The Regulation of Na^+/H^+ Exchanger Isoform 1 in Kidney Cells

by

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Abstract

The mammalian Na+/H+ exchanger isoform 1 (NHE1) is a ubiquitous plasma membrane protein that regulates intracellular pH by removing a proton in exchange for extracellular sodium. NHE1 is expressed in many tissues including the kidney where it has been demonstrated to play essential roles in pH and cell volume regulation. It has also been implicated in some pathological conditions of the kidney including ischemia and chronic kidney diseases. NHE1 has a large 315 amino-acid cytosolic regulatory domain that regulates the catalytic membrane domain. The cytosolic domain is mainly regulated by phosphorylation and protein interaction. This study examined how these regulatory mechanisms regulate NHE1 in kidney cells. The activation of NHE1 in myocardial cells by sustained intracellular acidosis (SIA) was shown to be mediated by ERK1/2 phosphorylation. In this study, we also demonstrate that SIA stimulates NHE1 activity in Mardin-Darby Canine Kidney (MDCK) cells. To characterize how SIA stimulates NHE1 in kidney cells, wild-type and mutant NHE1 cDNAs were stably expressed in MDCK cells and examined for activation and phosphorylation in response to SIA. All the cDNAs had a L163F/G174S mutation, which conferred a 100-fold resistance to EMD87580, an NHE1-specific inhibitor. This allowed us to assay exogenous NHE1 activity while inhibiting endogenous activity with EMD87580 and while inhibiting the NHE3 isoform of the Na^+/H^+ exchanger using the isoform-specific inhibitor S3226. We demonstrated that the amino acids Ser771, Ser776, Thr779, and Ser785 are important for NHE1 phosphorylation and activation after acute SIA. SIA also activated ERK-dependent pathways in MDCK cells, and this was blocked by treatment with the MEK inhibitor U0126. Treatment with U0126 also blocked activation of NHE1 by SIA. These results

suggest that acute acidosis activates NHE1 in mammalian kidney cells and that in MDCK cells this activation occurs through phosphorylation of a distinct set of amino acids in the cytosolic regulatory tail of NHE1 by ERK1/2. We also examined how protein interaction regulates NHE1 activity. Using affinity chromatography with the C-terminus of NHE1, we determined the NHE1 binding proteins in the kidney which includes 14-3-3 protein, heat shock proteins (Hsp90 and Hsp70) and Na^{+}/K^{+} ATPase. We also confirmed that 14-3-3 and heat shock proteins bind to or regulate NHE1 but could not confirm that $Na^{+/}K^{+}$ ATPase binds to the intact protein. The Hsp90 inhibitor 17-AAG decreased NHE1 activity and NHE1 phosphorylation in MDCK cells but did not decrease protein levels. Additionally, 17-AAG decreased phospho-AKT levels. Direct inhibition of AKT with MK2206 decreased NHE1 activity, though this effect was not additive with the effect of 17-AAG. These results are the first demonstration that in renal cells, NHE1 is associated with several regulatory proteins including Hsp90, and suggest that this interaction affect NHE1 function through altered phosphorylation of the protein via the AKT kinase.

Preface

Chapter 3 of this thesis has been published as Ayodeji Odunewu and Larry Fliegel, "Acidosis-mediated regulation of the NHE1 isoform of the Na^+/H^+ exchanger in renal cells" *American Journal of Physiology – Renal Physiology*, Vol. 305, No. 3, F370-F381. I was responsible for the data collection and analysis and was involved in the manuscript composition. Larry Fliegel was the supervisory author and responsible for concept formation and manuscript composition.

Dedication

To God and family

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List of Abbreviations

17-AAG	17-*N*-allylamino-17-demethoxygeldamycin
AE2	Anion Exchanger 2
AngII	Angiotensin II
BCECF-AM	2’,7-bis(2-carboxyethyl)-5(6) carboxyfluorescein-acetoxymethyl ester
CHPs	Calcineurin Homologous Proteins
CaM	Calmodulin
CAII	Carbonic Anhydrase II
CaMKII	Ca^{2+}/Calmodulin-dependent protein Kinase II
CCD	Cortical Collecting Duct
CD	Circular Dichroism
DMEM	Dulbecco’s Modified Eagle’s Medium
DNA	Deoxyribonucleic Acid
DSP	Dithiobis[succinimidylpropionate]
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ERK1/2	Extracellular signal-regulated kinases ½
ERM	Ezrin/Radixin/Moesin
FBS	Fetal Bovine Serum
GAM	Goat Anti-Mouse antibody
GST	Glutathione-S-transferase
HEK293	Human Embryonic Kidney Cells
HEPES	N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid
HGF	Hepatocyte Growth Factor
Hsp70	Heat shock protein 70
Hsp90	Heat shock protein 90
IPTG	Isopropyl ß-D-thiogalactopyranoside
IRI	Ischemia-Reperfusion Injury
Jak2	Janus Kinase
LC-CoA	Long-chain acyl-CoA
LPA	Lysophosphatidic Acid
MDCK	Mardin-Daby Canine Kidney
MEK1/2	MAPK/ERK Kinase
MTAL	Medullary Thick Ascending Limb
mTOR	Mammalian Target of Rapamycin
NGF	Nerve Growth Factor
NHEs	Na^+/H^+ Exchanger
NIK	Nck-interacting kinase

NMR	Nuclear Magnetic Resonance
p38MAPK	p38 mitogen-activated protein kinase
p90RSK	p90 ribosomal S6 kinase
PBS	Phosphate Buffer Saline
PCRB	C-Terminal of rabbit NHE1
PDGF	Platelet-derived growth factor
PIP_2	Phosphatidylinositol 4,5-biphosphate
PKA	Protein kinase A
PKB/Akt	Protein Kinase B
PKC	Protein Kinase C
PKD	Protein kinase D
PKD	Polycystic kidney disease
PI3K	Phosphoinositide-3-kinase
PMSF	Phenylmethylsulfonyl Fluoride
PP1	Protein Phosphatase 1
PP2A	Protein phosphatase 2A
PPAR	Peroxisome Proliferator-Activated Receptor
PTC	Proximal Tubule Cell
RIPA	Radioimmunoprecipitation Assay
ROCK	Rho Kinase
RVI	Regulatory Volume Increase
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrphoresis
Sgk1	Serum-and glucocorticoid-inducible Kinase
SHP2	Src homology 2 domain-containing protein tyrosine phosphatase
SIA	Sustained Intracellular Acidosis
TAL	Thick Ascending Limb
TM	Transmembrane segment
TBS	Tris Buffer Saline

Chapter 1

Introduction

1.1 Intracellular pH regulation in mammalian cells

The strict regulation of intracellular pH is of great importance, particularly to many cellular processes and enzyme activities with optimum pH within the physiological pH range. Most cells are subjected to acid influx from metabolic acid production and acid leakage from cellular organelles. However, changes in internal pH, even comparatively small ones, are capable of inducing dramatic and often adverse effects on cellular activities. Generally, within physiological range, metabolic activities increase with increasing pH*i* and vice versa (1).

A predominant mechanism of pH*i* regulation is through the activities of membrane-bound transporters, which include cation-H^+ exchangers, HCO_3^--dependent transporters and proton pumps (2). Mammalian Na^+/H^+ exchangers (NHEs) are a family of cation-H^+ exchangers that catalyze the exchange of one intracellular proton (H^+) for an extracellular sodium ion (Na^+).

1.2 Mammalian Na^+/H^+ exchangers

The Na^+/H^+ exchanger (NHE) is a membrane transport protein present in virtually all living organisms. It primarily functions to protect cells from intracellular acidification by catalyzing the electroneutral exchange of one intracellular H^+ for an extracellular Na^+.

Ten isoforms of NHE are currently known to exist (NHE1-10) with distinct tissue expression, cellular localization and physiological roles (3). Based on their subcellular localization, NHE1-5 are classified as plasma membrane proteins while NHE6 – 9 are the organellar NHEs being present in intracellular membranes such as Golgi (4). The more recently identified NHE10 appears to be exclusively expressed in osteoclasts (5). Sperm-

specific (sNHE) and testis-specific (mtsNHE) NHEs have also been reported and are thought to be involved in sperm motility and fertility (6,7).

The SLC9 gene family encodes mammalian NHEs and is generally made up of three subgroups – SLC9A, SLC9B and SLC9C. The isoforms NHE1-9 are encoded by the SLC9A subgroup and share a primary sequence identity of between 25 – 70% (8). sNHE and NHE10 (also known as NHA2), are encoded by *SLC9B1* and *SLC9B2* respectively; while mtsNHE is encoded by *SLC9C1* (9).

All NHEs are structurally similar, being made up of a N-terminus 12 transmembrane segment where ion exchange occurs, and an intracellular C-terminal domain that regulates the exchange activity. In addition, while NHEs function as monomers, they also appear to dimerize and this is thought to be important for the stability of the molecule (10).

1.2.1 The SLC9A Family

The evidence of cation/H^+ exchange was first demonstrated in liver mitochondria, in the 1960s by Peter Mitchell (11,12). Subsequently, a Na^+/H^+ exchange system was analyzed and described in the small intestine and proximal tubule of the kidney (13). Further studies by Pouyssegur *et al*. identified an amiloride-sensitive and growth factor-activated Na^+/H^+ exchange system that is involved in intracellular pH regulation (14). In 1989, the first NHE isoform, NHE1, was cloned (15).

NHE1 is often referred to as the "housekeeping" isoform being present in almost all mammalian cells with the exception of the macula densa and intercalated cells of the kidney (16). There have also been reports of the lack of NHE1 in certain subpopulations

of the brain (17). While NHE1 is almost always exclusively expressed on the plasma membrane, localization within the plasma membrane differs from one cell type to another. For example, in polarized epithelial cells, NHE1 is localized to the basolateral membrane with the exception of choroid plexus epithelia, where it is expressed in the apical membrane (18,19). In the myocardium, it is concentrated in the intercalated disks and transverse tubules while in fibroblasts it is localized to the lamellipodia (18,20-22). NHE1 is the most sensitive isoform to NHE inhibitors, amiloride and its derivatives and is even more sensitive to benzoylguanidine inhibitors (reviewed in (23)).

NHE2 is also expressed in several tissues including the gastrointestinal tract, kidney, skeletal muscle, heart, brain, uterus, testis, and lung (24). In polarized epithelial cells, it localizes to the apical membrane (25-27). While no overt disease phenotype was observed in NHE2 null mice, it appears to be important for the viability of gastric parietal cells, though not essential for net acid secretion in these cells (28). Absorptive defects have also been reported in NHE2-null cells in the kidney, colon and pituitary (29-31).

NHE3 and NHE4 are both relatively resistant to inhibition by amiloride and its derivatives. While NHE3 is highly expressed in the gastrointestinal tract and kidney, NHE4 is predominantly expressed in the stomach (32). When expressed in epithelial cells, NHE3 localizes to the apical membrane while NHE4 localizes to the basolateral membrane (33,34). NHE5 has 73% identity to NHE3 but is mainly expressed in non-epithelial cells such as the brain, spleen, testis and skeletal muscle (35).

NHE6-9 are intracellular NHE isoforms that are important for maintaining organellar pH. They are distributed across the Golgi network and recycling endosomes (36). NHE6 is localized in the recycling endosomes and is highly expressed in the brain,

skeletal muscle and heart (37,38). NHE7 localizes to the *trans*-Golgi network and predominantly expressed in the brain, skeletal muscles and secretory tissues (39). NHE8 is localized to the mid- and trans-Golgi and is expressed in the kidney, intestine and skeletal muscle and liver (36). Of particular note is its localization to the apical membrane of polarized epithelial cells of the proximal tubule and intestine (40,41). NHE9 localizes to late recycling endosomes and is fairly ubiquitous in tissue expression (4,36).

1.3 Structural Features of NHE1

NHE1 is made up of 815 amino acids separated into two domains: an N-terminal trans-membrane (TM) domain where ion transport is catalyzed and where pH sensing occurs, and a C-terminal cytosolic domain that regulates the ion transport activity.

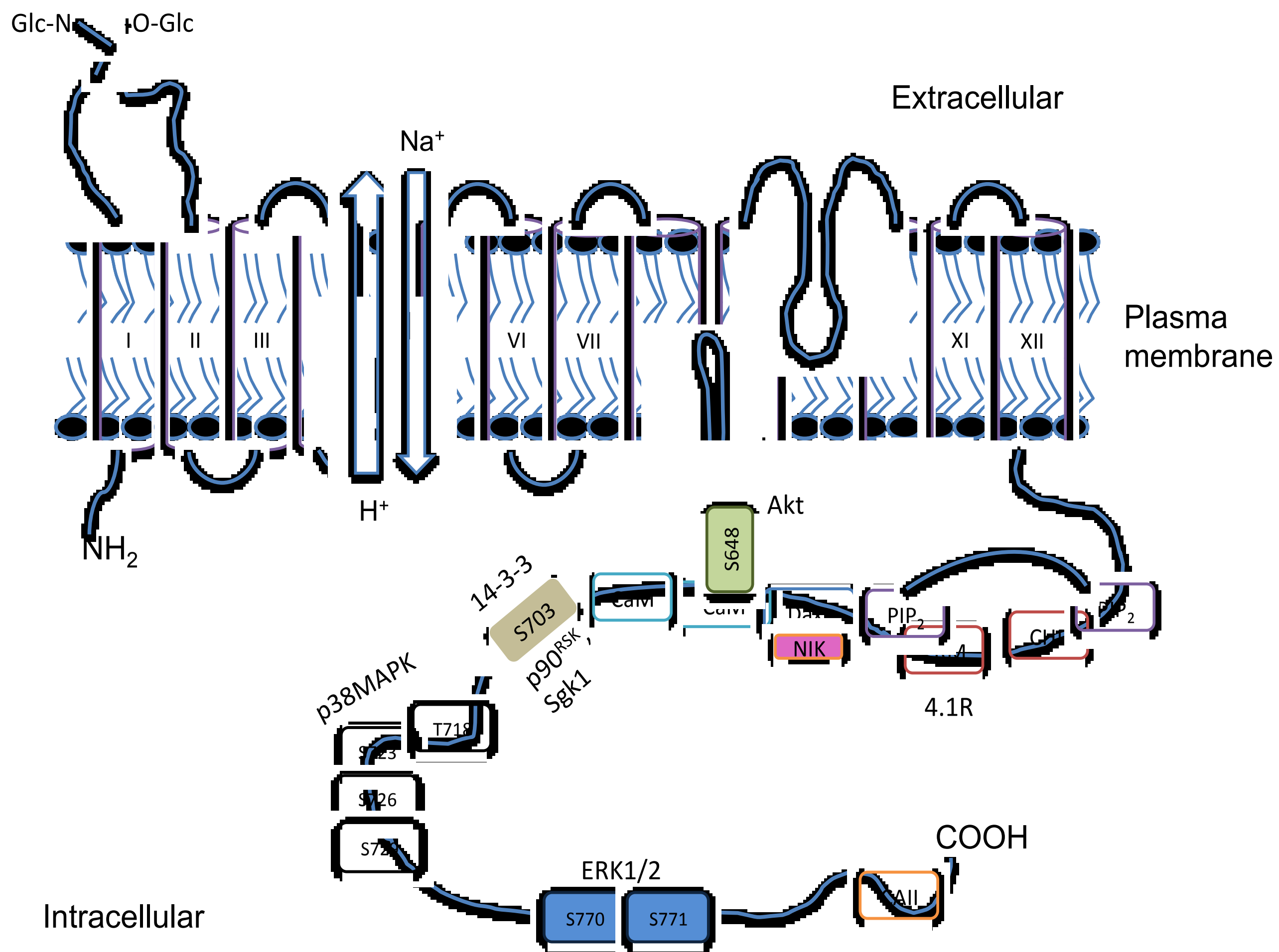

Figure 1.1:Schematic model of NHE1 structure: NHE1 consists of two main domains: the 12-transmembrane segment domain where ion exchange takes place (TMI-TMXII) and the regulatory cytoplasmic domain. The cytoplasmic region is phosphorylated by various kinases and interacts with a number of proteins that modulate NHE1 activity at the plasma membrane. Phosphorylation sites are represented by the amino acid abbreviation and residue numbers. Regulatory proteins are illustrated at approximate locations of action including calcineurin homologous proteins (CHP), calmodulin (CaM), carbonic anhydrase II (CAII), extracellular signal regulated protein 1/2 (ERK1/2), ezrin/radixin/moesin (ERM), Nck-interacting kinase (NCK), p38-mitogen-activated protein kinase (p38MAPK), phosphatidylinositol 4,5-biphosphate (PIP_2), protein kinase B (Akt/PKB), ribosomal protein S6 kinase (p90RSK), serum- and glucocorticoid-inducible kinase (Sgk1).

Although the complete structure of NHE1 has yet to be determined, models of the topology of NHE1 have been proposed. Using substituted cysteine accessibility analysis, a 12-TM model with a glycosylation site between TM1 and TM2 was proposed by Wakabayashi *et al*. (42). Subsequently, a slightly different model was proposed by Landau *et al.*, which also had 12 TM segments, but excludes the first two TM segments presented in the Wakabayashi model. The N-terminus was suggested to contain a signal sequence with a possible cleavage site occurring just behind TM1 (43). Although this model had its proponents, it has been demonstrated that glycosylation is seen consistently in immunoblots of NHE1 (44). In addition, the only N-linked glycosylation site is present on the first extracellular loop, consistent with the Wakabayashi model, and glycosylation of NHE1 has been suggested to play a role in targeting mature NHE1 to the basolateral membrane of polarized epithelial cells (45). Furthermore, our lab has been able to demonstrate that the Wakabayashi model is much more representative of the NHE1 structure by confirming that the amino acid residues 1 – 127 indeed form the first 2 TM segments of the protein (46). Further studies are however still required in deciphering the correct structure of NHE1.

