrine stimulated wild type NHE1 activity; however, the single NHE1 mutants Ser770Ala and Ser771Ala and the triple NHE1 mutant #3, Ser766/770/771Ala were not stimulated by phenylephrine. Furthermore treatment of cells with a MAPKK inhibitor (U0126) resulted in no increase in NHE1 activity upon phenylephrine stimulation. Taken together, these results elucidate the mechanism whereby NHE1 stimulation by phenylephrine requires phosphorylation of Ser^{770} and Ser^{771} and this occurs through an ERK-dependent pathway in the myocardium.

5.2 Results

5.2.1 Stimulation of NHE1 activity with phenylephrine

Sustained acidosis has been shown to activate the NHE1 protein in the myocardium, and we wanted to examine if hormonal induced activation of the NHE1 protein in the myocardium was also mediated by a similar mechanism. Therefore, we tested the effect of phenylephrine on the activity of wild type and phosphorylation site mutant NHE1 proteins. To test NHE1 stimulation by phenylephrine, single pulse activity assays were performed either under control or phenylephrine stimulated conditions. Control cells were pre-treated for 6 minutes in the absence of phenylephrine, and phenylephrine stimulated cells were treated with 100 μM of fresh phenylephrine (PE) for 6 minutes and 100 μM PE was maintained throughout the activity assay. For uninfected and GFP infected cardiomyocytes, assays were done in the absence of EMD87580, for control NHE1-IRM and all NHE1 phosphorylation mutants the activity assays (control and PE stimulated) were done in the presence of 10 μM EMD87580 throughout the entire assay. The rate of pH*i* recovery (ROR) for the control was compared with the ROR for the phenylephrine stimulated. Figure 5.1 illustrates the results of stimulation of NHE1 activity with phenylephrine. Phenylephrine stimulation resulted in a 35 to 55% increase in the rate of recovery from an acid load. In the absence of EMD87580, and without exogenous NHE1 protein, both the uninfected cells and cells infected with GFP protein had Na^+/H^+ exchanger activity stimulated by phenylephrine. In the presence of the NHE1

inhibitor EMD87580, exogenous Na^+/H^+ exchanger activity was activated by phenylephrine in all mutants except in mutant #3 (Ser766/770/771Ala) and in the single phosphorylation NHE1 mutants Ser770Ala and Ser771Ala.

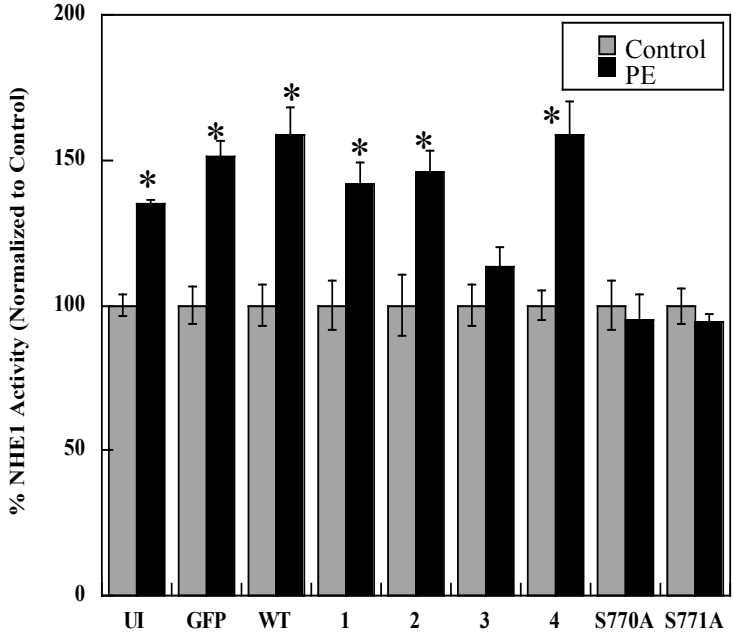


Figure 5.1 Effect of phenylephrine on activity of wild type and phosphorylation mutant NHE1 proteins. Isolated cardiomyocytes (described in section 2.5.1) were uninfected (UI) or infected with a MOI of 20 for 24 hrs with: GFP, cells infected with GFP-containing virus without NHE1; WT, infected with wild type inhibitor resistant (IRM) NHE1 adenovirus; 1-4, infected with IRM NHE1 adenovirus with group phosphorylation mutations 1-4 as indicated; S770A, S771A, cells were infected with mutant NHE1 proteins containing the Ser770Ala or Ser771Ala mutations. NHE1 activity was assayed using a single pulse assay, as described in section 2.5.6B. Cells were pretreated with or without 100 μM phenylephrine (PE) for 6 minutes and then maintained throughout the assay. For UI and GFP infected cellular NHE1 activity was in the absence of EMD87580, for other cells NHE1 activity was measured in the presence of 10 μM EMD87580. Grey bars indicate relative values of cells under control conditions (no PE) and Black bars indicate values after PE stimulation. Values are the mean +/- SE of 12 experiments. * indicates significantly elevated over the level of control at $p<0.001$.

5.2.2 Phenylephrine activation of ERK1/2

Previous reports have shown that phenylephrine activates NHE1 in parallel with activation of the protein kinase ERK1/2 (184). Therefore, we examined the effect of phenylephrine on activation of the protein kinases, ERK1/2 and $p90^{RSK}$.

Isolated cardiomyocytes were infected for 24 hours with the adenovirus expressing inhibitor resistant NHE1 protein (MOI 20). Cardiomyocytes were then treated under control or phenylephrine stimulation conditions for 6 minutes, and then the cardiomyocytes were lysed and proteins were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Western blot analysis was performed with the appropriate antibodies as described in Table III section 2.5.8.

Kinase activation of both ERK1/2 and $p90^{RSK}$ was assessed using antibodies specific to the phosphorylated form of the protein (Figure 5.2). The relative levels of phosphorylated ERK1/2 and $p90^{RSK}$ were compared to total protein levels of ERK1/2 and $p90^{RSK}$ using Image J Densitometry software.

ERK1/2 displayed significant activation by sustained intracellular acidosis (Figure 4.5 section 4.2.4). Furthermore, treatment of cells with the MAPKK inhibitor U0126 for 10 minutes prior to treatment with sustained acidosis abolished activation of ERK1/2 (Figure 4.5 section 4.2.4). Stimulation of isolated cardiomyocytes with 100 μM phenylephrine for 6 minutes also resulted in a large increase in the level of phosphorylated ERK1/2 (Figure 5.2). Phenylephrine-stimulated ERK1/2 was abolished by pretreatment with the MAPKK inhibitor, U0126 (Figure 5.2). Phenylephrine did not cause a significant increase in the level

of phosphorylated $p90^{RSK}$ (Figure 5.3). These results are consistent with what we observed for stimulation of these protein kinases with sustained acidosis.

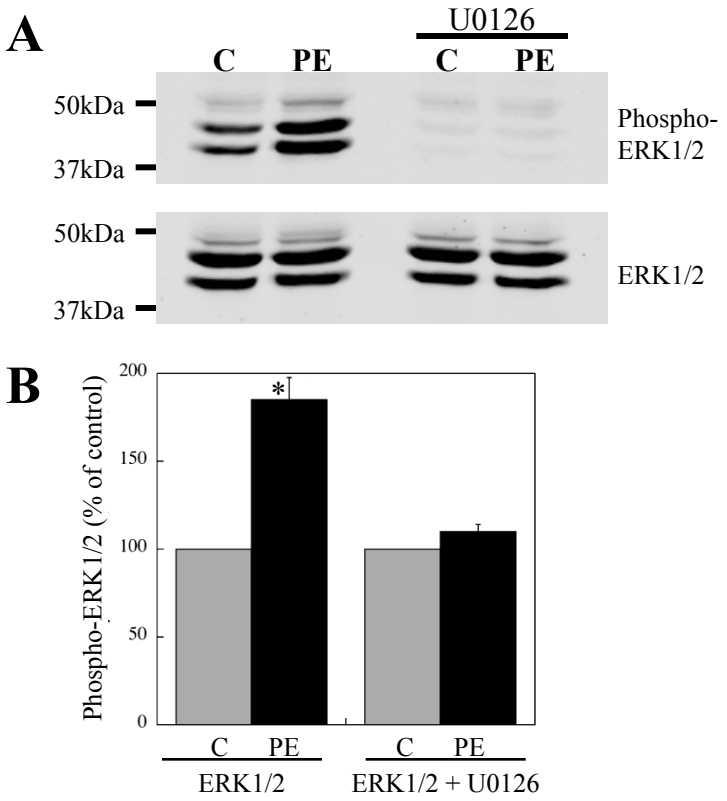


Figure 5.2 Effect of phenylephrine on activation of ERK 1/2. Isolated cardiomyocytes were treated with 100 μM phenylephrine (PE) as described in section 2.5.7. **(A)** Western blot with ERK1/2 (lower panel) and anti-phospho-ERK1/2 (upper panel) antibodies. Where indicated, cells were treated in the presence of 10 μM U0126. "C", control in the absence of PE; "PE", indicates cells were treated with 100 μM PE for 6 minutes. (**B)** Summary of effects of PE on ERK1/2 activation. The level of phospho-ERK1/2 was corrected for the level of protein and was measured in the presence or absence of U0126. Grey bars indicate values of control cells in the absence of PE. Black bars indicate values after PE stimulation. Control values were set to 100% and PE stimulated was normalized to controls. Values are the mean +/- SE of three experiments. * indicates significantly elevated over the level of control at $p<0.05$.