The specific features of the NHE1 structure (see Figure 1.1) are described as follows:

1.3.1 N-terminal tail

The short N-terminal tail of NHE1 is composed of 15 amino acids with no known function but it probably helps to anchor TM1 in the membrane (47).

1.3.2 The Transmembrane domain

The 12-transmembrane segment is made up of about 485 amino acids with the TMs being joined together by short intracellular and extracellular loops. Three re-entrant loops between TMIV-V (IL1), TMVIII-IX (IL4) and TMIX-X (EL5), were also identified (42).

While solving the complete structure of mammalian NHE1 still remains elusive, the Fliegel lab has given several reports on the NMR structure of the individual transmembrane segments, which have provided some insight on the structure and function of the protein.

The NMR structure of TMIV reveals three distinct regions made up of a series of β turns at the N-terminal, an extended central region and a helical C-terminal region (48). Further functional studies showed that TMIV is a major determinant of the sensitivity of NHE1 to inhibitors and contains residues that line the ion-transport pore (48-50). In addition, some amino acid residues in TMIV have been shown to be critical for NHE1 expression, membrane targeting and function (51,52).

TMVI consists of two helical segments surrounding a short central region. It also contains pore-lining residues that may be important for NHE1 activity (53,54). The structure of TMVII features an "interrupted helix" with amino acid residues that line the ion-transport pore of NHE1 and are crucial for its function and drug sensitivity (55,56). Structural studies of the TMVI-VII pair portrayed similar structures to the individual TM segments except that TMVII was a straight α-helix with no kinks. TMVII has actually been shown to alternate between the two reported conformations, that is, a kinked helix and a straight helix (57). Tryptophan scanning mutagenesis of the TM pair also revealed

that mutations, particularly at V240-V245 of TMVII, abolished NHE1 activity, which was not due to a lack of expression or mis-targeting. This suggests that these TM segments are closely associated and their interaction is crucial for NHE1 function (58).

TMIX has two helical regions, at right angle to each other, surrounding a kink at Ser351 (59). It also contains pore-lining residues and may be involved in NHE1 drug sensitivity (59,60). TMXI consists of two helical regions connected by a flexible segment. It is important for proper expression, targeting and function of NHE1 and possesses a pore-lining residue (61).

1.3.3 The Carboxy-terminal Domain

While the complete structure of the C-terminal domain remains unsolved, circular dichroism (CD) had been able to provide some preliminary information on the structure of the region. CD analysis suggests that the C-terminal domain is 35% α-helix, 16% β-turns and 49% random coil (62). Subsequent studies on the distal region of the C-terminus suggested it has a conformation that is affected by pH and calcium concentration; and contained more β-sheets and turns with little helical content (63). NMR studies also shows that this region is intrinsically disordered and may play a role in NHE1 trafficking (64).

1.4 Regulation of NHE1

Normally quiescent, NHE1 is activated in response to specific stimuli such as growth factors, hormones, osmotic stress, and intracellular acidosis. This occurs via

phosphorylation of the cytoplasmic domain and by phosphorylation-independent mechanisms such as the binding of various regulatory proteins (65).

1.4.1 Growth Factor and Hormonal Regulation of NHE1

Various hormones and growth factors regulate NHE1 activity and these include angiotensin II, thrombin, endothelin-1, α-adrenergic agonists, lysophosphatidic acid (LPA), epidermal growth factors (EGF) and insulin (65,66). About half of the growth factor and hormonal regulation of NHE1 is mediated by phosphorylation while the remaining half is mediated by the binding of regulatory proteins and co-factors to the cytosolic domain (67).

Angiotensin II has been shown to increase NHE1 activity in rat ventricular myocytes and distal nephron cells. This stimulatory action occurs via the AT1 receptor while the AT2 receptor mediates an opposing counteracting inhibition of NHE1 (68,69). The ionotropic effect of endothelin in various cell types has also been partly attributed to its stimulation of NHE1 activity (70-72). α_1-Adrenergic agonists, such as phenylephrine, stimulate NHE1 activity by shifting its pH*i* sensitivity to a more alkaline range. This is mediated by the α_1-Adrenoceptor subtype and requires the activation of the ERK pathway and PKC in myocytes and nephron cells respectively (73-75). Activation of NHE1 activity by thrombin (76), LPA (77,78) and EGF (79-81) have been reported in various cell types. Insulin has also been shown to stimulate NHE1 activity although an inhibitory effect has also been reported in endothelial and smooth muscle cells (82-84).

1.4.2 Phosphorylation

Phosphorylation of the cytoplasmic domain as a mechanism of NHE1 regulation was first identified by Sardet *et al.* (85) and occurs at the 180-amino acid distal region of the carboxyl terminal tail. It accounts for about half of the growth factor-induced regulation of NHE1 (67).

Several protein kinases have been shown to stimulate NHE1 activity by direct phosphorylation of the C-terminal domain. First to be identified was Ca^{2+}/calmodulin-dependent protein kinase II (Cam kinase II), which directly phosphorylates NHE1 *in vitro* (86). The ras-homolog A1 (Rho) kinase (ROCK) also directly phosphorylates NHE1 and plays a key role in cytoskeletal organization, cell migration and metastatic invasion (77,78,87).

The MAPK signalling pathway, particularly ERK1/2, has been shown to play an important role in the regulation of NHE1. Inhibition of the ERK1/2 signalling cascade significantly reduced growth factor-induced NHE1 activation in Chinese hamster fibroblast cells (88). Studies from our laboratory have also demonstrated that ERK1/2 and its downstream target, $p90^{RSK}$, directly phosphorylate and activate NHE1 in healthy rat myocardium (89). Additional studies in vascular smooth muscle cells showed that $p90^{RSK}$ phosphorylates NHE1 at Ser703 with demonstrated implications in cardiac ischemia-reperfusion injury and ischemic neuronal cell death (90,91). Interestingly, the serum- and glucocorticoid-inducible kinase has also been shown to phosphorylate myocardial NHE1 at Ser703 (92).

Stimulation of NHE1 activity in isolated cardiomyocytes and kidney cells by sustained intracellular acidosis (SIA) is mediated by the activation of ERK 1/2 (93,94).

Using mass spectrometry, our laboratory identified the phosphorylation sites for ERK 1/2 in NHE1 (95). These were grouped into four regions as follows: 1, Ser693; 2, Thr718, Ser723/726/729; 3, Ser766/770/771; and 4, Thr779, Ser785 (96). Subsequent studies showed that the activation of NHE by SIA is mediated by the ERK1/2 phosphorylation of region 3 in cardiomyocytes; and by Ser771 and region 4 in kidney cells (94,97).

Stress activated kinases such as p38MAPK and Nck-interacting kinase (NIK) have also been shown to regulate NHE1 activity. In vascular smooth muscle and human proximal tubule cells, p38MAPK negatively regulates NHE1 activity via a signaling pathway that also involves ERK1/2 (98,99). In lymphocytes however, it activates NHE1-induced alkalinization during apoptosis by phosphorylating Ser726 and Ser729 (100,101).

Protein kinase B (PKB or Akt) has also been identified as a regulatory kinase of NHE1. It phosphorylates NHE1 at Ser648 and is the only kinase known so far to have an inhibitory effect on cardiac NHE1 by blocking the calmodulin binding site (102). However, it appears to have a stimulatory effect on NHE1 in fibroblasts and renal epithelial cells where it promotes cell proliferation and cell migration (83,103,104). Protein Kinases A (PKA), PKC and PKD do not phosphorylate NHE1 directly but rather, regulate NHE1 activity indirectly (65,105).

Although the phosphorylation of NHE1 has been well studied, there has been much less work on the requisite dephosphorylation of NHE1. Protein phosphatases 1 and 2A (PP1 and PP2A) have been suggested to directly associate with NHE1 in myocardial cells (106,107). The Src homology 2 domain-containing protein tyrosine phosphatase (SHP-2) has also been shown to associate directly with NHE1 and regulate its activities (108). Conversely, insulin-mediated inhibition of SHP-2 in vascular smooth muscles and

endothelial cells results in a decrease in NHE1 activity (82).

1.4.3 Regulatory proteins of NHE1

A number of proteins have been shown to play significant roles in influencing NHE1 activity by binding to the cytosolic regulatory domain of NHE1. This regulatory mechanism accounts for about 50% of the hormonal regulation of NHE1 (67).

Calmodulin (CaM) is a calcium-binding second messenger protein known to mediate the Ca^{2+}-induced activation of NHE1. The interaction of CaM with NHE1 also mediates NHE1 activation by hormonal and growth factor stimuli (109-111). CaM binds to two affinity sites - a high and intermediate site located at amino acids 636 – 656 and 664 - 684 respectively, on the C-terminal tail of NHE1 (109). It modulates NHE1 activity by binding to the high-affinity region of the NHE1 tail thus preventing this auto-inhibitory domain from acting on the membrane domain (112).

Carbonic anhydrase (CAII) catalyzes the hydration of CO_2 to HCO_3 and H^+. It directly interacts with NHE1 at amino acids 790-802 of the cytosolic domain and modulates its activity in response to NHE1 phosphorylation (113,114). More recently, it was suggested that p90RSK phosphorylation of NHE1, increases CAII binding to NHE1, in response to myocardial stretch, and this constitutes an important component of slow force response (115).

Calcineurin homologous protein (CHP) exists in 3 isoforms - CHP 1, CHP2 and CHP3 (also known as tescalcin). CHP 1 is ubiquitously expressed in mammalian tissues and binds to NHE1 at amino acids 518 – 537 (116). Mutation of the CHP1-binding sites of NHE1 drastically reduced NHE1 activity and also shifted the pH-sensitivity of NHE1 to a more acidic range (117,118). Furthermore, binding of CHP1 to NHE1 is crucial for the stabilization and optimal plasma membrane expression of the exchanger (119,120). CHP2 interacts with NHE1 and this has been suggested to prevent serum deprivation-induced death in malignant tumors (121).

CHP3, sometimes known as tescalcin, binds to the cytosolic domain of NHE1 in a Ca^{2+} dependent manner (122). This association results in conformational changes in the cytosolic tail and promotes maturation and cell surface stability of NHE1 (122,123).

Ezrin, Radixin and Moesin (ERM) are a family of proteins that cross-link actin filaments with plasma membranes. ERM proteins associate directly with NHE1 at two binding motifs between 553-564 and this regulates cell shape, adhesion and motility (22). NHE1-ERM interaction also mediates Akt (PKB) signalling in cardiomyocytes and renal cells (104,124).

Daxx, a death-domain associated protein, translocates to the cytoplasm from the nucleus during ischemic stress, and binds to the ERM-interacting domain of NHE1 to stimulate its transport activity (125).

Heat shock protein (Hsp) 70 interacts with and acts as chaperones for many mammalian proteins. It has been shown to also interact with NHE1 and may be involved in protein folding (126). In lipopolysaccharide-stimulated macrophages and liver, disrupting Hsp70 and NHE1 association reduced the induction of pro-inflammatory factors, thus preventing liver injury (127).

Phosphatidylinositol 4,5-biphosphate (PIP$_2$) regulates the activity of several ion transporters including NHE1. Two PIP$_2$ binding motifs were identified on the cytosolic domain of NHE1 at amino acids 513-520 and 556-564. Mutation of these motifs or depletion of PIP$_2$ significantly reduced NHE1 activity (128). In proximal tubule cells, NHE1 interaction with PIP$_2$ resulting in cytoprotection and disruption of this interaction promotes apoptosis (129,130).

14-3-3 is an adaptor protein that keeps NHE1 in an active conformation after it has been phosphorylated at Ser703 by p90^{RSK} (131).

4.1R protein is a cytoskeletal protein in erythrocytes. Initial studies in mouse erythrocytes lacking 4.1R protein reported an increase in Na^+/H^+ exchange (132). Subsequent studies then demonstrated that 4.1R interacts with NHE1 cytoplasmic domain at the same residues as PIP$_2$. This interaction also appears to be modulated by pH*i*, [Na^+] and [Ca^{2+}] (133). Acidic pH or increased concentrations of Na^+ or Ca^{2+} promote the dissociation of 4.1R from NHE1 and this is thought to permit PIP$_2$ binding to NHE1, resulting in increased NHE1 activity (133).

1.5 Physiological and pathological roles of NHE1

While the primary functions of NHE1 are pH*i* and cell volume regulation, the exchanger also plays crucial roles in various cellular processes including growth, proliferation, differentiation, migration, and apoptosis (briefly described here, see (65,66,90,134) for reviews). One of the earliest evidence of the importance of NHE1 in cell growth was described in mutant cell lines, lacking Na^+/H^+ exchange activity, which failed to grow at neutral and acidic pHs (135). In mice, the genetic ablation of Na^+/H^+ activity resulted in postnatal growth retardation, ataxia and epileptic seizures (136,137).

NHE1 has been shown to be important in the progression of the cell cycle particularly the G_2/M entry and transition (138). In addition, cDNA microarray analysis of cells expressing mutant NHE1 revealed a significant change in the expression profile of genes associated with cell cycle progression, compared to wild type (139). The importance of NHE1 in cell differentiation has also been demonstrated in various cells types (140-143). NHE1 also affects cell migration through a variety of means including pH and cell volume regulation, cytoskeletal anchoring, and interaction with signalling proteins (144).

The role of NHE1 in apoptosis appears to differ depending on cell type. Inhibition of NHE1 in mouse pro-B lymphocytes and cardiomyocytes prevented the progression of apoptosis (101,145). In renal cells however, apoptosis is frequently associated with decreased NHE1 expression and activity; and the inhibition of NHE1 in these cells resulted in apoptosis (99,146-148).

Tumour cells have higher alkaline pH*i* values compared to normal tissues and this is primarily driven by NHE1 activity in these cells (149). NHE1 is constitutively active

in tumour cells and the ensuing dysregulated NHE1 activity has been implicated in tumour growth, cell migration and invasion resulting in metastasis (150,151). Inhibition of NHE1 in leukemic cells sensitizes them to apoptosis (152). Various studies have also shown evidence of the participation of NHE1 in inflammatory responses. Indeed, NHE1 is activated in response to many inflammatory signals and blocking NHE1 activity attenuates inflammatory responses that cause tissue injury (153,154).

Another major pathological role of NHE1 is in ischemia/reperfusion injury (IRI). The reduction in pH*i* during ischemia stimulates NHE1 activity, causing an accumulation of sodium ions in the cell. This leads to the reversal of activity of the Na^+/Ca^{2+} exchanger and results in an increase in intracellular calcium, triggering deleterious pathways that eventually lead to cell death. Support for the involvement of NHE1 in IRI is based on evidence from various studies showing that genetic ablation and/or pharmacological inhibition of NHE1 in animal models offers protection against IRI in the myocardium, neurons and liver (12,155-159). The activation of NHE1 regulatory kinases, in response to ischemia and reperfusion in neurons and myocardium, has also been demonstrated (91,160). In rat models, IRI in the kidney led to an increase in NHE1 expression in the renal cortex and medulla (161).

The activation of NHE1 has also been implicated in cardiac hypertrophy and its inhibition has been shown to reduce the progression of this phenotype (162-164). The involvement of NHE1 in hypertension has also been proposed in various studies (12). For example, overexpression of NHE1 in transgenic mice resulted in salt-sensitive hypertension (165). Furthermore, knockout of the NHE1 gene in mouse models prevented the development of pulmonary hypertension (166). There was also an increase

in the activity and phosphorylation of NHE1 in the proximal tubules of spontaneously hypertensive rat (167).

More recently, human mutations of NHE1 were identified, characterized and implicated in genetic diseases. Homozygous missense mutation of NHE1 at Glycine-305 (G305R) plays a causal role in Lichtenstein–Knorr syndrome, an autosomal recessive disorder characterised by ataxia and hearing loss (168). Glycine 305 is highly conserved in the eighth TM segment of NHE1. Analysis of the G305R mutation revealed significant reduction in glycosylation, cell surface targeting, and activity of NHE1 (168). Another mutation, N226H, was identified in another patient exhibiting some similar phenotypes to the patients with Lichtenstein–Knorr syndrome. This mutation completely annuls NHE1 activity without affecting expression or membrane targeting (169).

1.6 The role of Na^+/H^+ Exchange in Renal Function

The kidney plays a critical role in maintaining body acid-base homeostasis and extracellular volume by reabsorbing filtered bicarbonate and Na^+, for which NHEs are quite important. This is accomplished by the secretion of H^+ across the apical membrane into the lumen, and the transport of HCO_3^- across the basolateral membrane into the interstitium (170) (**Figure 1.2)**.

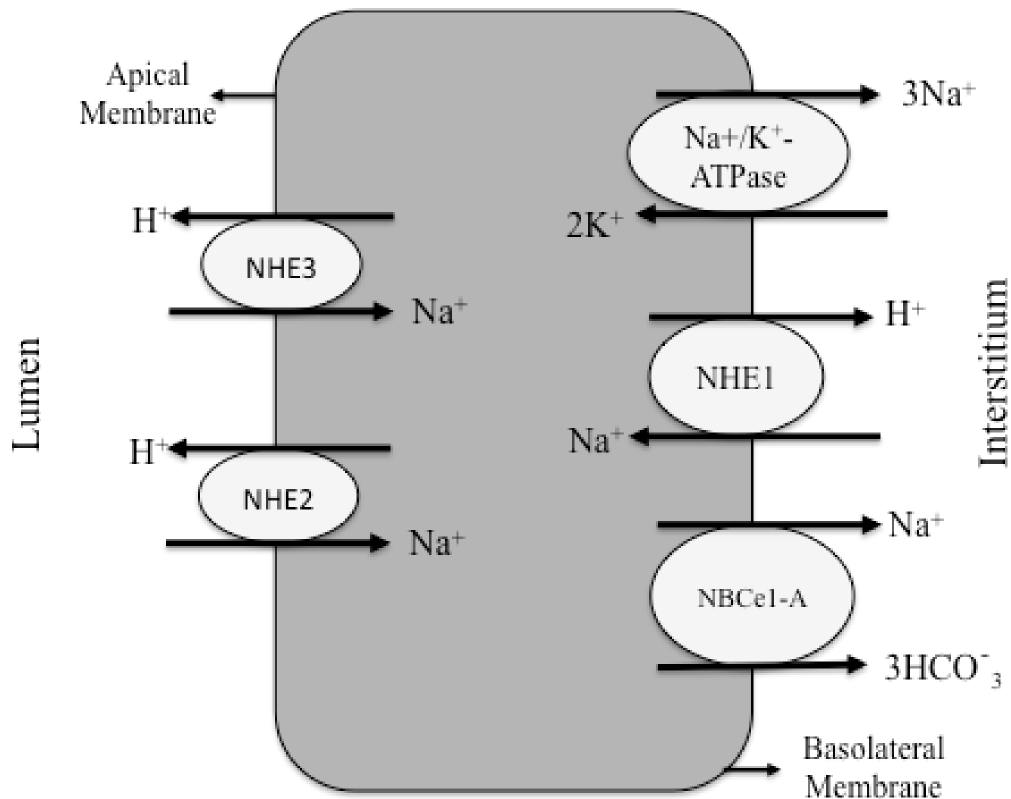

Figure 1.2: Simplistic representation of Na^+ and H^+ transport across renal epithelia membrane. *NHE* Na^+/H^+ exchanger, *NBCe1-A*, electrogenic Na^+/HCO_3^- co-transporter.

1.6.1 Distribution of NHEs in the kidney

Five NHE isoforms are expressed in renal epithelial cells (NHE1-4 and NHE8), each with specific distribution (16) (**Figure 1.3**). NHE1 is expressed in the basolateral membrane of most of the nephron segments including proximal tubule cells, cells of the thick ascending limb (TAL), distal tubule cells, and principal cells of the collecting tubule (18). NHE4 is also expressed in the basolateral membrane of the thick ascending limb and distal convoluted tubule (171). It is the only basolateral NHE isoform present in the macula densa and intercalated cells of the cortical collecting duct where NHE1 is absent

(8).

NHE2 is expressed on the apical membrane of the cortical distal tubule. Along with NHE3, it is also expressed in the TAL where both isoforms mediate bicarbonate reabsorption and acidification (25,172). In addition to the TAL, NHE3 is also expressed in the apical membrane of the proximal tubule where it serves as the major transporter for apical Na^+ and bicarbonate reabsorption (173). NHE3 also mediates the luminal transport of NH_4^+ and indirectly contributes to the absorption of other organic solutes such as oligopeptides and amino acids (174).

NHE8 was first identified in the kidney where it is expressed on the apical membrane of the brush border membrane of proximal tubule cells (40,175). While no overt phenotype was identified in NHE8-null mice, NHE3-null mice expressed higher levels of NHE8 at the BBM of proximal tubule cells compared to control, suggesting that NHE8 may be playing a compensatory role for NHE3 (176,177).

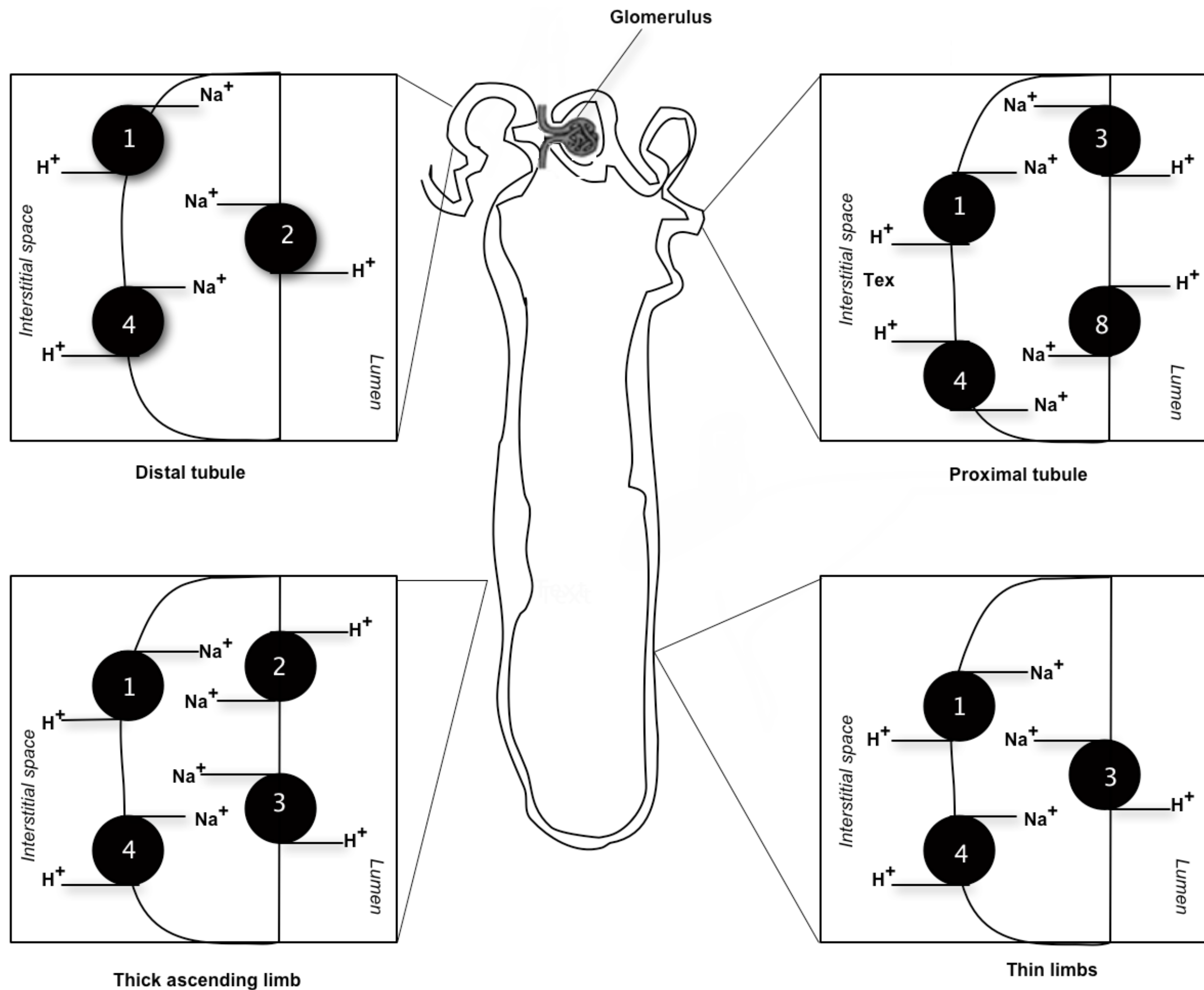

Figure 1.3: Distribution and localization of Na⁺/H⁺ exchangers in kidney. Apical isoforms, NHE3 and NHE8 are located in the proximal tubule. NHE2 and NHE3 are located in the thick ascending limb of the loop of Henle while only NHE2 is present in the distal convoluted tubule and connecting tubule. Basolateral isoforms, NHE1 and NHE4 are present in all the nephron segment except on the macula densa and intercalated cells of the cortical collecting duct where only NHE4 is the basolateral isoform.

1.6.2 Physiological and Pathological Role of NHE1 in the Kidney

NHE1 plays various roles in the kidney including maintaining intracellular pH and volume, cell motility and providing a platform for signalling complexes (16). Although, NHE3 accounts for most of the reabsorption of filtered Na^+ in the proximal tubule, NHE1 also makes significant contributions to the Na^+ transport and cell volume regulation in the proximal tubule.

Proximal tubule apoptosis mediates tubular atrophy, which is a hallmark of chronic kidney disease (CKD) progression (130). Epithelial cells are generally able to resist apoptosis through the restoration of intracellular volume by transporters such as NHE1, anion exchanger (AE2) and $Na^+/K^+/2Cl^-$ symporter. Hence, regulatory volume increase (RVI) has to be overcome or inhibited for a cell to undergo apoptosis. Neither AE2 nor the $Na^+/K^+/2Cl^-$ symporter are present in the proximal segment indicating that NHE1 plays a critical role in the resistance of renal tubule cells from apoptosis-induced cell shrinkage (147).

NHE1 contributes to proximal tubule cell survival through its association with PIP_2 and ERM, which elicits downstream Akt signalling that blocks apoptosis (104,129). Decreased NHE1 expression has been shown to be associated with apoptosis in renal cells and this is thought to be due to caspase-3-dependent degradation (99,146,147). In addition, animal models of unilateral ureteric obstruction and adriamycin-induced nephropathy showed increased renal tubular cell apoptosis when NHE1 was inhibited or in its mutant form (146,147). More recently, a model for lipotoxicity-induced tubular apoptosis was proposed which provides some insight into the biochemical mechanisms of NHE1 inactivation in tubular epithelial apoptosis. The study by Khan *et al*. indicated that

long-chain acyl-CoA (LC-CoA) competes with PIP_2 for NHE1 binding, resulting in the loss of NHE1 activity and the induction of apoptosis (130). In glomerular diseases, albumin carries long chain fatty acids to the proximal tubule at concentrations far above normal levels. The accumulation of these lipids at the proximal tubule results in apoptosis (178). Because of the structural similarity between LC-CoA and PIP_2, excess LC-CoAs are able to displace PIP_2 from NHE1 binding sites and bind NHE1 with a higher affinity, thereby disrupting the pro-survival signalling cascade normally initiated by NHE1-PIP_2 interaction (130).

The medullary thick ascending loop (MTAL) participates in acid-base and cell volume regulation by reabsorbing filtered HCO_3^- and Na^+-Cl^- not reabsorbed by the proximal tubule (179,180). The reabsorption of HCO_3^- in MTAL is dependent on the presence of Na^+ and the secretion of H^+ and this is primarily mediated by NHE3 activity at the apical membrane (181). However, this process is also sensitive to amiloride and nerve growth factor suggesting a secondary regulation of HCO_3^- reabsorption by basolateral membrane Na^+/H^+ exchange (182,183). NHE1 was conclusively identified to be responsible for the basolateral regulation of apical HCO_3^- reabsorption, based on evidence from NHE1 null mice showing defective transepithelial HCO_3^- absorption in the MTAL compared to wild type (179). A regulatory cross talk between NHE1 and NHE3 was proposed which appears to be mediated by the reorganization of actin cytoskeleton (184).

Conversely, the inhibition of NHE1 appears to be protective under certain pathological conditions, particularly those that cause acute kidney injury. One of such conditions is ischemia-reperfusion, which results in intracellular acidosis that stimulates

NHE1 activity. In animal models and various renal cell lines, an increase in the mRNA levels, expression and activity of NHE1 was observed following metabolic/chronic acidosis and IRI (161,185,186). Furthermore, treatment with an NHE1 inhibitor has been shown to protect the kidney from IRI in mouse models (187). In animal models of diabetic nephropathy and severe haemorrhage, reversal of kidney dysfunction and improved renal blood flow were reported when NHE1 was inhibited (188,189).

An explanation for these divergent outcomes may be based on the differing mechanisms of NHE1 activation under these conditions. In the case of tubular atrophy where NHE1 activation promotes cell survival, it is likely that cell volume shrinkage, induced by apoptosis, stimulates NHE1 activity resulting in the desirable restoration of cell volume. On the other hand, stimulation of NHE1 activity by intracellular acidosis under conditions such as ischemia, may promote pathological cell swelling and necrosis (47,134).

In other pathological conditions such as polycystic kidney disease (PKD), NHE1 is implicated in the Na^+ hyperabsorption that may be responsible for the hypertensive phenotype in PKD. According to studies in mouse models of the disease, there appears to be an increased apical localization of NHE1 in the kidneys of mouse models. The resulting increase in HOE-sensitive apical Na^+/H^+ exchange may therefore contribute to Na^+ hyperabsorption associated with PKD (190). NHE1 has also been implicated in salt-sensitive hypertension as reported by studies in renal tubules of mice overexpressing NHE1, which resulted in increased renal Na^+ reabsorption and salt-sensitive blood pressure (165). NHE1 activity was also found to be elevated in proximal tubule cells of spontaneously hypertensive rats (191).

1.6.3 The regulation of NHE1 in the kidney

Regulation of NHE1 in the kidney is mediated by diverse stimuli and molecular mechanisms that are described as follows.

Regulation by hormones, growth factors and receptors

Steroid hormones, including aldosterone, which is probably the most studied hormone that acts on the kidney, regulate renal function. It acts on the distal nephron segment and cortical collecting duct (CCD) to promote salt reabsorption through the regulation of ion transporters including NHE1 (192). In Madin-Daby canine kidney (MDCK) and mouse CCD cells, nanomolar concentrations of aldosterone induced rapid NHE1 activation in a Ca^{2+}-dependent manner. Activation of NHE1 by aldosterone also involves PKC and the MAPK cascade (193,194). A dose-dependent biphasic effect of aldosterone on NHE1 activity in the proximal S3 segment has also been reported; whereby, NHE1 activity is stimulated by low doses (10^{-12} – 10^{-8}M) of aldosterone and inhibited at high concentrations (10^{-6}M) of the hormone (195). In addition, aldosterone-induced NHE1 activation observed in the PTCs of SHR mice was absent in wild type mice indicating a potential role of this regulatory mechanism in the pathogenesis of hypertension (196). This genomic event also required the availability of excess H_2O_2 (196). NHE1 activity has also been implicated in the progression of aldosterone-induced glomerulosclerosis (197).

Angiotensin II (Ang II) is a peptide hormone that has many cellular functions including salt and water homoeostasis in the kidney. While Ang II activates signalling pathways by binding to AT_1 or AT_2 receptors, most of the effects of Ang II are mediated

through the AT_2 receptors (198). In MDCK cells, Ang II acts through the AT_1 receptor to stimulate NHE1 activity via the MAPK cascade (69).

The activation of α_1-adrenergic receptors by agonists such as phenylephrine, increases NHE1 activity in proximal tubule cells, via a PKC-dependent mechanism (75). A nuclear receptor, peroxisome proliferator-activated receptor (PPAR)-α has also been reported to promote cell survival in renal tubular cells by upregulating NHE1 expression and activity (148).

NHE1 is activated by various growth factors including EGF, NGF and Hepatocyte growth factor (HGF) in the kidney. In renal podocytes, EGF induces NHE1 activity through its receptor, EGFR, and signalling pathways that involve Janus kinase (Jak2) and CaM (199). A similar mechanism, induced by Bradykinin B_2 Receptors, has also been described in murine models of kidney inner medullary collecting duct (200). EGF-induced NHE activity has also been implicated in the progression of cell proliferation in CCDs of animal models of PKD (201). HGF was reported to increase NHE1 expression in human renal tubule cell lines and the inhibition of NHE1 prevented HGF-induced cell growth and proliferation (202). Interestingly, contrary to the classic stimulatory action of growth factors on NHE1, NGF inhibits NHE1 in MTAL through the activation of the PI3-K and ERK signalling pathways, consequently inhibiting HCO_3^- absorption (183,203).

Regulation by protein kinases

As earlier mentioned, about half of the regulation of NHE1 by hormones and growth factor is mediated by phosphorylation. Virtually all the regulatory mechanisms

described above involve protein kinases particularly the MAPK family. Specifically, ERK1/2 pathway mediates the regulation of NHE1 activity by aldosterone, Ang II, and NGF (69,193,194,204). Serum deprivation upregulates transcription, translation and activity of NHE1 in rat PTCs via a regulatory mechanism that involves ERK1/2 signalling cascade (205). Another study demonstrated that the susceptibility of immortalized human PTCs to apoptosis following mechanical stretch has been attributed to decreased NHE1 expression and activity as a result of RhoA-induced ERK1/2 inhibition and p38 activation (99). In MDCK cells, it has been shown that acute and chronic high glucose treatment activated NHE1 via the ERK1/2 and p38MAPK pathway respectively (206).

The MAPK family also appear to sometimes work in conjunction with other protein kinases in regulating NHE1. For example, tyrosine kinases, along with ERK1/2 cascade mediate the stimulation of NHE1 activity by serum deprivation (205). PKC was also shown to be required for MAPK signalling mediating aldosterone-induced NHE1 activity (194). Independently, PKC and a tyrosine kinase, Jak2, mediate activation of NHE1 by α_1-adrenergic receptors and EGF respectively (75,199).

Phosphoinositide 3-kinase (PI3K) has also been implicated in the regulation of NHE1 particularly in epithelial cells. The inhibition of NHE1 in the MTAL occurs through the activation of the PI3K/mammalian target of rapamycin (mTOR) signalling pathway (203,207).

Regulation by proteins

The cytoskeletal adaptor proteins, ERM, have been shown to play a crucial role in proximal tubule cell survival. In association with the cytoplasmic tail of NHE1, they recruit and activate pro-survival kinases such as PI3K and Akt (104). This interaction appears to be independent of Na^+/H^+ exchange, and is promoted by cellular stress. It also involves the translocation of ezrin from its typical localization at the apical membrane to the basolateral membrane where it interacts with NHE1 (208).