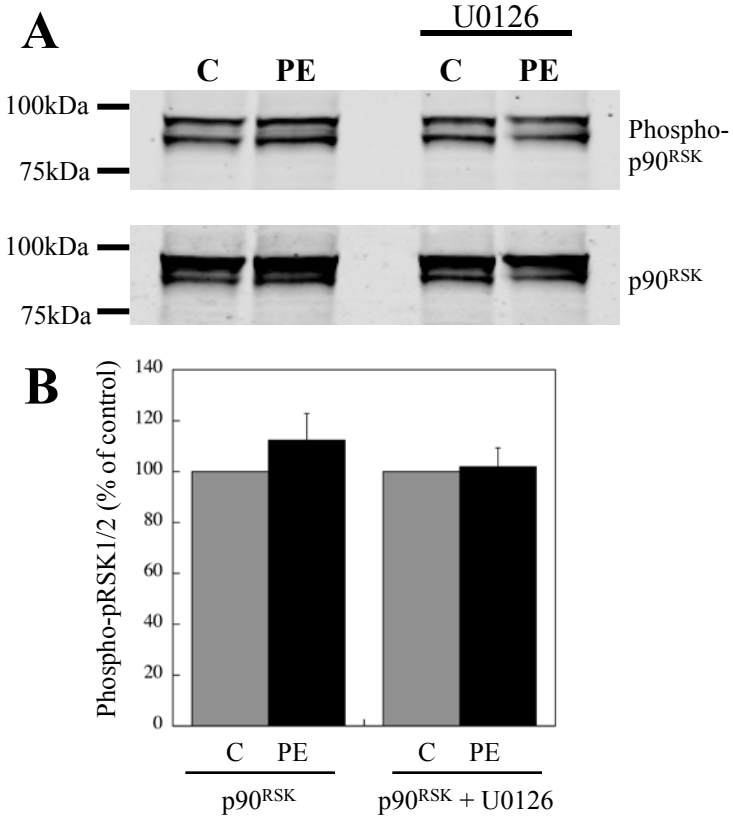


Figure 5.3 Effect of phenylephrine on activation of p90^{RSK}. Isolated cardiomyocytes were treated with 100 μM phenylephrine (PE) as described in section 2.5.7. **(A)** Western blot with p90^{RSK} (lower panel) and anti-phospho-p90^{RSK} (upper panel) antibodies. Where indicated, cells were treated in the presence of 10 μM U0126. "C", control in the absence of PE; "PE", indicates cells were treated with 100 μM PE for 6 minutes. **(B)** Summary of effects of PE on p90^{RSK} phosphorylation levels. The level of phospho-p90^{RSK} was corrected for the level of protein and was measured in the presence or absence of U0126. Grey bars indicate relative values of control cells in the absence of PE. Black bars indicate values after PE stimulation. Control values were set to 100% and PE stimulated was normalized to controls. Values are the mean +/- SE of three experiments.

5.2.3 U0126 inhibits NHE1 stimulation by phenylephrine

The amount of activated ERK1/2 increased upon treatment with 100 μM phenylephrine for 6 minutes (Figure 5.2). Therefore we examined the effect of inhibiting the kinases ERK1/2 and $p90^{RSK}$ on the ability of phenylephrine to stimulate NHE1 activity.

To assess NHE1 activity using the MAPKK inhibitor U0126 (inhibiting kinase MAPKK upstream of ERK1/2 in the MAPK pathway), single pulse assays were performed (223,224). Control assays were done in the presence of U0126 (dissolved in DMSO), with a pre-treatment of 3 μM U0126 for 10 minutes followed by an acute acid load. Phenylephrine stimulatory assays were performed with 3 μM U0126 in DMSO pre-treated for 10 minutes, and 4 minutes into that 100 μM phenylephrine was added and the combination was incubated for 6 minutes and both U0126 and phenylephrine were kept in all solutions throughout the assay. The rate of pH*i* recovery (ROR) for the control was compared with the ROR for the phenylephrine stimulated, and the phenylephrine stimulated activity was expressed as a percentage relative to the control.

When cells were treated with the MAPKK inhibitor U0126, both endogenous and exogenous NHE1 were not stimulated by phenylephrine (Figure 5.4). Thus, not only did phenylephrine stimulate NHE1 via amino acids S^{770} and S^{771}, it also acted via an ERK1/2 dependent pathway.

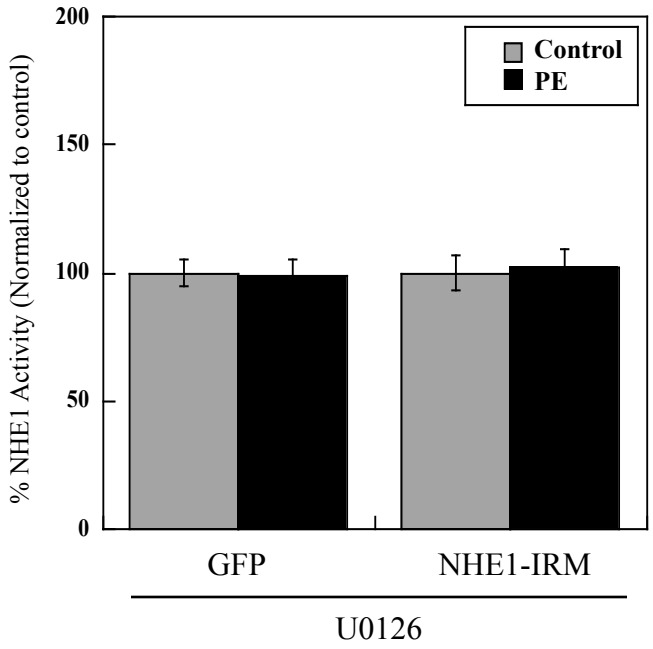


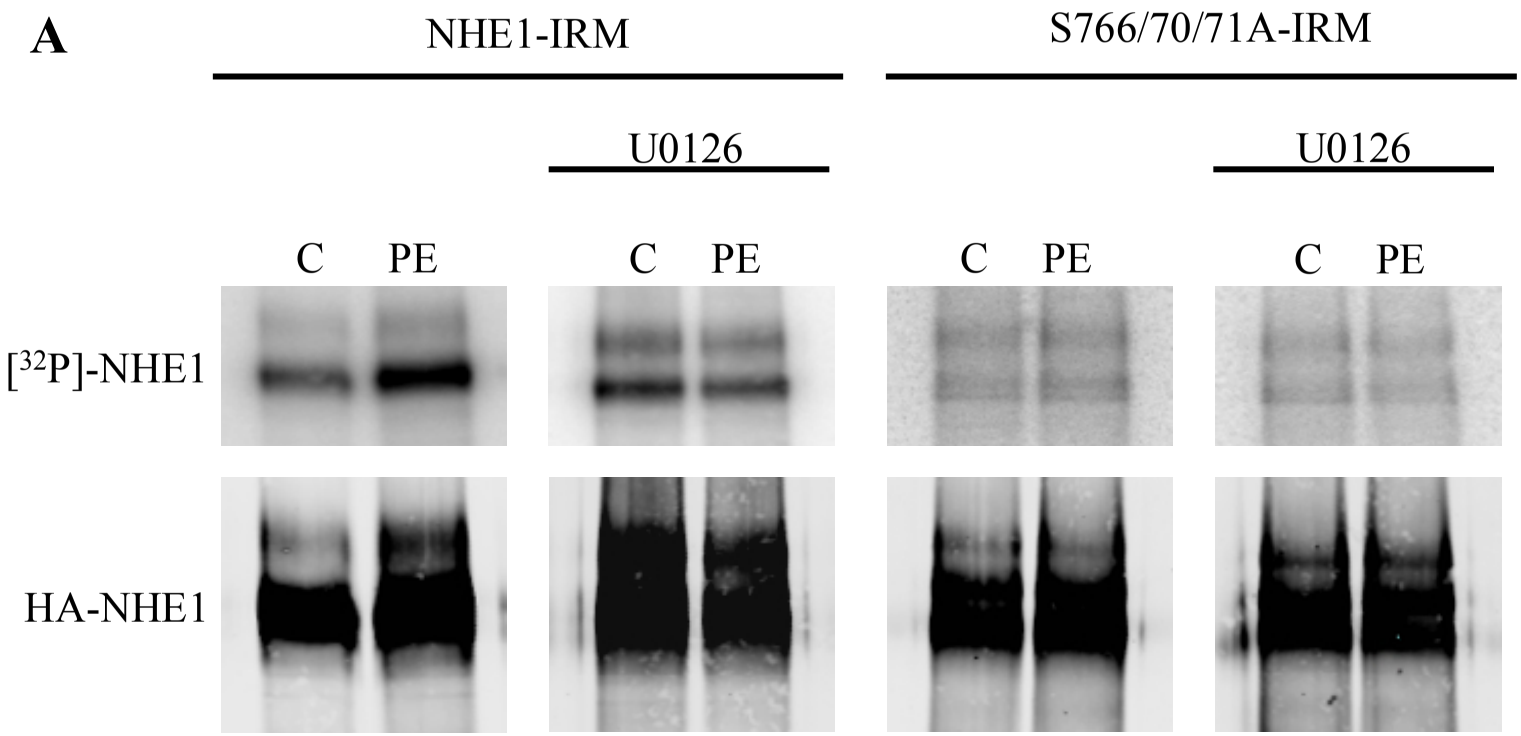
Figure 5.4 Stimulation of wild type NHE1 activity by phenylephrine is ERK-dependent. Cardiomyocytes were infected with adenoviruses at an MOI of 20 for 24 hrs; GFP, infected with GFP expressing adenovirus without NHE1. NHE1-IRM, infected with wild type inhibitor resistant NHE1 adenovirus. NHE1 activity was assayed using a single pulse assay, as described in section 2.5.6B. The cells were treated for 10 minutes with 3 μM U0126 and subjected to single pulse Na^+/H^+ exchanger activity assays and U0126 was present for the entire assay period. Assays for NHE1-IRM were done in the presence of 10 μM EMD87580. Grey bars indicate relative values of cells under control conditions (Control) and Black bars indicate values after 6 minutes of 100 μM phenylephrine stimulation (PE). Values are the mean +/- SE of 12 experiments.

5.2.4 *In vivo* phosphorylation of NHE1 with phenylephrine treatment

We examined the effect of phenylephrine stimulation on phosphorylation levels of NHE1 in cardiomyocytes *in vivo*. Since previous results showed that the phosphorylation site mutant Ser766/770/771Ala of NHE1 abolished the ability of NHE1 activity to be stimulated by phenylephrine, we then examined the differences in the actual level of phosphorylated NHE1 protein. Cardiomyocytes were infected for 24 hours with the appropriate adenoviruses (NHE1-IRM and NHE1-IRM phosphorylation site mutant #3). Cells were incubated in phosphate free buffer with radioactively labeled $H_3{}^{32}PO_4$. Cells were then treated with control or phenylephrine stimulatory conditions in the presence or absence of the MAPKK inhibitor (10 μM U0126); this was followed by cell lysis and immunoprecipitation of the HA-tagged NHE1 protein with an anti-HA antibody. Immunoprecipitated protein was run on a 10% SDS-PAGE, transferred to nitrocellulose membrane, and exposed on a phosphor-imaging screen for two weeks. Then Western blot analysis for total immunoprecipitated protein was performed. The relative levels of phosphorylated NHE1 in WT and NHE1 mutant #3 under control or sustained acidosis conditions, the absolute phosphorylation level of mutant #3 was 5% compared to wild type NHE1 (section 4.2.6 Table V).

Figure 5.5A shows a typical autoradiogram that demonstrates increased phosphorylation of the NHE1 protein in response to phenylephrine. This occurred for the wild type NHE1 with inhibitor resistant mutations (NHE1-IRM). There was no apparent increase in the level of phosphorylation of NHE1 mutant 3 (Ser766/770/771Ala) in response to phenylephrine (Figure 5.5 A and B). In

addition, U0126 abolished the increase in phosphorylated NHE1-IRM upon phenylephrine stimulation. The level of phosphorylation of mutant 3 was always much less than that of the wild type. Exposure times were increased greatly to facilitate accurate quantification of the levels of phosphorylation. In conclusion, phenylephrine stimulation increased the level of phosphorylated NHE1 and this occurred in an ERK dependent manner requiring the amino acids Ser^{766}, Ser^{770} and Ser^{771} of NHE1.


A
NHE1-IRM
S766/70/71A-IRM
U0126
U0126
C
PE
C
PE
C
PE
C
PE
[32P]-NHE1
HA-NHE1

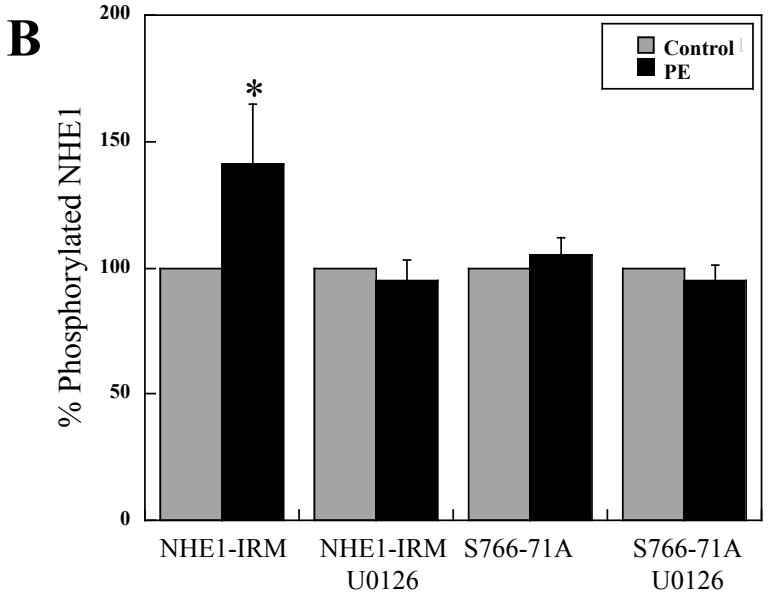


Figure 5.5 Effect of phenylephrine on phosphorylation of wild type and mutant NHE1 proteins. Isolated cardiomyocytes were infected with a MOI of 20 for 24 hrs with: NHE1-IRM, wild type NHE1 protein with inhibitor resistant mutations and mutant #3 NHE1 inhibitor resistant protein with mutations Ser 766/770/771 to Ala. **(A)** Examples of NHE1 protein immunoprecipitated from cardiomyocytes incubated with [^{32}P] +/- phenylephrine treatment in the absence or presence of 10 μM U0126 throughout the experiment as described in section 2.6. Upper part of each panel is the autoradiogram and illustrates phosphorylated protein. Lower part illustrates anti-HA Western blot used to correct for the amount of immunoprecipitated protein. "C", immunoprecipitated NHE1 protein from control cells; "PE", immunoprecipitated NHE1 protein from cells treated with 100 μM phenylephrine for 6 minutes. **(B)** Summary of effects of PE on phosphorylation levels of NHE1-IRM and mutant 3 NHE1 proteins in isolated cardiomyocytes. Values are the mean +/- SE of four experiments. * indicates significantly elevated over the level of control $p<0.01$.

5.3 Discussion

The cardiac plasma membrane Na^+/H^+ exchanger isoform 1 plays an integral role in myocardial damage by ischemia reperfusion (I/R) injury and cardiac hypertrophy (described in section 1.5.2). Previous studies from our laboratory have shown that I/R injury activates both NHE1 and the protein kinase ERK1/2 (83). Myocardial NHE1 has been shown to be acutely activated by both sustained intracellular acidosis and alpha-adrenergic stimulation (198,212). Earlier studies have shown that NHE1 activation by sustained acidosis occurs by activation of the classical Ras/Raf/MEK pathway (184). Our present studies (Chapter 4) elucidated the mechanism whereby sustained intracellular acidosis (SIA) activates NHE1 in the myocardium via an ERK1/2 dependent pathway specifically via the amino acids Ser^{770} and Ser^{771} of NHE1's cytosolic tail.

Therefore, we wanted to examine whether hormonal stimulation of the exchanger occurred via the same mechanism as that observed with sustained intracellular acidosis. We chose α_1-adrenergic stimulation, as it has been shown to acutely activate NHE1 and the protein kinase ERK1/2 (184,198). To test α_1-adrenergic stimulation, we used the selective agonist, phenylephrine. Previous reports have shown that 100 μM of phenylephrine was sufficient to activate NHE1 activity and ERK1/2 (184,185).

Our present studies tested the response of phosphorylation mutants of NHE1 to phenylephrine stimulation. We found that endogenous and control exogenous NHE1 was stimulated with treatment of 100 μM phenylephrine for 6 minutes. However, the phosphorylation mutant 3, which had Ser766/770/771

mutated to Ala, and also the single phosphorylation mutants, Ser770Ala and Ser771Ala, were not stimulated by phenylephrine. These results demonstrated that the amino acids Ser^{770} and Ser^{771} are essential for phenylephrine stimulation of NHE1 in the myocardium.

Previous studies demonstrated that phenylephrine stimulation of NHE1 occurred in parallel to the activation of ERK1/2 and our results showed that Ser^{770} and Ser^{771} are essential in stimulation of NHE1 by SIA in an ERK1/2 dependent manner. Therefore, we examined the activation of the protein kinases ERK1/2 and $p90^{RSK}$ in our system with phenylephrine stimulation. Experiments were completed in the presence of protease inhibitors to prevent protein degradation as well as phosphatase inhibitors to prevent dephosphorylation of the kinases analyzed. Western blot analysis revealed that 6 minutes of 100 μM phenylephrine treatment resulted in a very large increase in phosphorylated ERK1/2, but not $p90^{RSK}$. These results were consistent with our studies using sustained acidosis (Chapter 4 section 4.2.4). We postulate that both sustained acidosis and phenylephrine activate ERK1/2 acutely in our system whereas $p90^{RSK}$ activation required longer-term activation. A more detailed discussion of the potential role of $p90^{RSK}$ in NHE1 stimulation can be found in section 4.3.

The next step in our study was to examine the effects of inhibiting ERK1/2 on phenylephrine mediated NHE1 stimulation. Therefore we utilized the MAPKK inhibitor, U0126 that inhibits the kinase upstream of ERK1/2, MEK. We examined if NHE1 was stimulated by PE in the presence of 3 μM U0126. We found that treatment with U0126 abolished both endogenous and exogenous

NHE1 activation by phenylephrine. These results demonstrated that NHE1 activation by phenylephrine was dependent on the ERK1/2, MAPK cascade. A model of NHE1 regulation by phenylephrine is illustrated in Figure 5.6.