PIP_2 also binds to the juxtamembrane portion of the NHE1 cytoplasmic tail; an association that is not only required for Na^+/H^+ exchange but also protects the proximal tubule from apoptosis (128,129). NHE1 is compartmentalized in lipid rafts (209), which are also enriched with phosphoinositides including PIP_2 (210). Abu Jawdeh *et al.* (129) postulated a model of NHE1 regulation by PIP_2 to consist of an on-off switch mechanism. The model describes the stimulation of NHE1 by PIP_2 binding which results in the activation of PI3K. PIP_2 is then phosphorylated by PI3K, and the resulting PIP_3 stimulates the downstream Akt survival cascade and also negatively feeds back to reduce NHE1 activity by competing with PIP_2 for NHE1 binding (129).

Phosphorylation of CaM by Jak2 induces the association of CaM with NHE1 and causes an upregulation of NHE1 activity in renal cells (199,200).

1.7 Thesis Objectives

From the various studies highlighted, it is obvious that NHE1 plays more than a housekeeping role in the kidney. It is therefore important to understand and elucidate the

mechanisms of NHE1 regulation in renal cells. Hence, the objective of this doctoral research project was to study the regulation of NHE1 in the kidney. This was done by

1. Examining and characterizing the response of NHE1 to physiological stimuli
2. Identifying the amino acids involved in the regulation of NHE by phosphorylation
3. Identifying and elucidating as yet unknown regulatory interactions with NHE1 in the kidney.

The hypotheses for this research were as follows:

a) One or more amino acids in the cytoplasmic domain of renal NHE1 are phosphorylated by protein kinases in response to physiological stimuli, specifically, sustained intracellular acidosis.
b) Unidentified interactions with NHE1 in the kidney exist, which may involve known or unknown NHE1 interacting proteins.

Chapter 2

Materials and Methods

2.1 Cell culture

MDCK cells were passaged in Ham's F12/Dulbecco's Modified Eagle's Medium supplemented with 5% fetal bovine serum (FBS) and 50 μg/ml Gentamicin. HEK293 cells were passaged in Dulbecco's Modified Eagle's medium/High glucose supplemented with 10% FBS, 100 μg/ml Gentamicin and 25 mM HEPES. Cells expressing exogenous NHE1 were grown in the presence of 400 μg/ml Geneticin (G418). All cells were maintained at 37°C in 5% CO_2.

2.2 Characterization of NHE in MDCK and HEK cells

The presence and activity of endogenous NHE in MDCK and HEK293 cells were confirmed and characterized by western blotting and intracellular pH assay.

2.2.1 Extraction of endogenous NHE proteins from MDCK and HEK293 cells

NHE proteins were extracted from cell culture using Radioimmunoprecipitation Assay (RIPA) Lysis Buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 0.1% (v/v) Triton X-100, 1 mM EGTA, protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1% (v/v) protease inhibitor cocktail).

Cells were grown to confluency in 10 mm dishes, rinsed with ice-cold Phosphate Buffered Saline (PBS), and placed on ice. 100 μl of RIPA lysis buffer was added to each plate and incubated on ice for 1-3 minutes. Cells were scrapped on ice with disposable scraper and transferred to Eppendorf tube. This was followed by centrifugation at 1400

rpm at 4°C for 5 minutes. The supernatant was removed and tested for protein expression using SDS-PAGE.

2.2.2 Western blot analysis of cell extracts from MDCK and HEK293 cells.

Cell extracts were resolved on 10% SDS-PAGE gels. RIPA lysate supernatants were mixed with 4× 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 5:1 ratio. The sample was then loaded on 10% acrylamide SDS-PAGE gel, which ran for ~ 30 min at 80V and then at 120 V until the dye had run off. Proteins on the SDS-PAGE gel were transferred to a nitrocellulose membrane for 1.5 – 2 hours at 500 mA. Nitrocellulose membrane was blocked 1 hr (room temperature) – overnight (4°C) with 10% (w/v) milk in 1× Tris Buffered Saline (TBS).

Expression of NHE mutants was confirmed by immunoblotting using anti-NHE1 antibody (BD Biosciences) and anti-NHE3 antibody (gift from Dr. Todd Alexander, University of Alberta). The primary antibodies were diluted in 1X TBS + 1% milk (1:4000) and incubated with the nitrocellulose membranes for 2 hours (room temperature) or overnight (4°C). The membranes were washed 4X for 15 mins in 1X TBS. Horseradish peroxidase-conjugated goat anti-mouse (GAM) and goat anti-rabbit (GAR) secondary antibodies (Bio/Can, Mississauga, Canada) were diluted in 1X TBS + 1% milk (1:10000 and 1:4000 respectively) to detect NHE1 and NHE3 proteins respectively. The membranes were incubated with secondary antibodies for 1.5 h at room temperature (20ºC) with gentle shaking. This was also followed by washing in 1X TBS 4 times (15 min each).

Immunoreactive proteins were detected on x-ray film using the Amersham enhanced chemiluminescence western blotting and detection system.

2.2.3 Characterization of NHE activity in HEK293 and MDCK cells

The contributions of NHE1 and NHE3 to NHE activity in the two cell lines were characterized using inhibitors specific to each NHE isoform. EMD87580 and S3226 were used to specifically inhibit NHE1 and NHE3 activity specifically. The effect of each inhibitor on NHE activity was measured by a PTI Deltascan spectrofluorometer.

MDCK and HEK293 cells were grown to ≥90% confluency on coverslips and incubated overnight in reduced (0.5%) serum media. Before measuring activity, cells were incubated in serum free media containing 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 and is de-esterified once inside the cell, where it becomes impermeable and fluorescent. Intracellular pH measurements with BCECF were determined by the ratio between dual excitation wavelengths (425 nm and 503 nm) and the emission wavelength of 524 nm. The ratio of BCECF emissions is independent of dye concentration.

After loading cells with BCECF, the activity assay was carried out in solutions pre-warmed to 37°C. Coverslips containing cells were placed in 2.5 ml Na^+ normal Buffer (135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, 5.5 mM Glucose, 10 mM Hepes, pH: 7.3) for 3 minutes to stabilize the pH*i*. 50mM NH_4Cl was used to acidify cells for 3 mins. The coverslip was then placed in 2.5 mL Na^+ Free buffer (135 mM N-Methyl-D-glucamine, 5.0mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, 5.5 mM glucose, 10

mM Hepes, solution pH: 7.3) for 10 – 20 seconds at 37°C. Cells were allowed to recover from the acid load by incubating them in Na^+ normal Buffer for 3 – 4 mins. Following recovery, cells were equilibrated in a three-step pH calibration using Na^+ free pH buffer (5 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 Nigericin, at pHs of 6,7 and 8). Varying concentrations of EMD87580 (0.1 – 30 μM) or S3226 (0.1 – 10 μM) were present throughout the assay. Control experiments were also done in the absence of inhibitors.

The calibration measurements were used to convert fluorescent output to pH. The recovery rate of pH*i* to neutral pH was then measured as the activity of NHE and is calculated from the first 20 sec of recovery from acidification and expressed as ΔpH/sec.

2.2.4 Stimulation of NHE1 activity by sustained intracellular acidosis

To determine the effect of sustained intracellular acidosis (SIA) on NHE1 in kidney cells, MDCK and HEK293 cells, were subjected to a two-pulse acidification assay using the PTI Deltascan spectrofluorometer. Assays were done in the presence or absence of 3 μM S3226. Results from the previous experiments showed that this concentration effectively inhibited NHE3 in kidney cells (94).

In the first pulse, acidification of cells by NH_4Cl removal was followed by ~20 sec incubation in a Na^+-free buffer, after which the cells were allowed to recover in a normal Na^+ buffer as described above. The second pulse was the same except that acidification induced by NH_4Cl withdrawal was sustained for 3 mins in Na^+-free buffer prior to recovery in normal Na^+ buffer. SIA was not introduced in the second pulse for

control cells. This was followed by three-step calibration in calibration buffers of pHs 6, 7 and 8.

2.3 Site-directed mutagenesis

Mutations in the regulatory region of the cytosolic tail of NHE1 were made to the expression plasmid pYN4^{+} (Figure 2.1), which contains the cDNA coding for HA-tagged human NHE1 (51). The mutants used were as follows (Table 1): Mutant 3: S776/770/771A, Mutant 4: T779A/S785A and individual mutations to S770 and S771 to Alanine (S770A and S771A) and were described earlier (97). All mutants had an additional mutation L163F/G174S, which increased NHE1 resistance to inhibition by approximately 100-fold (211).

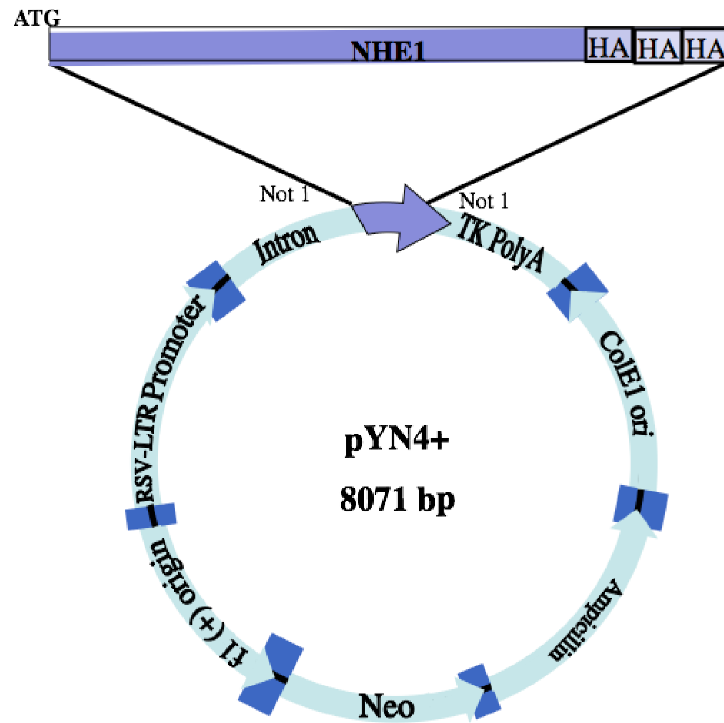

Figure 2.1: pYN4+ expression plasmid. pYN4^{+} was made from plasmid pOPRSVICat, from which the Cat gene was cut out at the Not1-Not1 sites, and replaced with NHE1-HA tag.

Table 1: List of mutations made to the NHE1 protein

Mutation	Name
L163F/G174S	IRM
S770A	S770
S771A	S771
S776/770/771A	IRM3
T779A/S785A	IRM4

2.3.1 Bacterial transformation

E. coli DH5α strain was transformed by electroporation using the following parameters1.8 kV, 25 μF capacitance, 150 Ω resistance (BioRad GenePulser II). Transformed cells were transferred to 0.5 ml LB medium, and placed in a shaker for 1 hour at 37ºC. Cells were then plated on LB agarose plates containing 100 μ g/ml ampicillin (Amp) and incubated overnight at 37ºC.

2.3.2 Plasmid DNA Isolation

Transformed E. coli colonies were picked individually and cultured in 125 mL of LB media containing 100 mg/mL Amp and incubated in a shaker overnight at 37ºC overnight. Plasmid DNA was isolated using the maxi-prep protocol described below.
Bacterial cell pellets were resuspended in 10 ml of resuspension buffer (50 mM Tris-Cl, pH 8.0; 10 mM EDTA; 100 μg/ml RNase A), followed by addition of 10 ml of Lysis Buffer (0.2 M NaOH, 1% SDS). The mixture was then left to stand at room temperature for 5 mins. 10 ml of ice cold Neutralization Buffer (3 M Potassium Acetate, pH 5.5) was added to the mixture, followed by thorough mixing and incubation on ice for 20 mins. The cell suspension was then centrifuged at 11,500 rpm for 30 min at 4°C. The supernatant, containing the plasmid DNA, was dispensed into a new container and centrifuged at 13,500 rpm for 15 min at 4°C. Plasmid DNA was extracted from the supernatant by passing the solution through a column. Bound plasmid DNA was then washed and eluted using an Elution Buffer (1.25 M NaCl; 50 mM Tris-Cl, pH 8.5; 15% isopropanol). Eluted DNA was precipitated by adding 10.5 ml of isopropanol to the

column, and the suspension was centrifuged at 13,500 rpm for 30 mins at 4°C. DNA pellet was washed by re-suspending it in 70% ethanol and centrifuging at 13,500 rpm for 10 mins. Supernatant was carefully decanted and the DNA pellet air-dried for ≈10 mins. Purified DNA was dissolved in 400 µl of 10 mM Tris-Cl, pH 8.5 and stored at -20°C.

2.4 Establishing NHE1 mutants in cell culture

For establishing NHE1 mutants in cell culture, Mardin-Darby Canine Kidney (MDCK) cells and Human Embryonic Kidney 293 (HEK293) cells were used. MDCK cells are derived from adult female cocker spaniel and are commonly used for epithelial studies due to their polarization, rapid growth rate, and suitability for confocal imaging (212). HEK293 cells were generated from normal human embryonic kidney transformed by sheared Adenovirus type 5 and are widely used for their transfectability (213) (**Graham, 1997**). Both cell lines express NHE1 and NHE3 proteins endogenously.

2.4.1 Stable transfection

The cells were stably transfected with the mutant plasmid constructs using Lipofectamine™ 2000 (LF2000) reagent (Invitrogen) in accordance with the manufacturer's instructions. About 24 hours before transfection, $\approx 1 \times 10^6$ cells were plated in 60 mm dish with growth media in the absence of any antibiotics. For each plate to be transfected, the following were prepared: 10 µg DNA per 500 µl Opti-MEM I Reduced Serum Medium (Invitrogen) and 20 µl of LF2000 reagent per 500 µl Opti-MEM Medium. Both solutions were incubated at room temperature for 5 mins, combined and

left at room temperature (20ºC) for 20 mins to allow for the formation of DNA-LF2000 reagent complexes. 1ml of the DNA-LF2000 reagent complexes was added to each dish, which contained growth medium without antibiotics or FBS. 10% FBS was subsequently added after 4 – 6 hours. The cells were incubated at 37°C in 5% CO_2 for 24 hours.

After 24 hours, cells were passaged at 1:10, 1:100, and 1:1000 dilutions in fresh growth medium with antibiotics and 10% FBS. For the next three days, 800 μg/ml Geneticin (G418) was added to each dish and cells were maintained at that concentration until colonies were formed. Once colonies began forming, G418 concentration was reduced to 600 μg/ml and subsequently reduced to 400 μg/ml. Colonies were isolated and transferred to 12-well plates containing 1.5 ml of growth medium and 15% FBS. 400 μg/ml G418 was added the next day and maintained until cells were confluent. Once confluent, cells were trypsinized and split into two; one set was grown in 60 mm dishes to be tested for protein expression while the other was grown in 6-well plate to maintain cell line.

2.4.2 NHE1 extraction and western blot analysis of NHE1 expression

The expression of HA-tagged NHE1 proteins in the kidney cells was confirmed by SDS-PAGE and Western blot as described previously (**Sections 2.2.2 and 2.2.3).** Immunoblotting was however done using anti-HA antibody (Boehringer Mannheim, Laval, Quebec) and horseradish peroxidase-conjugated GAM antibody.

2.5 Characterization of NHE1 mutants in kidney cells

2.5.1 Effects of SIA on NHE1 mutants kidney cells

To determine the effect of sustained intracellular acidosis (SIA) on NHE1 mutants in kidney cells, MDCK and HEK293 cells, were subjected to a two-pulse acidification assay as described above. Assays were done in the presence or absence of 10 μM EMD87580 and 3 μM S3226 to inhibit endogenous NHE1 and NHE3 respectively. Where indicated, a dual-pulse assay was done in the presence of U0126. U0126 was dissolved in DMSO and added to a final concentration of 10 μM. U0126 or control (DMSO) was added for 10 min before assay and present throughout the assay. A single pulse acidification assay was also done to determine the effect of 10 μM S3226 on NHE1 in AP1 cells stably expressing inhibitor-resistant mutant form of NHE1.

2.5.2 Cell surface biotinylation

MCDK cells stably expressing NHE1 mutants were grown to confluence on 60 mm plates and washed twice with PBS, followed by washing once with 4ºC borate buffer (154 mM NaCl, 7.2 mM KCl, 1.8 mM $CaCl_2$, 10 mM boric acid, pH 9.0). Cell surface proteins were labeled with biotin by incubating the cells in 3 ml Sulpho-NHS-SS-Biotin (Pierce Chemical, Rockford, IL, U.S.A.), prepared at 0.5 mg/ml in borate buffer, for 30 min. The reaction was stopped by washing the cells in cold quenching buffer (192 mM glycine, 25 mM Tris, pH 8.3) three times and solubilizing them on ice with 500 μl of IP buffer (1% (w/v) deoxycholic acid, 1%(v/v) Triton X-100, 0.1% (w/v) SDS, 150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5, 0.1 mM benzamidine, 0.1 mM PMSF,

0.1% (v/v) protease inhibitor cocktail). Cells were scrapped off the plates and centrifuged at 14,000 rpm for 20 min at 4°C. Half of the supernatant was collected and retained as "total" fraction of proteins. The other half was incubated with immobilized streptavidin overnight at 4°C with gentle rocking. The following day, the incubated solution was centrifuged at 10,000 rpm for 2 min at 4°C. Supernatant was collected and retained as "unbound" fraction of proteins. Samples from total and unbound fractions were analyzed by SDS-PAGE and immunoblotting. The amount of bound protein was calculated from densitometric analysis by subtracting unbound protein from total protein. The calculated bound protein indicates the amount of protein localized to the plasma membrane.