Since our studies found that sustained acidosis increased the level of phosphorylated NHE1 in an ERK dependent manner, we examined the level of phosphorylated NHE1 protein when treated with phenylephrine. Stimulation of NHE1 by phenylephrine resulted in an increase in phosphorylated NHE1 protein, and was also found to be ERK1/2 dependent. However, NHE1 phosphorylation site mutant #3 (Ser766/770/771Ala) displayed a low basal level of phosphorylation and this was not increased with phenylephrine treatment.

It is notable that studies in isolated adult ventricular cardiomyocytes revealed that the $p90^{RSK}$ selective inhibitor (fmk), inhibited phenylephrine-induced increases in NHE1 activity and phosphorylation (185). However, the studies of NHE1 phosphorylation by Cuello *et al*. utilized an antibody against the adaptor protein 14-3-3 to assess the level of phosphorylated NHE1 (185). The protein 14-3-3 has been previously shown to bind to phosphorylated Ser^{703} (104). Therefore, these studies did not assess all phosphorylation sites of NHE1. In contrast, our present studies examined all phosphorylation sites, including both the known ERK1/2 and $p90^{RSK}$phosphorylation sites on NHE1. Cuello *et al.* found that when assessing endogenous myocardial NHE1 activity, inhibition of $p90^{RSK}$ by fmk resulted in the disappearance of phenylephrine-induced increases in proton flux at acidic pH*i* (185). However, it is notable that the minimum pH*i* was significantly lower in fmk-treated cells than in either control or

phenylephrine-treated cells, and could have increased NHE1 activity by decreasing pH*i*, as this has been shown in previous studies to stimulate NHE1 (195).

In conclusion, the studies of NHE1 activity and phosphorylation showed that the amino acids Ser^{770} and Ser^{771} of the cytosolic tail of NHE1 are crucial in the activation of NHE1 by phenylephrine stimulation and this occurs in an ERK1/2 dependent manner. The mechanism whereby sustained acidosis stimulates NHE1 is the same as phenylephrine stimulated NHE1, acting on a common set of amino acids Ser^{770} and Ser^{771}.

These studies confirm that the amino acids Ser^{770} and Ser^{771} of NHE1 are crucial sites of phosphorylation by ERK1/2 and that they play an essential role in stimulation of the Na^+/H^+ exchanger in the myocardium. This is the first report that demonstrates a precise mechanism of phenylephrine stimulation of NHE1.

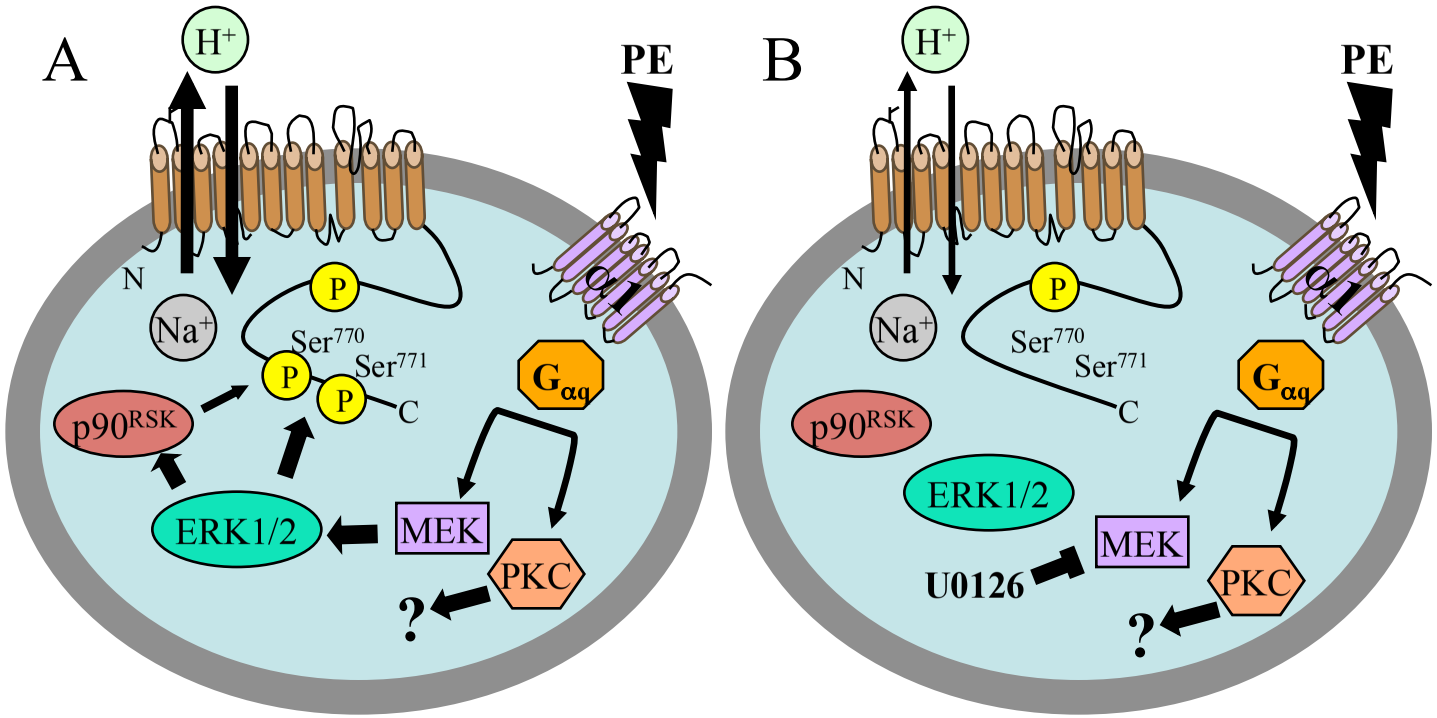


Figure 5.6 Model of NHE1 regulation by α_1-adrenergic stimulation. Residues phosphorylated are indicated Ser^{770} and Ser^{771}. **(A)** Model with α_1-adrenergic stimulation by agonist phenylephrine (PE). (**B)** Model with PE and MEK inhibitor, U0126. PE, phenylephrine; U0126, MEK inhibitor; MEK, mitogen activated protein kinase kinase; ERK1/2, extracellular regulated kinase isoform 1/2; $p90^{RSK}$ p90 ribosomal protein S6 kinase; PKC, protein kinase C; Gαq, heterotrimeric guanine nucleotide -binding protein alpha q subunit; α_1, α_1-adrenergic G protein coupled receptor.

Chapter 6:

Conclusions and Future Directions

6.1 Conclusions

The mammalian Na^+/H^+ exchanger (NHE) is a membrane protein that removes one intracellular hydrogen ion in exchange for one extracellular sodium ion. NHE is crucial in maintaining intracellular pH (pH_i) while protecting cells from acidification, as well as regulating cell volume and sodium fluxes (reviewed in (252)). Ten isoforms of NHE exist, however, NHE isoform 1 (NHE1) is the only plasma membrane isoform in the myocardium (4,6,7,144,253,254). The role of NHE1 in the myocardium is of particular importance as it has been implicated in myocardial damage from ischemia/reperfusion injury and hypertrophy (reviewed in (232)). NHE1 consists of two domains, a membrane domain that transports ions and a cytosolic domain that regulates the membrane domain and is a target of protein interactions and phosphorylations (reviewed in (31)). The exact mechanisms whereby protein kinases and phosphatases regulate NHE1 in the myocardium are not well characterized. To understand how NHE1 is regulated in heart disease, it is crucial to examine and elucidate the mechanisms whereby this protein is activated.

Therefore, the objective of my thesis was to examine the regulation of NHE1 in the myocardium. Specifically, I wanted to identify and characterize the exact amino acids which are phosphorylated on the cytosolic tail of NHE1 and to determine the precise protein kinases which regulate NHE1 directly in the myocardium. The hypothesis of this research is that specific protein kinases phosphorylate one or more amino acids of the cytosolic tail of NHE1 that are critical to pH regulation of NHE1 in the myocardium. Not only did we examine

the precise amino acids which are phosphorylated on NHE1 but we also characterized the regulation of NHE1 in the myocardium by treating myocardial cells with physiological stimuli.

The first goal of my thesis was to establish a method of overexpressing NHE1 in isolated myocardial cells. We used isolated neonatal rat ventricular cardiomyocytes, which have been extensively used in our laboratory, and are a well established model to study NHE1 (83,109,222). Since primary cultures of cardiomyocytes are very refractory to lipid or calcium based transfection systems, we chose to utilize the adenoviral expression system developed by Bert Vogelstein's laboratory (217). In primary cultures of cardiomyocytes, the adenoviral expression system results in almost 100% transfection efficiency and has been widely used to study exogenous proteins in the myocardium (255-257). We used the Stratagene adenoviral vector system as described by He *et al*. (217), and introduced our cDNA of interest, an inhibitor resistant mutant of NHE. This system resulted in a robust expression and activity of exogenous NHE1. We were able to distinguish exogenous NHE1 from endogenous NHE1 by its resistance to NHE1 inhibitors (amiloride analogs). The inhibitor resistant mutant of NHE1 (IRM NHE1) had a 100-fold difference in the IC_{50} for the NHE1 inhibitor EMD87580 in isolated cardiomyocytes (wild type NHE1 IC_{50} 0.3 μM and IRM NHE1 IC_{50} 30 μM). Furthermore, we demonstrated that in isolated cardiomyocytes we could use 10 μM of EMD87580 to completely inhibit the endogenous NHE1 while retaining exogenous NHE1 activity (222). In conclusion,

we established an expression system that would enable us to introduce NHE1 constructs in cultured isolated neonatal rat ventricular cardiomyocytes.

The main goal of my thesis was to examine the exact sites of phosphorylation of NHE1 that are important in regulation of the exchanger. For this purpose I constructed multiple adenoviruses, which contained the cDNA of the inhibitor resistant NHE1 as well as the various phosphorylation site mutations. Earlier studies in non-myocardial cells examined four groups of phosphorylation site mutants of NHE1: Ser693Ala; Thr718Ala, Ser723/726/729Ala; Ser766/770/771Ala; & Thr779Ala, Ser785Ala (Figure 2.3A). These were identified as targets of ERK2 phosphorylation *in vitro* (113). Since we knew regulation of NHE1 in the myocardium was ERK-dependent, we began our study with these same mutants. The phosphorylation mutants were performed in groups because, in other transport proteins such as cystic fibrosis transmembrane conductance regulator (CFTR), groups of phosphorylatable amino acids have been shown to work together in an overlapping manner (235). Experiments in non-myocardial cells showed that the amino acids $Ser^{766/770/771}$ are important in the regulation of NHE1 activity (114). Therefore we also tested this mutant.

To examine the role of phosphorylation in NHE1 regulation, we used two physiological stimuli: sustained intracellular acidosis and α_1-adrenergic stimulation. Sustained intracellular acidosis (SIA) has been previously shown to rapidly stimulate NHE1 activity and phosphorylation via an ERK-dependent pathway in neonatal and adult ventricular cardiomyocytes and non-myocardial cells (114,212). Therefore, we also used these stimuli in myocardial cells.

Primary cultures of isolated neonatal cardiomyocytes were made and infected with adenoviruses expressing inhibitor resistant NHE1 alone or with phosphorylation site mutations. Western blotting revealed that we were able to overexpress all mutant and wild type NHE1 proteins to similar levels with adenoviral infection of cardiomyocytes using a multiplicity of infection (MOI) of 20 for 24 hours. When we examined NHE1 activity in response to SIA, we found that all mutants, except mutant 3 (Ser766/770/771Ala) could be stimulated by SIA. Immunoprecipitation of radioactively [^{32}P]-labeled NHE1 protein corroborated these results. All mutants, but mutant 3 (Ser766/770/7771Ala) showed increases in the level of phosphorylated NHE1 protein in response to SIA. Furthermore, the absolute level of phosphorylated mutant 3 was only 5% of wild type under basal (unstimulated) conditions. In conclusion, amino acids Ser^{766}, Ser^{770} and Ser^{771} are important in the regulation of NHE1 by SIA and are important sites, which are phosphorylated under basal as well as stimulated conditions.

The next step in my thesis was to examine the amino acids in mutant 3 individually to understand which ones specifically regulated NHE1 by SIA. I constructed two more adenoviruses each with the single phosphorylation site mutations, Ser770Ala and Ser771Ala. We chose not to examine Ser766Ala because previous studies in CHO cells showed that this amino acid was not important in NHE1 regulation (114). Our studies showed that both NHE1 mutants, Ser770Ala and Ser771Ala were unable to be stimulated by sustained intracellular acidosis (SIA). Additionally, they displayed decreased levels of

phosphorylated NHE1 protein, which was not increased with SIA treatment. Therefore, our studies found that both the amino acids Ser^{770} and Ser^{771} of NHE1 are essential in stimulation of the exchanger by SIA.

Previous studies in adult ventricular cardiomyocytes revealed that SIA activates the protein kinase ERK1/2 through proximal activation of the classical Ras/Raf/MEK pathway (184). Therefore, my next thesis goal was to examine the role of ERK1/2 and its downstream target $p90^{RSK}$ in this regard. We found that ERK1/2 was activated rapidly with SIA, however, this was not the case for $p90^{RSK}$, which displayed a much slower rate of activation under SIA conditions. This increase in phosphorylated ERK1/2 was abolished by treatment with the MEK inhibitor, U0126. The use of U0126 also abolished the ability of the Na^+/H^+ exchanger to be stimulated by SIA, as well as decreased the level of phosphorylated NHE1 protein. Taken together, these results indicate that the protein kinase ERK1/2 is essential for stimulation of NHE1 by sustained acidosis.

NHE1 activity and ERK1/2 activation have also been shown to be stimulated by the α-adrenergic agonist, phenylephrine (195,198). The final goal of my thesis was to examine NHE1 regulation by phosphorylation when myocardial cells were hormonally stimulated. The question was, does phenylephrine act through the same amino acids as SIA? I found that mutation of NHE1 amino acids Ser^{770} and Ser^{771} to Ala abrogated the ability of phenylephrine to stimulate NHE1 activity. Furthermore, mutant 3 of NHE1 with $Ser^{766/770/771}$ mutated to Ala displayed no increase in phosphorylated NHE1 protein with phenylephrine stimulation. We also showed that ERK1/2 is rapidly activated by phenylephrine

stimulation in our system. Our studies demonstrated that phenylephrine stimulation of NHE1 required both the amino acids Ser^{770} and Ser^{771}, and their phosphorylation is ERK1/2 dependent. Clearly hormonal stimulation of NHE1 by phenylephrine, acts through the same amino acids as SIA.

In conclusion, this study examined the mechanism of sustained intracellular acidosis and phenylephrine mediated stimulation of NHE1. I demonstrated for the first time that in the myocardium both mechanisms of NHE1 activation rapidly affect NHE1 via a common pathway. I conclude that phosphorylation of Ser^{770} and Ser^{771} plays a critical role in NHE1 regulation in the myocardium by both sustained acidosis and phenylephrine stimulation. And both activation of NHE1 by sustained acidosis and phenylephrine occurs in an ERK1/2 dependent manner.

6.2 Future Directions

Our present study has elucidated the mechanism of NHE1 regulation by sustained acidosis and phenylephrine stimulation in the myocardium. We found that NHE1 is phosphorylated on its cytoplasmic tail at amino acids Ser^{770} and Ser^{771} by the protein kinase ERK. Future studies outlined will continue to examine the role of phosphorylation and regulation of NHE1.

6.2.1 Structure of NHE1 C-terminal tail with phosphomimetic sites using NMR

Circular dichroism analysis of the cytoplasmic tail of NHE1 has revealed that the membrane proximal region is compact and structured while the distal region is flexible and non-structured (57). In addition, a mechanism was postulated whereby phosphorylation of NHE1 alters the activation state of NHE1 by inducing conformational changes in the C-terminal tail of NHE1, thus altering the association of the tail with the membrane domain H^+ sensing region (108). Therefore, to understand how NHE1 activation is stimulated by phosphorylation, future studies will examine the structure of the NHE1 cytosolic tail with phosphomimetic sites.

We will overexpress a portion of the C-terminal tail (amino acids 546 to 778) with either control amino acids or with phosphomimetic mutant sites (Ser770/771Asp) and analyze the structure using one-dimensional proton NMR spectroscopy. The region of the C-terminal tail selected, amino acids 546-778, contains the phosphorylation sites Ser^{770} and Ser^{771}, but also the positively

charged patch of amino acids at 613-651, which we believe may interact with one another (Figure 6.1). Studies have shown that Ser^{770} and Ser^{771} are essential for the phosphorylation and regulation of NHE1 when stimulated by sustained acidosis and phenylephrine (258). The cDNA of the C-terminal tail control or C-terminal tail phosphomimetic mutant (Ser^{770} and Ser^{771} mutated to Asp) will be extracted from a plasmid containing the sequence of NHE1 using PCR and then cloned into the appropriate plasmids for use in the Gateway expression system (Life Technologies). The native proteins will be overexpressed in *E. coli* and purified using the six His tags, which we have attached to the N-termini of these NHE1 C-terminal tail proteins. Purified protein can then be analyzed by one-dimensional NMR.

We believe that there may be an interaction of the phosphorylated amino acids, which are negatively charged, with the upstream positively charged patch of amino acids (613-651 contains eleven basic residues). These studies will help explain the mechanism whereby phosphorylation stimulates NHE1 on a structural level.

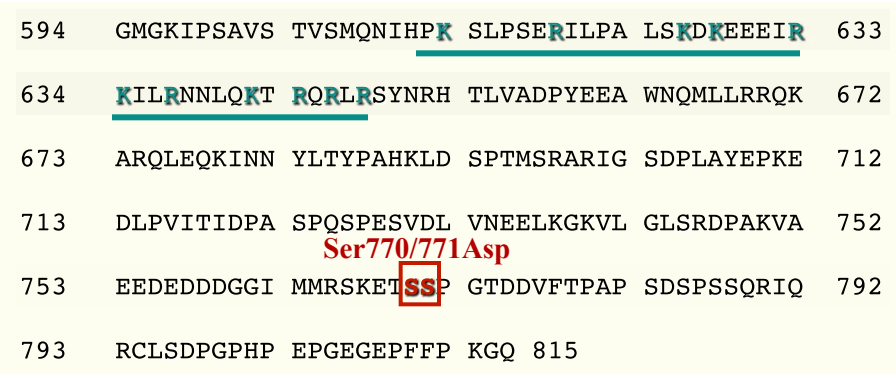


Figure 6.1 Cytosolic domain of NHE1 and positive amino acid rich region and phosphorylation mutation sites. The sequence of the final 221 amino acids of the cytosolic domain of human NHE1. Boxed, labeled and highlighted in red are the sites of the mutations of the important phosphorylation sites Ser^{770} and Ser^{771} mutated to Aspartate (D). Highlighted and underlined in green is the region 613-651 of the cytoplasmic tail which is rich in the positively charged amino acids Arginine (R) and Lysine (K).