2.5.3 *In vivo* phosphorylation assay

To determine the effect of SIA on NHE1 phosphorylation, MDCK cells expressing NHE1 mutants were labelled with [^{32}P] inorganic phosphate. Cells were grown to ~ 100% confluence, washed and incubated with 8 ml phosphate free DMEM (Gibco) for 30 mins at 37°C and 5% CO_2. The medium was removed and plates were washed 2 times with phosphate-free DMEM. 2 ml of phosphate-free DMEM was added with $H_3{}^{32}PO_4$ (Perkin Elmer) added to a final concentration of 100 μCi/ml media. Following incubation with [^{32}P] for 3 hrs, the cells were subjected to SIA for 3 min using Na^+ normal and Na^+-free buffers as described in Section 2.2.3.

2.5.4 Cell lysis and Immunoprecipitation of phosphorylated NHE1s

Cell lysates were first prepared using RIPA buffer containing 150 mM NaCl, 80 mM NaF, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1 mM EGTA, 5 mM Na pyrophosphate, 1 mM Na orthovanadate and protease inhibitors). Where indicated, detergents were added as follows: 1% (v/v) NP-40, 0.5% (w/v) deoxycholate and 0.1% (w/v) SDS. Ice-cold RIPA buffer without detergents was added to cells and placed on dry ice for 5 mins. After thawing on ice for about 10 – 15 min, cells were scraped and transferred to Eppendorf tubes and strongly vortexed. The cells were centrifuged at 14,000 rpm for 30 mins at 4°C. The supernatant was removed and resuspended in RIPA buffer with detergents and further centrifuged at 10,000 rpm for 30 min at 4°C.

To immunoprecipitate NHE1 via the HA-tag, the supernatant was incubated with 1.4 µg/ml of rabbit HA-probe (Y-11) sc-805 (Santa Cruz) antibody and rocked overnight at 4ºC. Then 100 µl of prepared protein-A-Sepharose beads were added to the supernatant with the antibody, and the mixture was rocked for 1 h at 4ºC. The beads were collected by centrifugation at 4000 rpm for 30 s and washed twice with RIPA buffer. The bound proteins were eluted from the beads by solubilizing the proteins in 50 µl of 1X SDS-PAGE sample-loading buffer at 37ºC for 15 min. The beads were spun down, and the supernatant containing immunoprecipitated NHE1 was analyzed on SDS-PAGE gel. After transfer to nitrocellulose membrane, radioactivity was detected using Typhoon 9400, variable mode imager (GE Healthcare, Piscataway, NJ).

2.6 MAPK phosphorylation assay

To determine the pathways involved in mediating MDCK cells, time course activation of ERK and $p90^{RSK}$ following SIA was examined. Cells were incubated in Na^+ normal buffer for 3 min followed by acidification with 50 mM NH_4Cl for another 3 min. SIA cells were then incubated in Na^+ free buffer for 1, 3 and 5 min while controls were not subjected to SIA. In some experiments, 10 μM U0126 was used before and during MAPK phosphorylation assay. All the steps were carried out in a 37ºC room.

2.6.1 Cell lysis for MAPK assay

Cell were incubated with 1ml MAPK 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, protease inhibitors) on dry ice for 5 mins and thawed on ice for~15 min. Cell were scrapped and transferred to Eppendorf tubes and sonicated on ice for 20 sec at constant duty with an output of 30 (Branson Sonifier). This was followed by centrifugation at 14,000 rpm for 30 mins at 4ºC. Supernatant was removed and retained for further analysis.

2.6.2 Western blotting of ERK1/2 and p90RSK proteins

Supernatant from MAPK lysis was resolved on 10% SDS-PAGE and proteins transferred to nitrocellulose membrane as described in Section 2.2.2. The nitrocellulose membrane was blocked with 10% (w/v) skimmed milk in 1X TBS for 1 h at room temperature (20ºC).

Total amount of ERK1/2 and $p90^{RSK}$ proteins in sample was detected using appropriate antibodies (Cell Signaling Technologies) diluted in 1% skim milk in TBS + 0.1% Tween-20 (1:2000). Following incubation with the primary antibodies overnight at 4°C, the membrane was washed 4 times with 1X TBS + 0.1% Tween-20 for 5 min each at room temperature. The membrane was then incubated with the appropriate horseradish-peroxidase conjugated secondary antibodies diluted in 1X TBS + 0.1% Tween 20 (GAR: 1:4000). The antibodies were stripped from the membrane by incubation in 10 ml of Stripping buffer (0.2 M Glycine, pH 2.8) for 10 mins at room temperature. The membrane was washed for 10 min with ddH_20 and blocked for 2 h with 10% milk in 1X TBS.

The membrane was then re-blotted with antibodies against phospho-ERK1/2 and phospho-$p90^{RSK}$ (Cell Signaling Technologies) to detect activated/phosphorylated ERK1/2 and $p90^{RSK}$ proteins followed by the appropriate secondary antibodies diluted in 1X TBS + 0.1% Tween 20 (GAM - 1:10000; GAR -1:4000). Immunoreactive proteins were visualized on x-ray film using ECL (Amersham).

2.6.3 Inhibition of MAPK pathway

To confirm the role of MAPK pathway, the MEK1/2 inhibitor, U0126 was used to inhibit the MAPK pathway upstream of ERK1/2 and $p90^{RSK}$. To determine the effect of inhibiting MAPK on NHE1 activity, dual-pulse assay with or without SIA was done in the presence of 10 µM U0126 or DMSO (vehicle control). Another set of pH*i* assay was done without U0126, with or without SIA. 3 µM S3226 was maintained in both sets of assays to inhibit endogenous NHE3.

For MAPK phosphorylation assays, cells were pre- incubated in 10 μM U0126 for 10 min at 37°C, which was also maintained throughout SIA experiment while DMSO was used for control.

2.7 Screening for NHE1-interacting partners

To screen for proteins that interact with NHE1 in the kidney, an affinity chromatography technique, similar to that used earlier for potassium channel (Kir2.x) was used (214).

2.7.1 Protein purification for affinity chromatography

The C-terminus of rabbit NHE1 (amino acids 545-815), referred to as PCRB, was produced as a fusion protein with glutathionine-S-transferase (GST) using PGEX-3X plasmid as described earlier (215). *E. coli* Topp2 strain was transformed with PCRB by electroporation as earlier described. LB medium containing ampicillin was inoculated with PCRB-containing bacteria and induction was at 30°C with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 5 hours. After induction, the cells were lysed by sonication in lysis buffer (20 mM Tris, pH 7.5; 200 mM NaCl, 1 mM EDTA) and centrifuged at 11500 rpm for 15 min at 4°C. Supernatant was removed and filtered through a 0.45 μm filter to clear cellular debris.

Fusion protein in the supernatant was purified by affinity chromatography using glutathione agarose (Sigma Aldrich). 2 ml of glutathione agarose (50% v/v) in PBS was added to the supernatant on column under gravity flow and incubated for gentle mixing

for 30 min at 4°C. Resin was washed four times with PBS containing 1% Triton X-100 at 4°C. Bound protein was eluted from the resin with Elution Buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 9.5) at 4°C. The wash and elute fractions were analyzed on 10% SDS-PAGE. GST was produced and purified using the PGEX plasmid without the insert.

2.7.2 Kidney Extract Preparation

Kidney extracts were prepared from 10 frozen mouse kidneys cut into appropriately sized pieces for homogenization. Kidneys were homogenized with equal volume 0.5 mm zirconium oxide beads and 2 volumes of homogenization buffer (20 mM Hepes, pH 7.6, 125 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM dithioreitol, 1X Protease Inhibitor Cocktail (Roche Applied Science), 1 mM PMSF). Homogenization was done in a Bullet Blender (Next Advance, Inc.). Homogenate was centrifuged twice, first at 4,000 rpm for 10 min and then at 35,000 rpm for 1 h at 4°C in an ultracentrifuge. Supernatant was collected from the second spin and stored for affinity chromatography.

2.7.3 Preparation of Affi-Gel-GST-PCRB affinity matrix

To prepare affinity columns for affinity chromatography, GST and GST-PCRB was coupled to Affi-Gel 10 (Bio-Rad). Specifically, 5 mg of purified GST or 2.5 mg of purified GST-PCRB was coupled to 1 ml and 500 µl Affi-Gel 10 beads respectively. Prior to coupling, Affi-Gel 10 slurry was washed with cold deionized water. In addition,

the amount of the ligands, GST or PCRB, was calculated before coupling at Ab_{280}. The ligands were incubated overnight with Affi-Gel overnight at 4ºC with coupling buffer (0.1M MOPS + 80 mM $CaCl_2$). Coupling efficiency was determined by calculating the amount of ligand left in solution after coupling reaction. The reactive esters were blocked with 100 µl of 1 M ethanolamine (pH 8.0) per ml of gel slurry for 1 h. The resins were then washed with coupling buffer and homogenizing buffer to prepare columns for affinity chromatography.

2.7.4 Affinity chromatography

Kidney extracts were incubated with GST and PCRB-bound columns overnight at 4ºC with gentle agitation. Sample solution was drained and resins washed with 50 volumes homogenizing buffer + 0.5% Nonidet P-40, 500 mM and 1 mM PMSF. This was followed by washing with 50 volumes homogenizing buffer + 1 mM PMSF. Bound proteins were eluted with homogenizing buffer + 1% SDS at room temperature. Eluted proteins were precipitated with 20% trichloroacetic acid. Protein pellet was air-dried with vacuum drier. Dried proteins were resuspended in 50 µl of 4X SDS sample buffer and boiled for ~10 min. The sample was resolved by SDS-PAGE and analyzed by mass spectrometry.

2.8 Analysis of NHE1-bound proteins

To independently assess the association of NHE1-interacting proteins identified by mass spectrometry, co-immunoprecipitation experiments were done. Four proteins were examined based on previous studies shown to interact with the cytosolic tail of NHE1.

2.8.1 Mass spectrometry

Proteins from control (GST) and NHE1-GST were analyzed by mass spectrometry. In-gel trypsin digestion was performed on samples that were run on SDS-PAGE. Briefly, the excised gel bands were destained twice in 100 mM ammonium bicarbonate/acetonitrile (50:50). The samples were then reduced (10 mm BME in 100 mm bicarbonate) and alkylated (55 mM iodoacetamide in 100 mM bicarbonate). After dehydration enough trypsin (6 ng/ul) was added to just cover the gel pieces and the digestion proceeded overnight (~16 hrs.) at room temperature. Tryptic peptides were initially extracted from the gel using 97% water/2% acetonitrile/1% formic acid. This was followed by a second extraction using 50% of the first extraction buffer and 50% acetonitrile.

Fractions containing tryptic peptides were dissolved in aqueous 25% (v/v) acetnonitrile and 1% (v/v) formic acid were resolved and ionized by using nanoflow HPLC (Easy-nLC II, Thermo Scientific) coupled to the LTQ XL-Orbitrap hybrid mass spectrometer (Thermo Scientific). Nanoflow chromatography and electrospray ionization were accomplished by using a PicoFrit fused silica capillary column (ProteoPepII, C18) with 100 µm inner diameter (300 Å, 5 µm, New Objective). Peptide mixtures were

injected onto the column at a flow rate of 3000 nl/min and resolved at 500 nl/min using 45 min linear gradients from 0 to 45% v/v aqueous ACN in 0.2% v/v formic acid. The mass spectrometer was operated in data-dependent acquisition mode, recording high-accuracy and high-resolution survey Orbitrap spectra using external mass calibration, with a resolution of 60 000 and *m*/*z* range of 400–2000. The fourteen most intense multiply charged ions were sequentially fragmented by using collision induced dissociation, and spectra of their fragments were recorded in the linear ion trap; after two fragmentations all precursors selected for dissociation were dynamically excluded for 60 s. Data was processed using Proteome Discoverer 1.4 (Thermo Scientific) and the Uniprot mouse database was searched using SEQUEST (Thermo Scientific). Search parameters included a precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.8 Da. Peptides were searched with carbamidomethyl cysteine as a static modification and oxidized methionine and deamidated glutamine and asparagine as dynamic modifications.

2.8.2 Co-immunoprecipitation of NHE1-bound proteins.

MDCK cells grown to confluence in 100 mm dishes, were washed with ice-cold PBS and treated with crosslinker, 25 mM dithiobis[succinimidylpropionate] (DSP), to a final concentration of 2 mM and incubated on ice for 1.5 h. The reaction was stopped by adding 10 mM Tris, pH 7.5 for 15 min.

Cells were then incubated on ice for 10 min with 500 µl RIPA lysis buffer, collected and centrifuged at 10,000g for 10 min at 4ºC. Cell lysate was pre-cleared with agarose at 4ºC for 30 min followed by centrifugation at 1,000g for 30 s at 4ºC.

Supernatant was incubated with goat polyclonal anti-HA agarose-conjugate antibody (Santa Cruz) with gentle agitation overnight at 4ºC. Beads were collected by centrifugation at 1000 g for 30 s at 4ºC and washed 3 times with PBS. The bound protein was eluted from the beads by incubating with 40 µl of 2X SDS-PAGE sample loading buffer at 37ºC for 15 mins. Eluted proteins were resolved on SDS-PAGE and analyzed by immunoblotting with antibodies against 14-3-3 (Santa Cruz), Na^+/K^+-ATPase (Developmental Studies Hybridoma Bank, University of Iowa), Hsp90 and Hsp70 (Enzo Life Sciences).

2.8.3 Inhibition of Hsp90 and p-Akt

Specific inhibitors were used to inhibit Hsp90 and p-Akt activity. 17-AAG (17-*N*-allylamino-17-demethoxygeldanamycin) and MK-2206 (Selleck Chemicals LLC) were used to inhibit Hsp90 and p-Akt respectively. Both inhibitors were dissolved in DMSO. 17-AAG was used at concentration between 0.1 – 1 µM while MK2206 was used at concentrations between 1 – 10 µM. Unless otherwise stated, MDCK cells were generally treated overnight (~18 h) with these inhibitors.

2.8.4 Hypoxia/Reoxygenation

MDCK cells stably expressing HA-NHE1 were treated with simulated ischemia and reperfusion as follows. Cells were placed into an anaerobic chamber (GasPac system, BD Biosciences) at 37°C for ~16 h. The chamber contained a disposable hydrogen and carbon dioxide generator envelope and an oxygen-consuming palladium

catalyst. On activation, it created a hypoxic condition of 25–35 mm $HgPO_2$ and the anoxic condition inside the chamber was ensured with a methylene blue anaerobic indicator. After hypoxia, the chamber was opened and cells were placed in a standard incubator (95% air, 5% CO_2) for 2 h for reoxygenation prior to assay. Some experiments were in the presence of 10 μM EMD8750, 1 μM 17-AAG or 10 μM MK2206, which was maintained throughout the experiment.

2.8.5 Cell viability Assays

Cell viability was assessed using cell viability assay kits (Promega). Cells were cultured on 384-well plates and left untreated (control) or treated with 10 μM EMD87580 with or without 1 μM 17-AAG or 10 μM MK-2206 in phenol red-free high-glucose modified DMEM (HyClone) media and subjected to hypoxia/reoxygenation as described above. Inhibitors and vehicle controls were added to the appropriate wells to achieve a final volume of 25 μl. After overnight incubation with or without hypoxia, an equal volume of the CellTiter-Fluor Reagent was added to the wells and incubated for 30 mins at 37°C. Fluorescence was measured at $400_{EX}/505_{EM}$nm using BioTek Synergy MX microplate reader. All viability data were normalized to untreated controls.

2.9 Statistics

All data are expressed as means ± SE and plotted with KaleidaGraph 4.1 (Synergy Software, PA, US). For intracellular pH measurements, the reported results reported are those of 6 -10 experiments per treatment/condition. The results of western blot analyses

for MAPK assays and treatments with 17-AAG and MK2206; and *in vivo* phosphorylation assays, are those of a minimum of three experiments per treatment/condition. Statistics for all experiments was calculated using Wilcoxon Mann-Whitney rank sum test. A *P*-value less than 0.05 was considered to be statistically significant.

Chapter 3

Acidosis-mediated regulation of renal NHE1

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3.1 Introduction

The Na^+/H^+ exchanger isoform-1 (NHE1) is a ubiquitously expressed plasma membrane glycoprotein which functions to protect cells from intracellular acidification by extruding one intracellular proton in exchange for a single extracellular sodium (216). Ten isoforms of Na^+/H^+ exchanger have been discovered (NHE1-NHE10). NHE1 was the first isoform discovered and is ubiquitously distributed (217). Other isoforms have more restricted tissue distributions and some have predominantly intracellular localization. In mammals, aside from its role in regulation of intracellular pH, NHE1 is also important in regulation of cell volume, cell proliferation, differentiation and in metastasis of some types of tumor cells (149,216,218).

Na^+/H^+ exchanger proteins have two major domains. The N-terminal membrane domain is approximately 500 amino acids and transports ions. This is followed by a hydrophilic, carboxyl-terminal cytosolic domain that is 315 amino acids in human NHE1. The cytosolic domain regulates the membrane domain and much of the regulation is by phosphorylation occurring in the distal 178 amino acids of the cytosolic domain (67,89). We demonstrated that the mitogen-activated protein kinases (MAPK) extracellular regulated kinases 1 and 2 (ERK1/2) are implicated in growth factor activation of NHE1 in the myocardium (89) and in CHO cells (219). The ERK-dependent pathway was activated in several models of ischemic heart disease which cause acidosis and this resulted in further activation of the Na^+/H^+ exchanger in the myocardium (160). We also demonstrated that ERK phosphorylates the NHE1 cytosolic domain at one or more of the following amino acids, Ser693, Ser766, Ser770, Ser771, Thr779, and Ser785 (220) *in*

vitro. In CHO cells (96) and in heart cells (97), we showed that amino acids Ser770 and Ser771 are critical to NHE1 activation by acidosis.