6.2.2 The role of $p90^{RSK}$ and regulation of NHE1

Previous studies, by our laboratory and others, have shown that the MAPK cascade is crucial in the phosphorylation-dependent regulation of NHE1 (83,109,114,184,212). Our present study demonstrated that NHE1 stimulation by sustained intracellular acidosis (SIA) and phenylephrine (PE) occurs by a common pathway in an ERK dependent manner (258). Our results further demonstrated that use of the MAPKK inhibitor, U0126 resulted in: a) inhibition of stimulation of endogenous NHE1 and inhibitor resistant NHE1 activity in isolated cardiomyocytes and, b) no increase in phosphorylated inhibitor resistant NHE1 upon treatment with SIA or PE. These results are in accordance with previous findings, which show that the effects of sustained acidosis on NHE1 activity are MAPK pathway-dependent (184). One must consider that these MAPKK inhibitors not only inhibit ERK1/2, but also its downstream kinase $p90^{RSK}$, so the effect of these two kinases cannot be fully differentiated. For this reason, it would be of great use to have specific $p90^{RSK}$ inhibitors.

Recently, two specific $p90^{RSK}$ inhibitors have been described, SL0101 and fmk (259,260). The fmk inhibitor has previously been used in neonatal and adult rat ventricular cardiomyocytes to examine the effects on NHE1 activity. In future studies we will examine the activity of control NHE1 and phosphorylation site mutants of NHE1 in cardiomyocytes pretreated with fmk (3 μM) (261). We will test the fmk inhibitor in infected cardiomyocytes subjected to stimulation (SIA or PE). This will enable us to differentiate the role of ERK1/2 and $p90^{RSK}$ in the activation of NHE1.

Pharmacological inhibitors are invaluable tools in research, however, their specificity, may by limited for their targets. Therefore, we propose to examine the role of specific protein kinases through the use of dominant negative (DN) kinases. However, the DN kinases must be introduced into the myocardial cells. A great advantage of using the adenoviral expression system is the versatility of being able to infect cells of interest such as cardiomyocytes with more than one adenovirus simultaneously. For example, we could examine the effects of DN kinases with either control NHE1-IRM or phosphorylation site mutant NHE1-IRM proteins. Future studies include making an adenovirus that contains DN $p90^{RSK}$ (mutations K94/447A) to examine the specific effects of $p90^{RSK}$ depletion (262). This will enable us to evaluate how the phosphorylation site mutants of NHE1 respond to stimulation by sustained acidosis and phenylephrine in the absence of $p90^{RSK.}$ (211). Ultimately, these studies will enable us to identify the true role of both ERK1/2 and $p90^{RSK}$ in the phosphorylation and regulation of NHE1. Preliminary studies from our laboratory in isolated neonatal cardiomyocytes indicate that NHE1 continues to be stimulated (both activity and level of phosphorylated protein) by sustained intracellular acidosis when treated with DN $p90^{RSK}$.

6.2.3 Myocardial NHE1 regulation and phosphatases

Section 1.4.4 discusses the role of phosphatases in NHE1 regulation. Previous studies from our laboratory have shown that PP-1 is an important regulatory phosphatase of NHE1. However, PP-1 has not been shown to directly dephosphorylate ERK1/2- and $p90^{RSK}$-mediated phosphorylation of NHE1. To complete these studies, neonatal rat isolated cardiomyocytes will be infected with the appropriate adenoviruses (control or phosphorylation site mutant NHE1), and then [^{32}P] labeled. Cardiomyocytes will be untreated, or treated with stimulators (hormonal and SIA) and then immunoprecipitated, using the HA tag. The immunoprecipitated [^{32}P] labeled NHE1 will be subjected to *in vitro* dephosphorylation by the phosphatases as previously described (137). Future studies will examine the ability of PP-1, PP-2A and PP2-B to dephosphorylate different phosphorylation site mutants of NHE1 in a time-dependent manner.

PP-2C is also found in the myocardium and may be important in the regulation of NHE1 (128). Using the same procedures as in our previous studies (for PP-1, PP-2A and PP-2B), we will examine the *in vitro* ability of PP-2C to bind to and to dephosphorylate the C-terminal 178 amino acids of NHE1 (137). We will further examine the effects of overexpression of PP-2C on NHE1 activity, by making stable cell lines in CHO cells, which express PP-2C, and then examine its effects on NHE1 activity. Adenovirus expressing PP-2C is commercially available and could alternatively be used to examine overexpression of PP-2C in the myocardium.

These studies still fail to address how phosphatases such as PP1 and PP2A bind to NHE1. To examine this, we will use truncated mutants of the cytoplasmic tail of NHE1, NHE1C-67 (748-815), NHE1C-178 (637-815), NHE1C-272 (543-815), NHE1C-343 (472-815) and NHE1C-515-630; some of these have been previously used by our laboratory (110). We will examine their ability to bind to either PP1 or PP2A. We will do this by using microcystin (PP-1 and 2A inhibitor)-bound Sepharose beads, which bind covalently to either PP-1 or PP-2A (catalytic subunits). As controls we will use Tris-Sepharose resins (unbound to microcystin) as well as microcystin-Sepharose resins alone (unbound to PP-1 or 2A), incubated with our NHE1 proteins (segments of the C-terminal tail). Beads will be washed and then analyzed by SDS-PAGE for the absence or presence of binding. Binding would result in both the presence of either PP-1 or PP-2A and the protein corresponding to NHE1 C-terminal tail segments.

Taken together, these studies will enable us to examine which phosphatases are required for specific phosphorylation sites on NHE1, and which phosphatases dephosphorylate specific ERK1/2 or $p90^{RSK}$ phosphorylation sites. Analysis of the binding of PP-1 or PP-2A to the C-terminal tail of NHE1 will enable us to examine the known phosphatase structure and model binding of PP-1 or PP-2A to the cytoplasmic tail of NHE1. This will allow us to examine the realistic areas where the phosphatases may function to dephosphorylate the known phosphorylation sites of NHE1.

6.2.4 *In vivo* model of NHE1 Ser770/Ser771Ala mutant

Our previous studies have shown that the amino acids Ser^{770} and Ser^{771} are essential in the activation of NHE1 activity in response to both sustained acidosis and phenylephrine stimulation. NHE1 has been shown to play an essential role in ischemia/reperfusion injury and myocardial hypertrophy (reviewed in (231,263)). More recently it has been suggested that the detrimental role of NHE1 in the myocardium under such conditions is a result of increased NHE1 activity due to regulation rather than increased NHE1 expression (226). Our present studies in cultured isolated neonatal rat ventricular cardiomyocytes revealed for the first time a mechanism whereby NHE1 activity is regulated by the protein kinase ERK1/2 at amino acids Ser^{770} and Ser^{771} (258).

The next step in understanding the importance of NHE1 regulation in myocardial damage due to I/R injury would be to assess our NHE1 phosphorylation mutant *in vivo*. For this we propose transgenic mice be made with a cardiac specific knockout of endogenous wild type NHE1 while simultaneously introducing cardiac specific NHE1 phosphorylation mutant Ser770/771Ala. Expression of NHE1 in transgenic mice will be confirmed by Western blot analysis of cardiac expression of our HA-tagged NHE1 mutant, with lack of expression of endogenous NHE1. Future studies would include examining how the transgenic mice, which express only NHE1 with mutations Ser770/771Ala in the myocardium, respond to ischemia reperfusion injury. Our present studies have shown that NHE1 phosphorylation site mutant Ser770/771Ala is resistant to stimulation by acidosis and therefore we

hypothesize this mutant of NHE1 would be more resistant to ischemia reperfusion injury. These experiments would allow us to examine the exact mechanisms whereby NHE1 regulation plays a crucial role in ischemia/reperfusion injury in the intact myocardium.

6.2.5 Summary

NHE1 plays an important role in both physiological and pathological pathways in the myocardium. My present studies, as well as future studies, will elucidate mechanisms whereby NHE1 is regulated in the myocardium. The salutary benefits of NHE1 inhibitors have not been promising in clinical trials, and thus a better understanding of the mechanisms which regulate NHE1 activity will lead to new ad hopefully improved approaches to manipulating NHE1 activity in cardiac disease.

Chapter 7:

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Appendix 1:

Overexpression of CHP1 and NHE1 regulation

A.1 Introduction

The hydrophilic carboxy terminal domain of NHE1 is approximately 315 amino acids and is responsible for the regulation of NHE1 ion exchange. This domain is a site of many protein and cofactor interactions as well as phosphorylations. This is discussed in detail in section 1.4. Calcineurin homologous protein (CHP) is a phosphoprotein that has been shown to interact with the cytosolic tail of NHE1 and affect NHE1 regulation.