Isoform one of the Na^+/H^+ exchanger is ubiquitous in mammalian tissues. One tissue in which it is present is the kidney. NHE1 has been identified in renal tissues by Western blotting, characterization of activity, or identification of its mRNA in various renal cell lines and tissues including Madin-Darby canine kidney (MDCK) cells (221), rat proximal tubule cells (205), in M-1 cortical collecting duct cells (194) in human embryonic kidney cells (222) in renal tissues including renal tubules (223) in inner medullary collecting duct (224) and in whole renal cortex (225). It has been localized on both the apical and on the basolateral membrane of polarized renal cells MDCK cells (226) though more frequently reported to be on the basolateral membrane only (227,228). In renal cells the Na^+/H^+ exchanger is involved in a variety of functions including $NaHCO_3$ absorption, NaCl absorption, pH regulation and maintenance of cell volume (reviewed in (228)). The NHE3 isoform of the Na^+/H^+ exchanger is also present in renal cells and is found on the apical membrane of polarized renal cells (228). Several studies have shown earlier that acidosis activates NHE1 in the myocardium via ERK-mediated phosphorylation (97,229). The kidney is subject to chronic acidosis which results in a host of adaptive changes including elevated NHE1 message (230). Long term metabolic or respiratory acidosis has been reported to elevate messenger RNA levels of NHE1 (230,231). However, the effects of short term acidosis on the activity of NHE1 in renal cells have not been well examined. In addition, though ERK 1/2 has been suggested to regulate NHE in kidney cells (232) the molecular mechanisms of ERK-dependent regulation of NHE1 have not been elucidated in renal tissues. In this study, we examine

if acidosis acutely activates the NHE1 isoform of the Na^+/H^+ exchanger in renal cells. We determine the mechanism of activation and amino acids involved in regulation of the protein by phosphorylation. Our results are the first demonstration of the amino acids and mechanisms involved in regulation of this protein in the mammalian kidney.

3.2 Results

We have previously (96,97,233) demonstrated that sustained intracellular acidosis activates the NHE1 isoform of the Na^+/H^+ exchanger. Initial experiments characterized the Na^+/H^+ exchanger in two cell lines derived from renal tissues, MDCK and HEK293 cells. They also examined whether sustained intracellular acidosis could stimulate Na^+/H^+ exchanger activity. EMD 87580 is a specific inhibitor of the NHE1 isoform of the Na^+/H^+ exchanger. At a concentration of 10 μM the NHE1 isoform is largely inhibited while the NHE3 isoform of the Na^+/H^+ exchanger is resistant to inhibition until much higher concentrations are used (211,234). Figure 3.1 A,B illustrate the effect of addition of the inhibitor EMD87580 on the Na^+/H^+ exchanger activity of MDCK cells. Concentrations of EMD87580 of 1 μM or higher inhibited approximately 30% of the NHE activity, consistent with a significant, though minority presence of the NHE1 isoform of the Na^+/H^+ exchanger.

Figure 3.2 A,B illustrates similar experiments with HEK cells. At concentrations above 1 μM EMD87580 inhibited approximately 50% of the Na^+/H^+ exchanger activity of these cells consistent with the presence of the NHE1 isoform of the Na^+/H^+ exchanger.

S3226 is a Na^+/H^+ exchanger inhibitor reported to have specificity for the NHE3 isoform of the Na^+/H^+ exchanger (235). To determine if this isoform was present in MDCK and HEK cells we tested the effect of this inhibitor on Na^+/H^+ exchanger activity

in these cell types. Figures 3.1C and 3.2C show that the presence of S3226 potently inhibited up to 80% and of Na^+/H^+ exchanger activity in MDCK cells while in HEK cells the effect plateaued at 60% inhibition.

To confirm the presence of the NHE1 and NHE3 isoforms of the Na^+/H^+ exchanger western blotting was used. Fig. 3.3A demonstrates that the NHE1 isoform of the protein was present. There was a characteristic larger molecular weight isoform and a smaller size protein corresponding to the fully and partially or de-glycosylated forms of the NHE1 protein respectively that we have described earlier (236). Fig. 3.3B demonstrates the presence of the NHE3 isoform of the Na^+/H^+ exchanger. A characteristic ≈80-85 kDa immunoreactive band was present.

We next examined whether a sustained intracellular acid load, stimulated activity of the Na^+/H^+ exchanger in MDCK and HEK293 cells. We (96,97) and others (229) have demonstrated this phenomenon earlier in other cell types. The results are shown in Fig. 3.4. Cells were subjected to a two pulse assay with acidosis induced by ammonium chloride withdrawal. In the second pulse, acidosis was maintained for 3 minutes prior to reintroduction of NaCl and recovery of pH_i by the Na^+/H^+ exchanger. Fig. 3.4B illustrates a summary of the results. In MDCK cells, the recovery from the second pulse was slightly reduced, compared to the first pulse in the absence of a sustained acidosis. In contrast, after 3 minutes of sustained acidosis, the second recovery was elevated compared to the first pulse and was significantly greater than the value obtained in the absence of acidosis. Similar results were obtained with HEK293 cells though to a lesser extent. Sustained intracellular acidosis (SIA) caused a smaller, but significant, increase in NHE activity (Fig. 3.4C, D).

For our experiments, we included S3226 as an inhibitor of the NHE3 isoform of the Na^+/H^+ exchanger. To confirm that the dose of S3226 that we used does not inhibit the NHE1 isoform of the exchanger we tested the effect of 10 μM S3226 on AP-1 cells that were transfected with the inhibitor resistant isoform of the Na^+/H^+ exchanger that we used earlier in our experiments (237). AP-1 cells have their endogenous Na^+/H^+ exchanger deleted and only possess that Na^+/H^+ exchanger which we add to the cells (238). Fig. 3.5 demonstrates that 10 μM S3226 did not inhibit activity of the exogenous NHE1 protein.

Since we demonstrated that SIA produced an enhancement of NHE1 activity (Fig. 3.4), we examined which amino acids of the regulatory cytosolic domain of NHE1 might be involved. We had previously demonstrated that two regions containing phosphorylatable amino acids are potentially important in this regard. Ser766, Ser770 and Ser771 were in one region (called region 3) and amino acids Thr779 and Ser785 of region 4 (97). Ser703 (233) and the phosphorylatable amino acids Ser693 and Thr718, Ser723, Ser726 and Ser729 (96,97) were not involved in the SIA response in several cell types. We therefore made stable cell lines of cells that contained plasmids that express NHE1 with mutations in region 3 and region 4. The NHE1 isoform that was expressed contained mutations in transmembrane segment IV that made the protein resistant to inhibitors. EMD87580 inhibitor (and S3226) were added to the assay so that only exogenous NHE1 activity was measured. The results are shown in Fig. 3.6. Fig. 3.6A demonstrates that the various stable cell lines in MDCK cells expressed the HA tagged NHE1 protein. Similar results were obtained in HEK cells (not shown). In MDCK cells, cell lines with NHE1 with a mutation in Ser771 or with the mutations in IRM3 and

IRM4, were not stimulated by sustained intracellular acidosis (Fig. 3.6B). However, the wild type NHE1 protein and mutation of Ser770 to Ala, did not prevent stimulation of activity by SIA. In contrast in HEK cells (Fig. 3.6C), none of the mutations prevented SIA from stimulating NHE1 activity, suggesting that the mechanism of stimulation of NHE1 activity was different in these cell types.

To examine the pathways that were involved in mediating the SIA in MDCK cells we examined a time course of activation of ERK and $p90^{rsk}$ that had previously been suggested to be involved in activation of NHE1 in other tissues (96,97). Fig. 3.7A illustrates the results which are summarized in Fig. 3.7B and 3.7C. Treatment of cells with SIA resulted in increased amount of P-ERK and P-RSK which peaked at 3 minutes in MDCK cells. The levels were increased significantly for pERK at 3 min and were not significantly elevated for P-RSK. P-ERK levels in HEK293 cells increased, but the changes were variable and not statistically significant (not shown).

To directly determine which amino acids may be involved in regulation of NHE1 by phosphorylation we examined the effect of mutation of several putative phosphorylation sites, on the level of phosphorylated protein in the presence or absence of SIA. Initially we compared the basal level of phosphorylation of the wild type NHE1 protein and of several mutants in MDCK cells. The results (Fig. 3.8A,B) showed that mutation of amino acids Ser770, Ser771 or of Thr779 and Ser785 in the IRM4 mutant, caused large decreases in the basal level of phosphorylation of the NHE1 protein.

To determine which amino acids may be involved in activation of NHE1 by SIA we examined the relative levels of phosphorylation of the NHE1 protein in Wt and mutant NHE1 proteins, in the presence or absence of SIA. The results are shown in Fig.

3.8C, D. SIA induced increased phosphorylation in the wild type NHE1 protein and in the protein with a mutation at Ser770. In contrast, the other mutants, S771A, IRM3 and IRM4 all showed no increase in phosphorylation in response to SIA, and in fact showed a slight decrease in phosphorylation levels.

To further confirm the role of the Erk pathway in acidosis mediated activation of NHE1 in MDCK cells we tested the effect of the inhibitor U0126 on the activation of the Erk pathway and on activation of the NHE1 protein. Fig. 3.9A, and 3.9C demonstrate that treatment with U0126 dramatically reduces the level of phosphor-Erk protein and prevented activation of the Erk by SIA. The levels of phopho-$p90^{rsk}$ were less affected, declining only slightly (Fig. 3.9B, D). Treatment of MDCK cells with U0126 also prevented activation of the cells by sustained intracellular acidosis (Fig. 3.10).

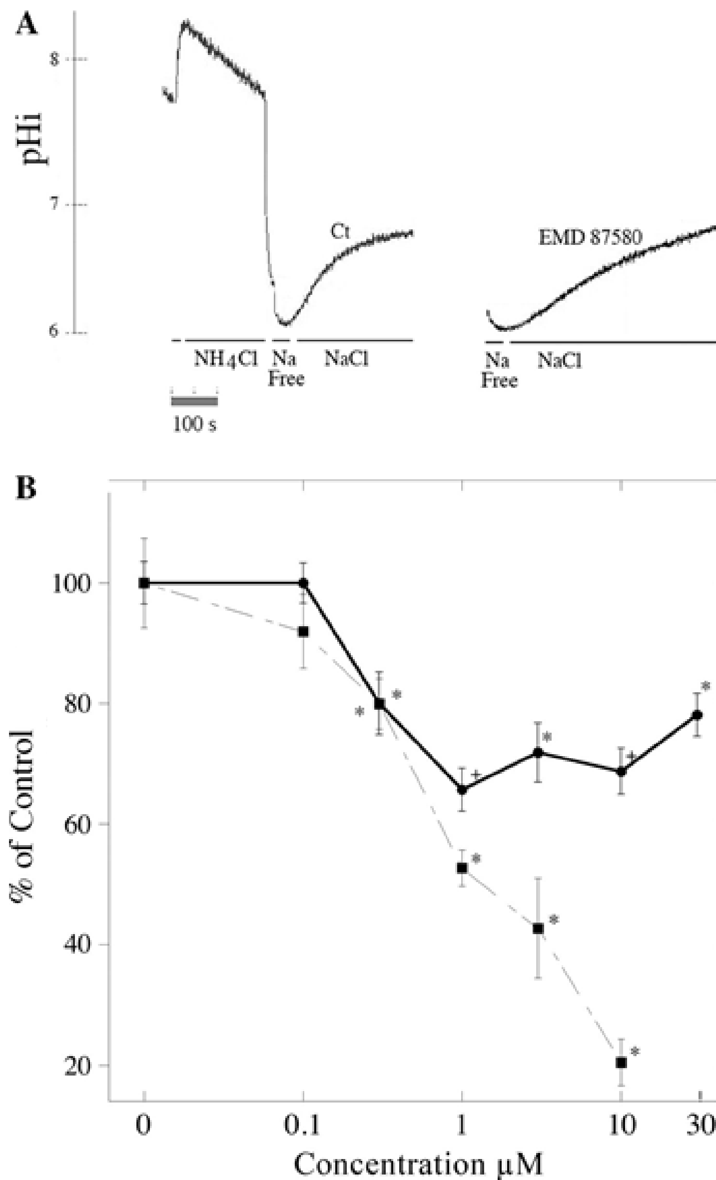


Figure 3.1: Characterization of the Na+/H+ exchanger in MDCK cells. *A*: example of traces illustrating the effect of EMD87580 (10 µM) on recovery from an acute acid load. Intracellular pH was examined in cells that were transiently acidified using NH_4Cl as described in Section 2.2.3 in the presence of varying concentrations of inhibitors present throughout the experiment. Periods of NH_4Cl, NaCl, and Na^+-free solution as indicated. An entire example of the recovery in the absence of EMD87580 is indicated. For treatment with EMD87580, only the recovery after acidification is shown. *B*: summary of endogenous NHE activity in the presence of varying concentrations of EMD87580 (●) or S3226 (■). Values are means ± SE of at least 6 experiments. $^*P < 0.05$, $^+P < 0.01$, significantly different from the control. Absolute value of control activity of NHE1 in MDCK cells was 0.21 ± 0.023 ΔpH/min.

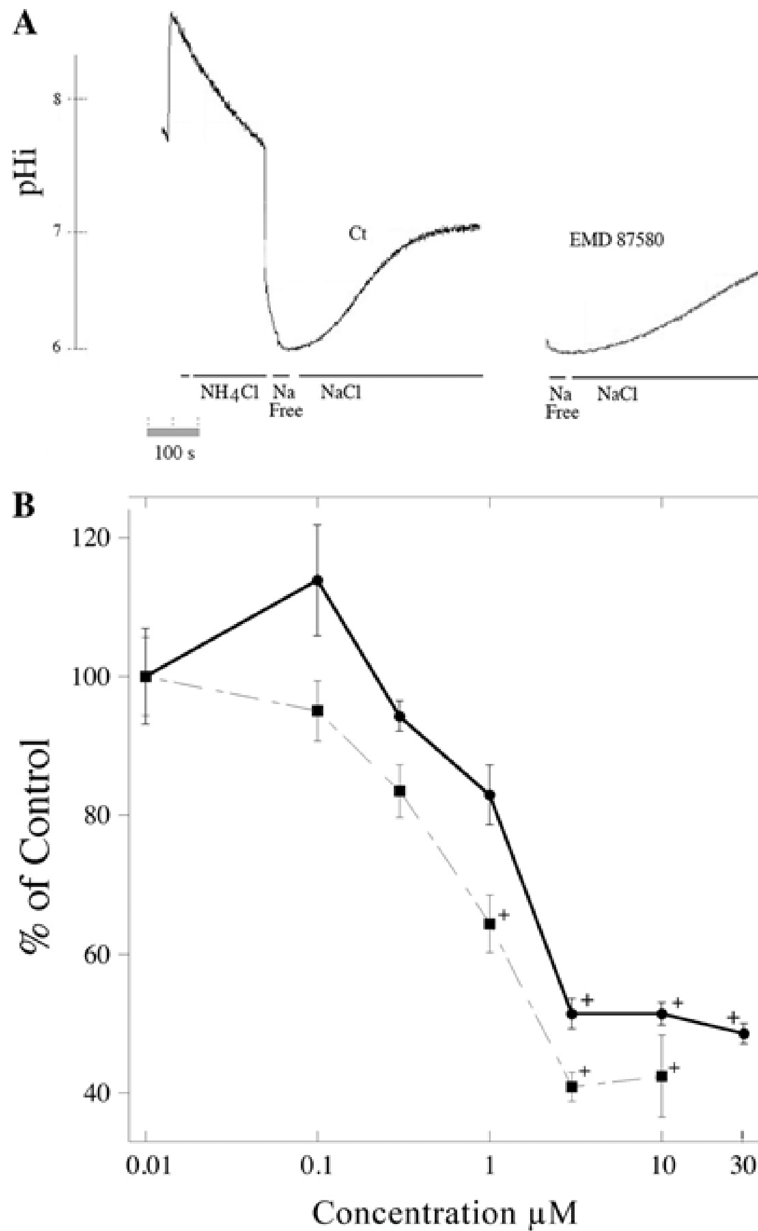


Figure 3.2: Characterization of the Na^+/H^+ exchanger in HEK293 cells. A: examples of traces illustrating the effect of EMD87580 (10 µM) on recovery from an acute acid load. Intracellular pH was examined as described in Fig 3.2. B: summary of endogenous NHE activity in the presence of varying concentrations of EMD87580 (●) or S3226 (■). Intracellular pH was measured as described in Fig. 3.2, and recovery from acidosis examined was in the presence of varying concentrations of EMD87580, which was present in the entire recovery stage. Values are means ± SE of at least 6 experiments. $^*P < 0.01$, significantly different from the control. Absolute value of control activity of NHE1 in HEK cells was 0.25 ± 0.012 ΔpH/min.