CHP exists in two isoforms, 1 and 2, which share 61% amino acid identity (1,2). CHP possesses four EF-hand motifs, although only EF 3 and 4 bind and coordinate Ca^{2+}. Mutation of EF 3 or 4 results in reduced NHE1 activity and mutation of both EF 3 and 4 greatly decreases binding to NHE1 (3). CHP1 is a ubiquitously expressed protein and has been found in the myocardium. CHP1 has been shown to bind to NHE1 between amino acids 510-530 and mutations that inhibit binding of CHP1 to NHE1 results in decreased NHE1 activity (4,5). This suggests that CHP1 is an essential cofactor of NHE1. A number of other studies characterized the effect of CHP overexpression on NHE1, though the results are not conclusive. Studies in the CCL39 cell lines showed that overexpression of CHP1 resulted in the inability of NHE1 to be stimulated by serum (6). More recent studies showed that, in chicken B lymphoma DT40 cells, a deficiency of CHP1 resulted in destabilization of the NHE1 protein, both in expression and plasma membrane targeting (7). Reintroduction of CHP1 resulted in NHE1 protein and plasma membrane targeting being restored (7). Therefore, the role of

CHP1 in NHE1 regulation is controversial; however, there have been no studies examining NHE1 regulation by CHP1 in the myocardium.

CHP2 is highly expressed in tumor cells and the association of CHP2 with NHE1 was shown to protect cells from serum deprivation-induced death (2). Thus, it is proposed that the NHE1-CHP2 interaction maintains tumor cells in a malignant state. The role of CHP2 in the myocardium remains unknown, although CHP2 mRNA has been detected (2).

The role of CHP1 in the myocardium is unclear. Our laboratory has shown that the CHP1 protein is present in the myocardium (unpublished data). We therefore decided to examine the role of CHP1 in NHE1 regulation. To examine overexpression of CHP1 in myocardial cells we constructed an adenovirus which expresses CHP1 (described in section 2.7). We then examined the activity of NHE1 in cardiomyocytes overexpressing CHP1 as well as overexpressing both CHP1 and NHE1. Our present studies found that NHE1 activity was not increased with overexpression of CHP1.

A.2 Results

A.2.1 Production of adenovirus expressing CHP1

Production of recombinant adenoviruses is a technique that involves transferring the cDNA of interest (CHP1) into a series of vectors, followed by production of adenovirus in HEK293A cells. This is described in detail in section 2.1-2.4 as well as chapter 3. We used the same techniques as we had for adenovirus production with NHE1 expression but now inserted the C-terminal V5 tagged CHP1 gene instead (described in 2.7). CHP1 was initially cloned into the pAdTrack-CMV vector to begin adenoviral construction. For this construct, we confirmed that the clone was being expressed in this vector by transfecting AP-1 cells, and Western blotting was used to confirm that the CHP1 protein was being expressed (data not shown). We were able to differentiate endogenous from exogenous CHP1 by their different molecular weights. The exogenous CHP1 contains a C-terminal V5 tag, its molecular weight is approximately 24 kDa, whereas endogenous CHP1 is approximately 22 kDa. Additionally, all pAdTrack-CMV constructs were sequenced for the entire CHP1 gene to confirm its identity.

A.2.2 Overexpression of CHP1 in isolated cardiomyocytes

We examined the expression of CHP1 from the adenoviral vector in isolated cardiomyocytes. Isolated cardiomyocytes were made from neonatal rat hearts as described in section 2.7.2. Equal numbers of cardiomyocytes were then either uninfected (Mock), or infected with an MOI of 20 for 24 hours with the

following adenoviruses: adenovirus expressing the previously described HA-tagged inhibitor resistant NHE1 protein (Chapter 3), adenovirus expressing GFP protein alone, adenovirus expressing CHP1 or a combination of two adenoviruses expressing both CHP1 and inhibitor resistant NHE1. Cell lysates were made and proteins separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane and Western blot analysis was performed as described in section 2.7.2. Western blot of cell extracts blotted with anti-HA antibody was performed to detect exogenous inhibitor resistant NHE1 (upper panel Figure A.1). Western blot analysis of cell extracts blotted with anti-CHP1 antibody (lower panel Figure A.1) was used to detect both endogenous and exogenous CHP1. These studies showed that we were able to detect exogenous NHE1 when cardiomyocytes were infected with adenovirus expressing inhibitor resistant NHE1, as was previously shown in chapter 3. Furthermore, we detected overexpression of exogenous CHP1 when cardiomyocytes were infected with adenovirus expressing CHP1 that was V5 tagged. This was noted by its larger molecular weight of approximately 24 kDa compared to endogenous CHP1 of 22 kDa (1). Therefore, we were able to detect both exogenous NHE1 and CHP1 in isolated cardiomyocytes.

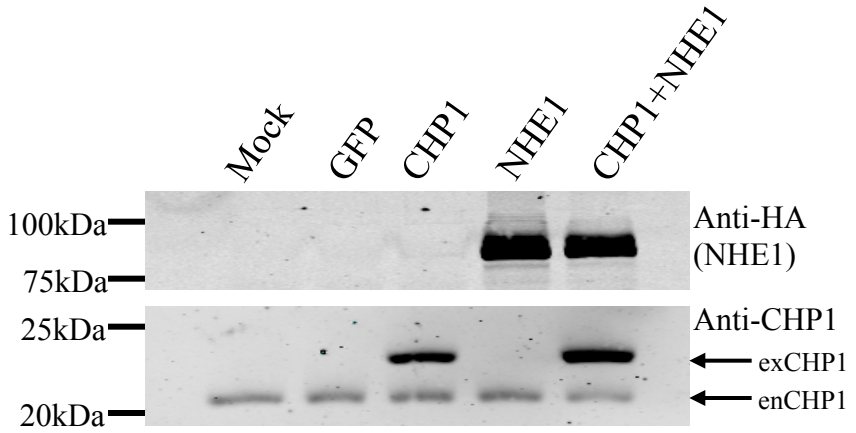

Figure A.1 CHP1 overexpression in isolated cardiomyocytes. Isolated cardiomyocytes were made from neonatal rat hearts as described in section 2.7.2. Equal numbers of cardiomyocytes were then either uninfected (Mock), or infected with a MOI of 20 for 24 hours with the following adenoviruses: NHE1, containing the HA-tagged inhibitor resistant NHE1 protein; GFP indicates cell extracts from isolated cardiomyocytes infected with adenovirus expressing GFP protein alone.;CHP1 indicates cell extracts from isolated cardiomyocytes infected with adenovirus containing CHP1; CHP1+NHE1 indicates adenoviral infection with both adenoviruses containing NHE1 and CHP1. Upper panel, western blot of cell extracts blotted with anti-HA antibody. Lower panel, western blot of cell extracts blotted with anti-CHP1 antibody. Arrows indicate either endogenous CHP1 (enCHP1) or exogenous CHP1 (exCHP1). ExCHP1 has a slightly larger molecular weight as it has a C-terminal V5 tag.

A.2.3 Buffering capacity of myocardial cells overexpressing CHP1

Isolated cardiomyocytes were infected with adenovirus with or without the CHP1 or NHE1 cDNA insert. Over 99% of the cells appeared to be infected as shown by fluorescence of the GFP marker protein. Previous work with adenoviral infected cardiomyocytes showed that fluorescence from the GFP marker protein was only a small fraction of that of BCECF and did not interfere with the measurement of intracellular pH (Chapter 4 section 4.2.2).

Our earlier studies examining the buffering capacity of cardiomyocytes infected with adenovirus found that there was no difference between cells infected with GFP alone or NHE1 inhibitor resistant adenoviruses (Chapter 4 section 4.2.2, and Figure 4.2). However, we needed to examine whether buffering capacity was altered with overexpression of CHP1. Therefore, we examined the buffering capacity of isolated cardiomyocytes infected with the following adenoviruses: GFP infected, CHP1 infected, inhibitor resistant NHE1 infected, and the combination of dual infected cells with both CHP1 and inhibitor resistant NHE1 expressing adenoviruses. Assays were performed as described in materials and methods section 2.5.10. Ammonium chloride (NH_4Cl) was added in a stepwise gradient with different concentrations. Beginning with 30 mM NH_4Cl then 20 mM, 15 mM, 10 mM, 5 mM, 1 mM, and 0. Each NH_4Cl solution was allowed to equilibrate for 30 seconds at 37°C. Following stepwise NH_4Cl treatment, cells were equilibrated in a three-step pH calibration, using Na^+-free calibration buffer. Buffering capacity (*B* in mM/pH unit) was estimated as the amount of acid loaded divided by the observed change of cell pH produced by this load (as described

earlier (8)). Values of *B* were determined at various pH*i* by varying the amounts of NH_4Cl and then plotted with buffering capacity, *B* versus pH. Table VIII shows the buffering capacity equations and R^2 values that were derived from the plots of β vs. pH. Our results indicate that buffering capacity was similar in all treatments and thus overexpression of CHP1 did not alter the intracellular buffering capacity of the cardiomyocytes.

Cardiomyocyte Conditions	Buffering Capacity Equation	R^2
Uninfected	$y= 8.625x^2 - 128.26x + 497.63$	0.3924
GFP Infected	$y= 9.0602x^2 – 141.12x + 549.19$	0.3075
CHP1 Infected	$y= 7.285x^2 – 115.11x + 454.48$	0.5123
NHE1 Infected	$y= 10.691x^2 – 165.46x + 638.19$	0.7202
CHP1 and NHE1 Infected	$y= 8.2732x^2 – 128.27x + 495.25$	0.5358

Table A1: Buffering capacity of cardiomyocytes overexpressing CHP1. The following table is a summary of the buffering capacity equation and R^2 values as described in section 2.7.4. Equal numbers of cardiomyocytes were either uninfected or infected with an MOI of 20 for 24 hours with the following adenoviruses: GFP infected, indicates isolated cardiomyocytes infected with adenovirus expressing GFP protein alone; CHP1 infected indicates isolated cardiomyocytes infected with adenovirus containing CHP1; NHE1 infected indicates adenovirus containing the HA-tagged inhibitor resistant NHE1 protein; CHP1 and NHE1 infected indicates adenoviral infection with both adenoviruses containing NHE1 and CHP1.