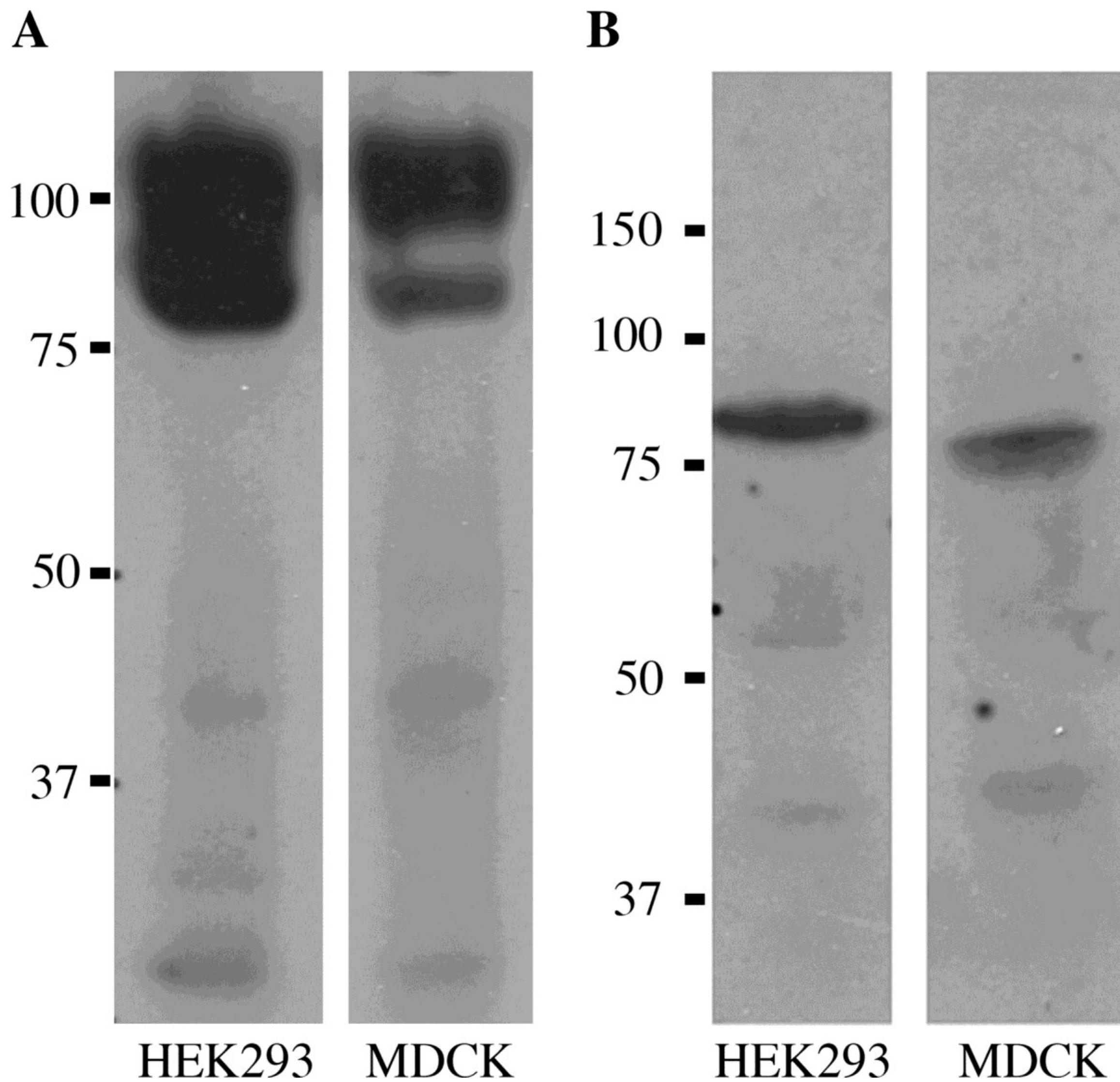


Figure 3.3: Western Blot Analysis of endogenous NHE1 and NHE3 expression in MDCK and HEK293 cells. Cell extracts were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies with antibodies against NHE1 (A) and NHE3 (B).

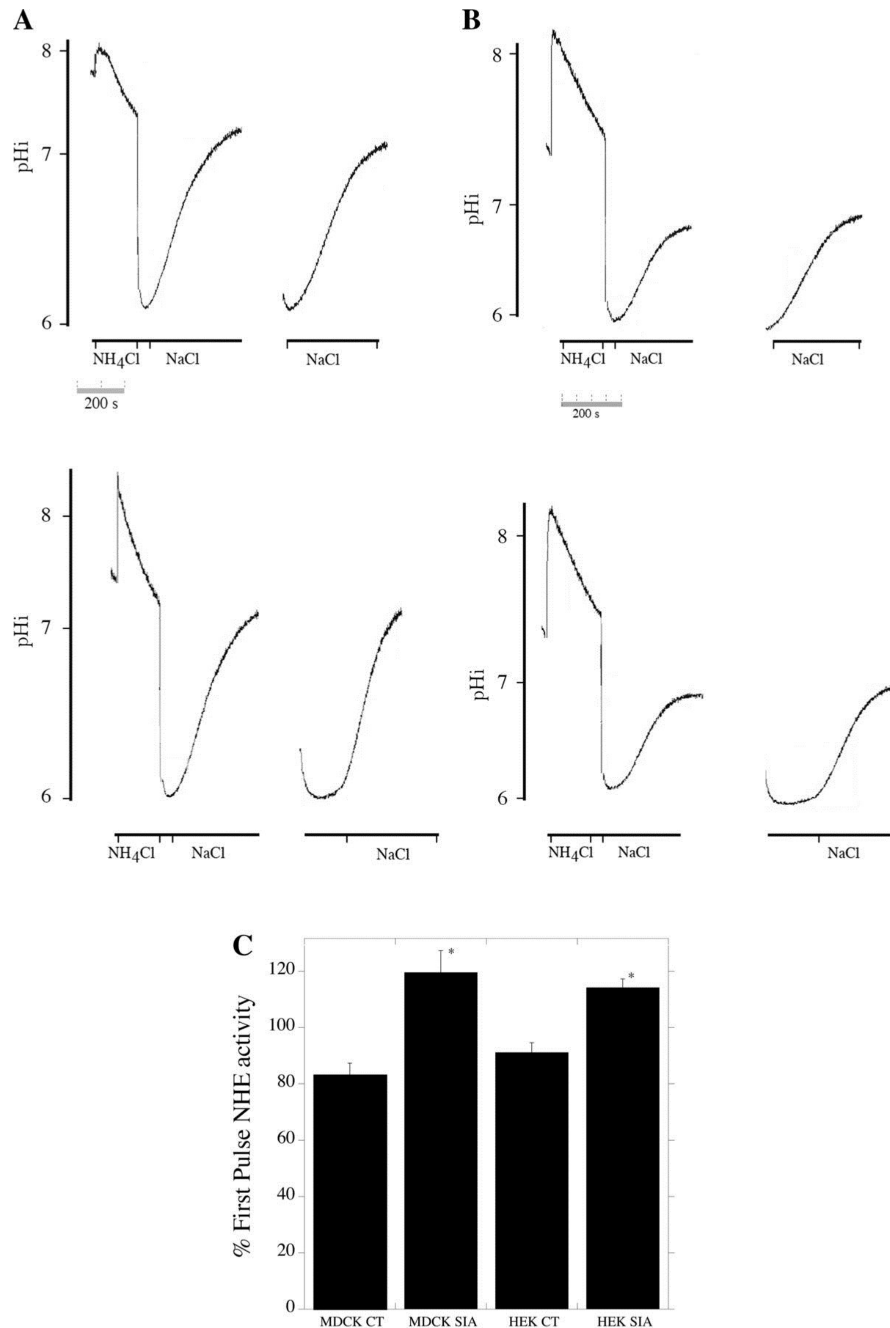


Figure 3.3: Effect of SIA on NHE activity in MDCK and HEK293 cells. Cells were subjected to a 2-pulse pHi assay and the activity of the exchanger in the 2nd pulse was compared with that of the 1st pulse. The 2nd pulse was done either with or without 3 min period of SIA immediately prior to recovery. *A* and *B*: example of traces of dual pulse assay on MDCK (A) and HEK293 (B) cells. C: summary of NHE1 activity in dual pulse assays of MDCK and HEK293 cells. Values summarized are means ± SE of 6 – 10 experiments. *P < 0.05, significantly different from the control. Absolute value of control activity of NHE1 in MDCK and HEK cells was 0.25 ± 0.023 and 0.23 ± 0.01 ΔpH/min, respectively.

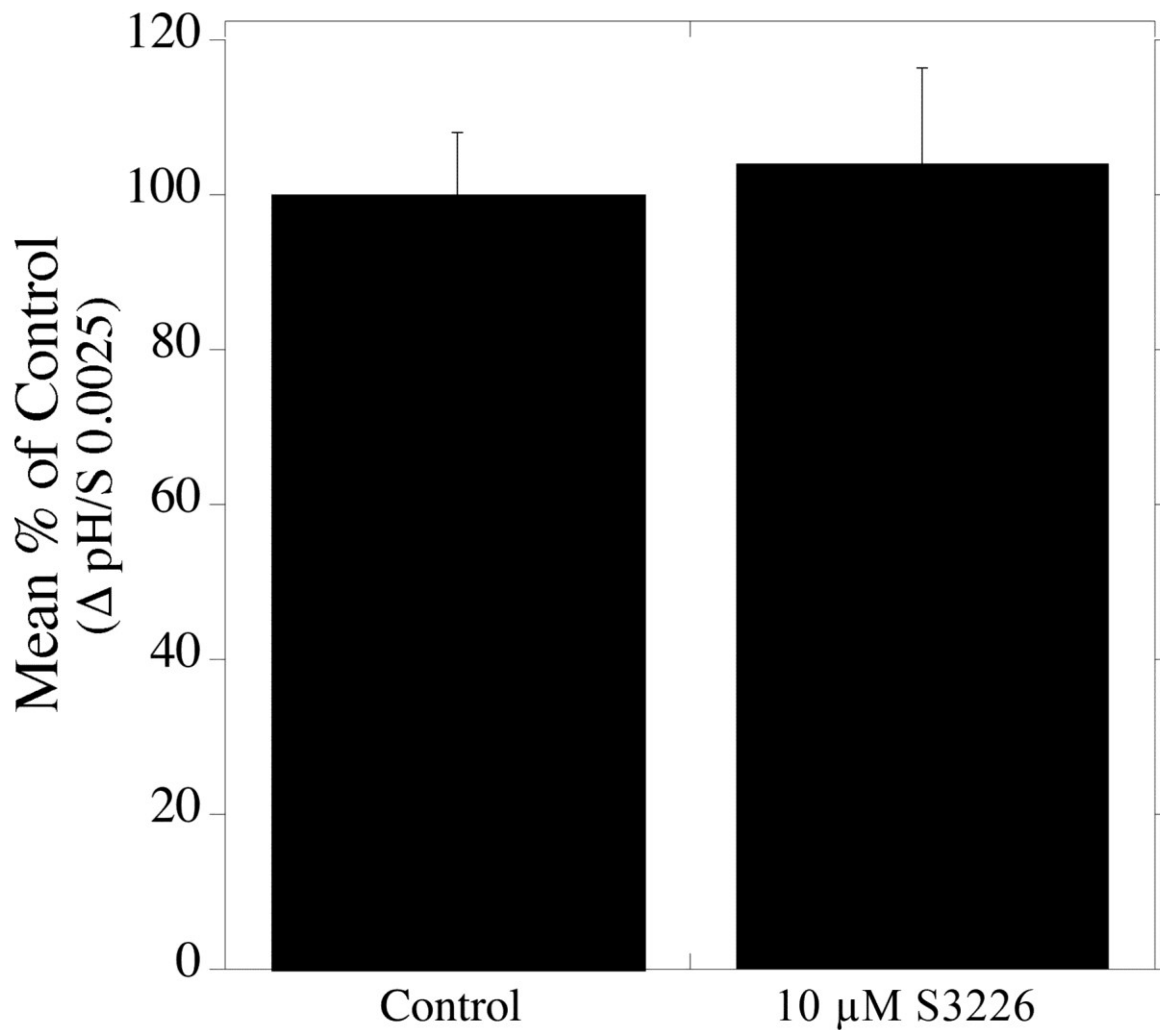
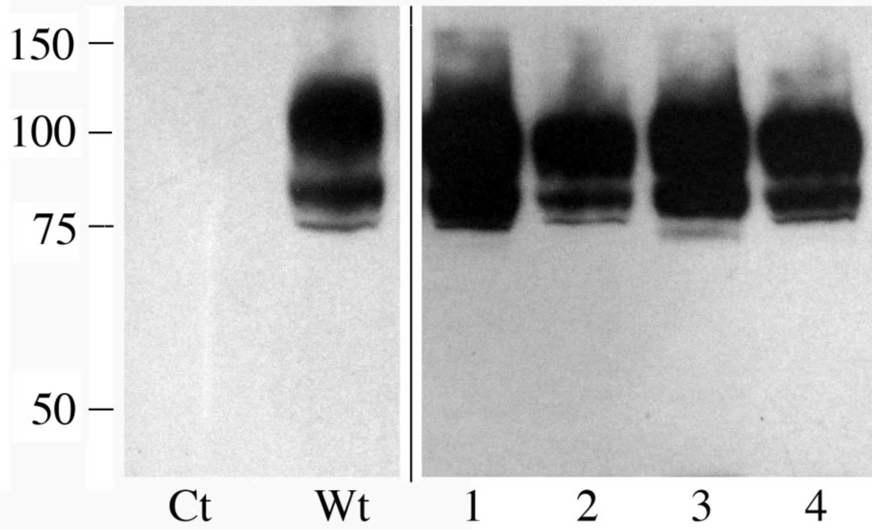


Figure 3.5: Effect of 10 μM S3226 on activity of the NHE1 protein in AP-1 cells. An inhibitor-resistant mutant form of the NHE1 protein was introduced into AP-1 cells. Using a single-pulse assay, the activity of the NHE1 isoform of the protein was determined in the presence or absence of S3226 as described in Section 2.2.3. S3226 was present throughout assay.


A
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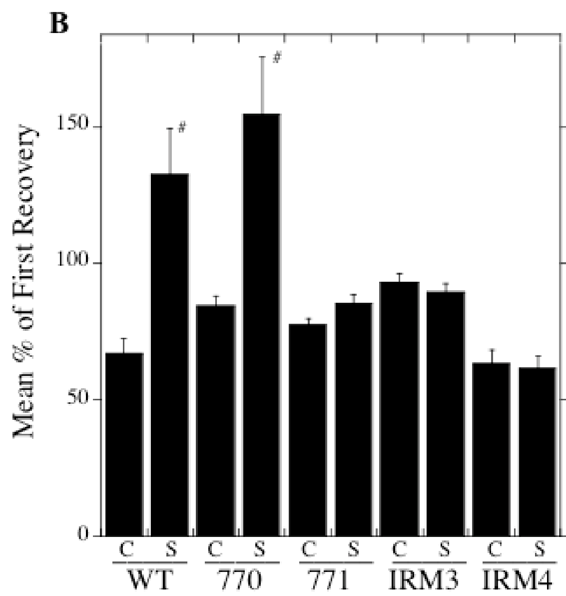
Ct
Wt
1
2
3
4


B
Mean % of First Recovery
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50
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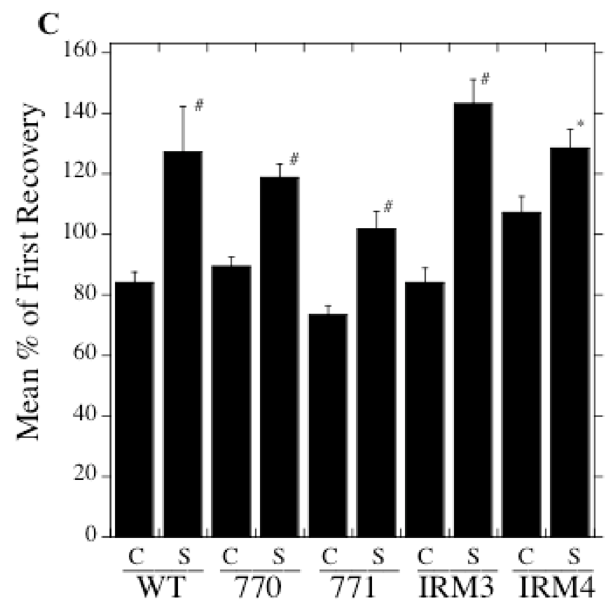
150
#
#
C S C S C S C S C S
WT 770 771 IRM3 IRM4


C
Mean % of First Recovery
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20
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C S C S C S C S C S
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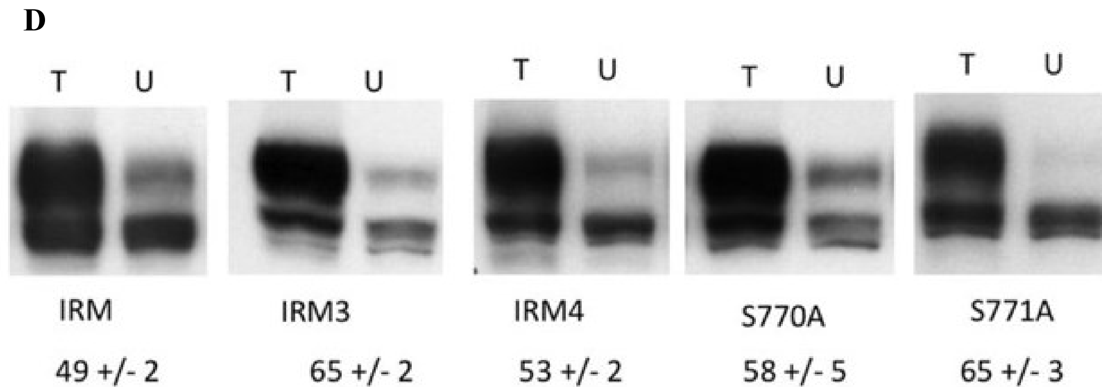


Figure 3.6: Analysis of mutated and wild-type NHE1 protein in MDCK and HEK cells. *A*: Western blot analysis of whole cell lysates of stable MDCK cell lines expressing HA-tagged NHE1 protein. Ct, control, whole cell lysate of MDCK cells mock transfected; Wt, lysate of MDCK cells stably transfected with wild-type NHE1 protein. *Lanes 1–4*: stable cell lines of NHE1 protein containing the mutants, S770A, S771A, IRM3, and IRM4, respectively. (Cells were not normalized for protein concentration.) *B* and *C*: effect of mutations of phosphorylation sites on induction of activity of NHE1 by SIA. *B*: stable cell lines of wild-type (WT) or indicated mutant NHE1 proteins in MDCK cells. Cells lines were subjected to dual pulse NHE activity assay essentially as described in Fig. 4. NHE1 activity was assayed in the presence of 10 μM EMD87580 and 3 μM S3226 as indicated. Value of the 2nd recovery from acidosis was compared with the 1st recovery, and results are expressed as percent of the 1st pulse. "C" indicates a dual pulse assay control in the absence of sustained intracellular acidosis. "S" indicates the 2nd pulse was after a period of sustained intracellular acidosis. *C*: as in *B* except in HEK 293 cells. Results are means ± SE of at least 6 experiments. $^{*}P < 0.05$, $^{\#}P < 0.01$, significantly different from the control. Absolute values of ΔpH change/min for all MDCK cell types uncorrected for levels of protein expression were from 0.23 to 0.43. For HEK cells, the values were between 0.34 to 1.2 ΔpH/min. *D*: surface localization of NHE1 of wild-type and mutant proteins. Sulfo-NHS-SS-biotin-treated MDCK cells were lysed proteins were solubilized and subsequently treated as described in the materials and methods. Equal samples of total lysates (T) and unbound (representing intracellular) lysates (U) were resolved on SDS-PAGE and probed with anti-HA antibody to identify NHE1 protein. Amount of surface localized NHE1 was calculated from densitometric analysis by taking the (total protein) − (unbound protein) = (membrane surface localized protein). Percentage of the total NHE1 protein localized to the plasma membrane is indicated. Results are means ± SE; *n* = at least 3 experiments.