A.2.4 Proton flux by NHE1 in cardiomyocytes overexpressing CHP1

The effect of overexpression of CHP1 on NHE1 activity was analyzed by measuring intracellular pH (pH*i*) as described in section 2.5.4. Single pulse assays were performed for the following treatments: GFP, CHP1, NHE1, and CHP1/NHE1 infected cardiomyocytes (MOI 20 for 24 hrs).

Although our previous studies demonstrated that buffering capacity of cardiomyocytes overexpressing CHP1 was not markedly different from controls, we chose to examine proton flux, which takes into account any alterations in buffering capacity between the various treatments (calculations described in section 2.7.5). Briefly, proton flux is the measurement of the movement of protons out of the cell. Calculation of proton efflux is as follows: Buffering capacity, *B* (mM/pH units) x ΔpH/Δtime (min) and results in mM protons/Δtime (min). Figure A.2 is the graph of proton flux versus intracellular pH for the various treatments indicated in the figure legend. There was no marked change in proton flux between the various treatments. Therefore, overexpression of CHP1 did not alter NHE1 activity above the level of the controls.

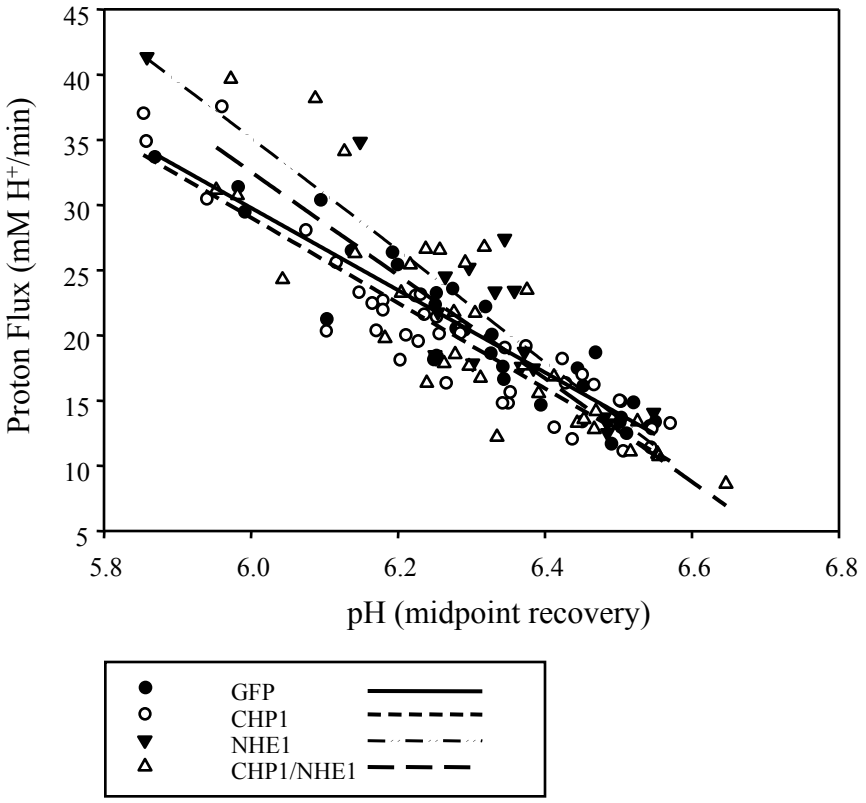

Figure A2 Proton efflux of cardiomyocytes overexpressing CHP1. Isolated cardiomyocytes were infected for 24 hrs with the following adenoviruses at an MOI of 20: GFP, adenovirus expressing GFP; CHP1, adenovirus expressing CHP1; NHE1, adenovirus expressing inhibitor resistant NHE1; and the combination of dual infected cells with both CHP1 and NHE1 expressing adenoviruses. Single pulse assays were performed for the following treatments and expressed as ΔpH/Δtime (min). Proton efflux (JH^+) was calculated by Buffering capacity, *B* (mM/ pH units) multiplied by ΔpH/Δtime (min) and results in mM protons/Δtime (min). Above is the graph of proton efflux with JH^+ on the Y axis and pH*i* of the midpoint recovery of NHE1 activity on the X axis. The figure legend indicates the graph for each treatment.

A.3 Discussion

CHP1 is a phosphoprotein that interacts with the cytoplasmic tail of NHE1 and regulates NHE1 activity. Previous studies have shown that altering CHP1 binding to NHE1 results in decreased NHE1 activity (4,5). CHP1 has been shown to be expressed in the myocardium; however, there have been no studies of CHP1-mediated regulation of NHE1 in myocardial cells. Therefore, we examined the role of CHP1 in isolated cardiomyocytes. To introduce CHP1 into cardiomyocytes, we constructed an adenovirus, which contained the CHP1 gene. The use of adenovirus for overexpression of cellular proteins in isolated cardiomyocytes has been well documented. Furthermore, we have characterized NHE1 overexpression in cardiomyocytes using this method in previous studies (Chapter 3) (9).

Western blot analysis of isolated cardiomyocytes infected with adenovirus overexpressing CHP1 revealed that we were able to detect CHP1 overexpression. Furthermore, we could differentiate between exogenous CHP1 (from the adenovirus) and endogenous CHP1 by their slight differences in molecular weight. We then examined NHE1 activity in cardiomyocytes overexpressing CHP1.

Our present study showed that overexpression of CHP1 did not alter NHE1 activity above the level of the controls. This was a surprising result as we had hypothesized that CHP1 overexpression would increase NHE1 activity. We postulate that there may be a minimal amount of CHP1 that is required for NHE1 activity and anything beyond that does not alter NHE1 activity. This would

explain why overexpression of the protein had no effect on NHE1 activity. We then hypothesized that if we overexpressed both CHP1 and NHE1 we would see an increase in NHE1 activity, however we did not. Thus even enlarging the pool of available NHE1 along with overexpression of CHP1 did not increase NHE1 activity. Studies in chicken B lymphoma DT40 cells revealed that CHP1 has an important role in NHE1 protein stabilization (7). Matsushita *et al*. found that CHP1 depletion resulted in significant reduction of NHE1 protein, and this was not the result of decreased mRNA levels but rather likely caused by alteration of post-translational processes (7). It is plausible that overexpression of CHP1 may also be altering the stabilization of the NHE1 protein and its plasma membrane targeting.

Our studies were unable to show a relationship between overexpression of CHP1 and NHE1 activity. In future studies we will use an adenovirus expressing siRNA for knockdown of CHP1 in cardiomyocytes and then test for the effect of the absence of CHP1 in cardiomyocytes on NHE1 expression and activity. Other studies may identify and examine the specific amino acids of CHP1, which are phosphorylated, and identification and characterization of the kinases and phosphatases, which regulate CHP1 phosphorylation. Together, these studies will provide valuable information on the regulation and role of CHP1 in NHE1 activation.

A.4 Future Directions

CHP1 is a phosphoprotein, which has been shown to bind to NHE1 and inhibit its activity. Future studies will examine the role of CHP1 in NHE1 regulation. Our laboratory has developed antibodies against CHP and found that CHP1 is present in the myocardium (unpublished data). We have constructed an adenovirus which expresses CHP1 for use in neonatal rat ventricular cardiomyocytes in order to study the effects of overexpression of CHP1 on NHE1 activity. Our studies to date have shown that we are unable to detect any changes in NHE1 activity with overexpression of CHP1 in isolated neonatal rat cardiomyocytes (Chapter 6). Therefore in future studies we will take the opposite approach. We will knockdown CHP1 in cardiomyocytes and then test NHE1 activity. To do this we will utilize siRNA against the CHP1 transcript. Once the appropriate siRNA has been tested in non-myocardial cells to knockdown CHP1, an adenovirus could then be produced containing the cDNA of the siRNA. The use of adenoviruses as a tool to introduce cDNA into primary cell cultures of cardiomyocytes has been widely studied and used. We will then be able to test NHE1 activity in isolated cardiomyocytes in which the CHP1 protein has been knocked down. Additionally, we will examine the expression of NHE1 in myocardial cells when CHP1 expression is knocked down, as previous studies have shown in non-myocardial cells that depletion of CHP1 results in decreased NHE1 expression (7). Other studies will also examine the effect of CHP1 knockdown in the presence of NHE1 overexpression by co-infection with an

adenovirus expressing NHE1. These studies would complement our previous studies and pinpoint the role of CHP1 in NHE1 regulation in the myocardium.

Earlier studies in non-myocardial cells have shown that mutation of the CHP1 binding site in the cytosolic tail of NHE1 results in decreased NHE1 activity (3). CHP1 is a phosphoprotein and therefore future studies should include the identification of the specific sites of phosphorylation on CHP1, and how mutation of these phosphorylation sites affects NHE1 activity. We could use the same adenovirus system as was used for analysis of phosphorylation site mutations in NHE1. Furthermore, it would be important to identify and characterize the kinases and phosphatases, which regulate CHP1 specific phosphorylation. Together, these studies will provide valuable information on the regulation and role of CHP1 in NHE1 activation in the myocardium.

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