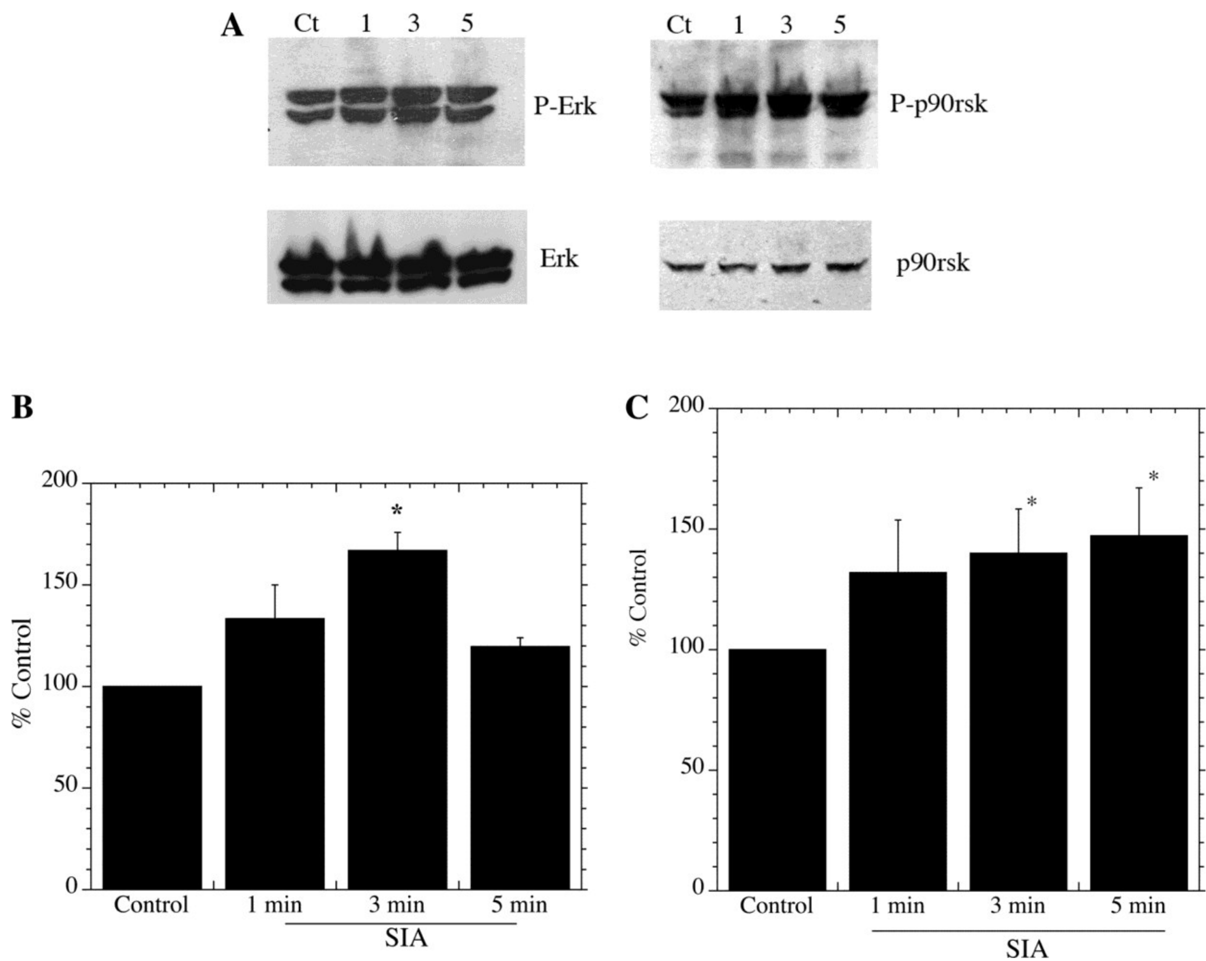
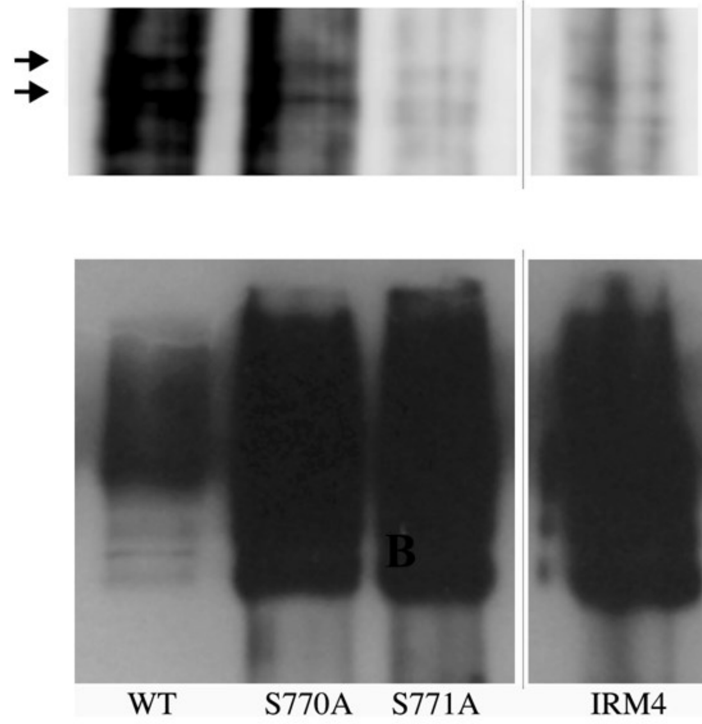
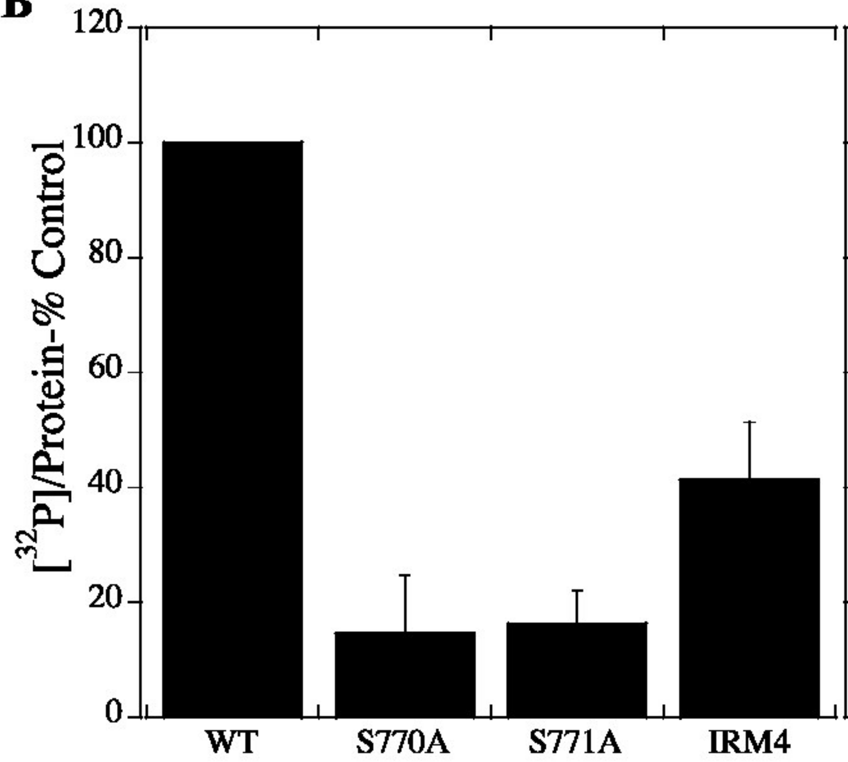


Figure 3.7: Time course of activation of ERK and p90rsk by sustained intracellular acidosis in MDCK cells. Cells were subjected to SIA for the times indicated and the level of phospho-ERK and phosphor-p90^{rsk} was examined by Western blotting. *A*: examples of Western blotting for phospho-ERK and phospho-p90^{rsk}. ERK and p90^{rsk} were immunoblotted to control for the absolute level of the protein. *B* and *C*: summary of experiments examining phospho-ERK (*B*) and phospho-p90^{rsk} (*C*) levels relative to the levels of ERK and p90^{rsk}. Results are means ± SE of 3–5 experiments. *$P < 0.05$, significantly different from the control.


A
B
WT
S770A
S771A
IRM4


B
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[32P]/Protein-% Control
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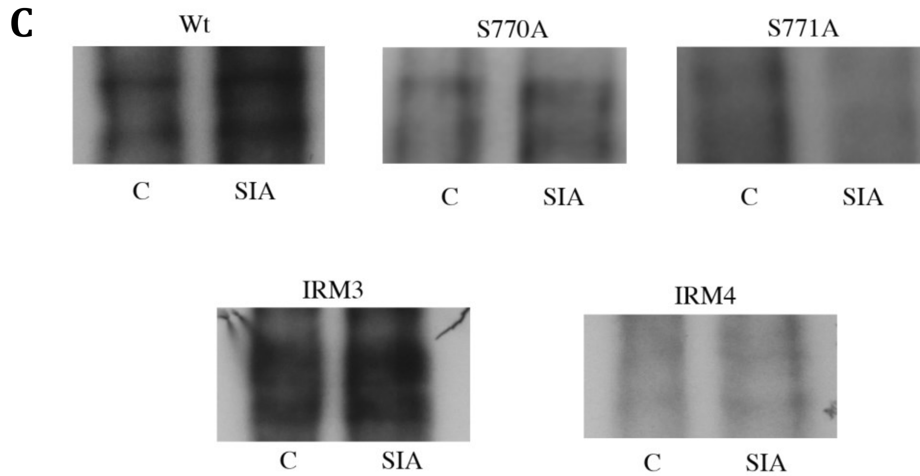
S770A
S771A
IRM4



D

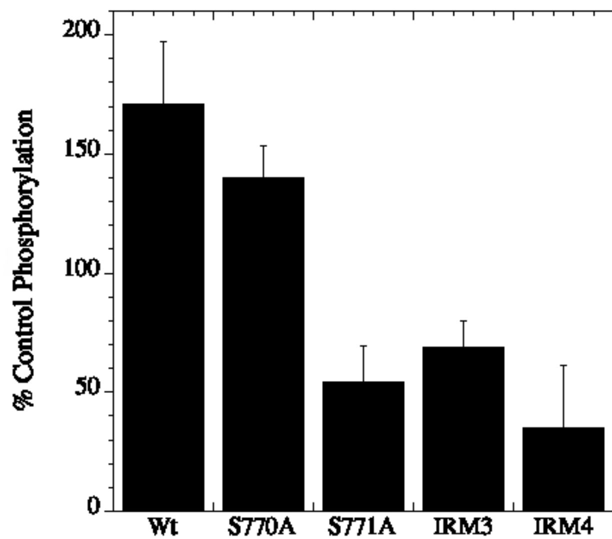


Figure 3.8: Effect of mutation of phosphorylation sites on phosphate incorporation in the NHE1 protein. *A*: stable cell lines of wild-type or NHE1 mutant-containing MDCK cells. Cells were incubated in [^{32}P]-containing media and immunoprecipitated as described in materials and methods. Cells were not stimulated by SIA. *Top*: [^{32}P] profile of immunoprecipitate. *Bottom*: immunoblot of immunoprecipitate using anti-HA tag antibody. *B*: summary of experiments in *A*. Results are means ± SE of at least 3 experiments. *C*: effect of SIA on phosphorylation levels of wild-type or NHE1 mutant-containing MDCK stable cell lines. Cells were incubated in [^{32}P]-containing media, and NHE1 was immunoprecipitated as described in materials and methods after SIA treatment. Examples of [^{32}P] levels of immunoprecipitates are shown. *D*: summary of experiments in *C*. Phosphorylation levels were corrected for the levels of immunoprecipitated protein which was determined using anti-HA tag antibody. Results are means ± SE of at least 3 experiments.

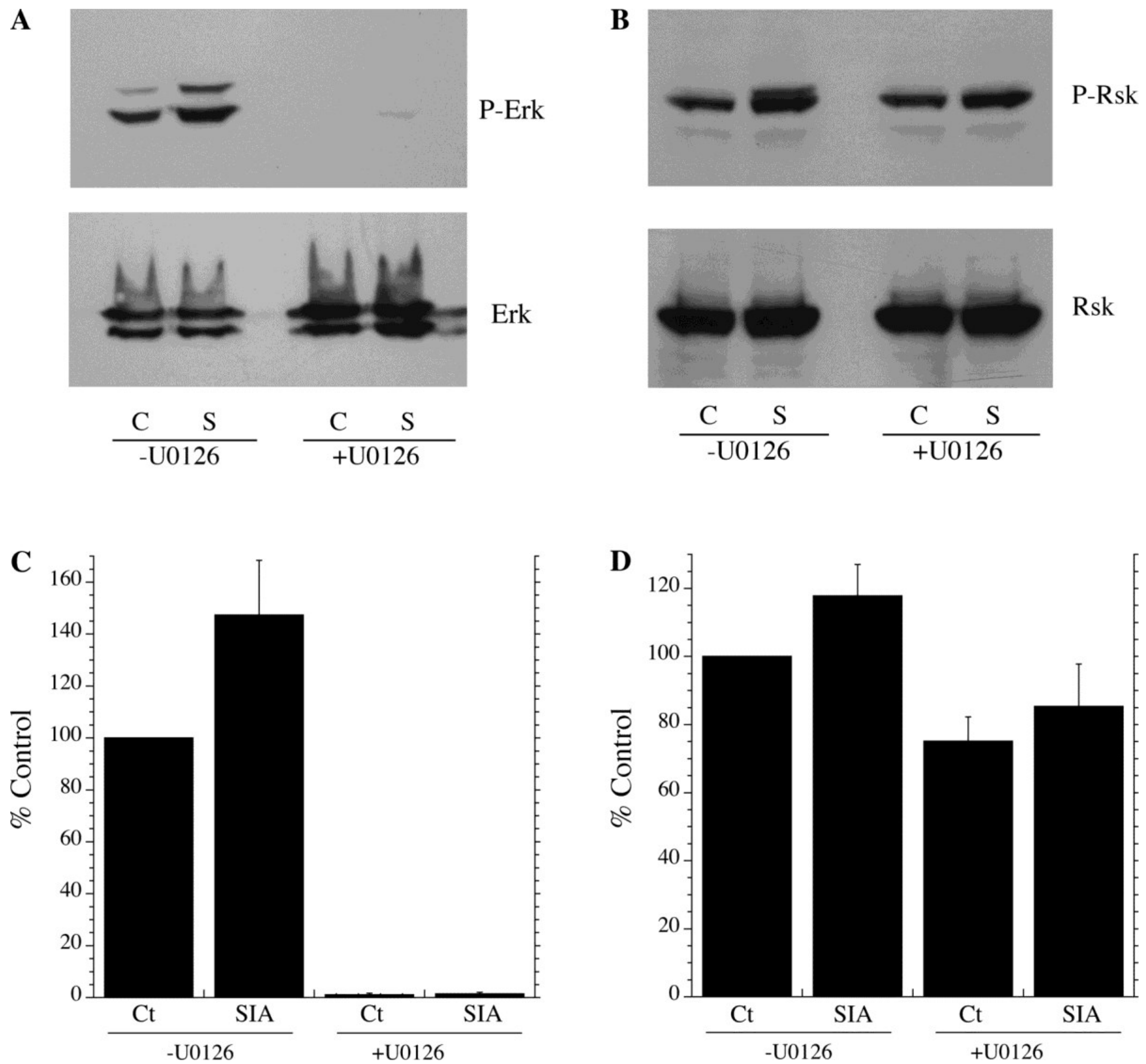


Figure 3.9: Effect of U0126 on phosphorylation levels and activation of ERK and p90rsk by sustained intracellular acidosis. **A**: example of Western blotting for ERK and phospho-ERK. *B*: example of Western blotting for RSK and phospho-RSK. *C* and *D*: summary of experiments in *A* and *B*, respectively. Results are means ± SE of at least 3 experiments.

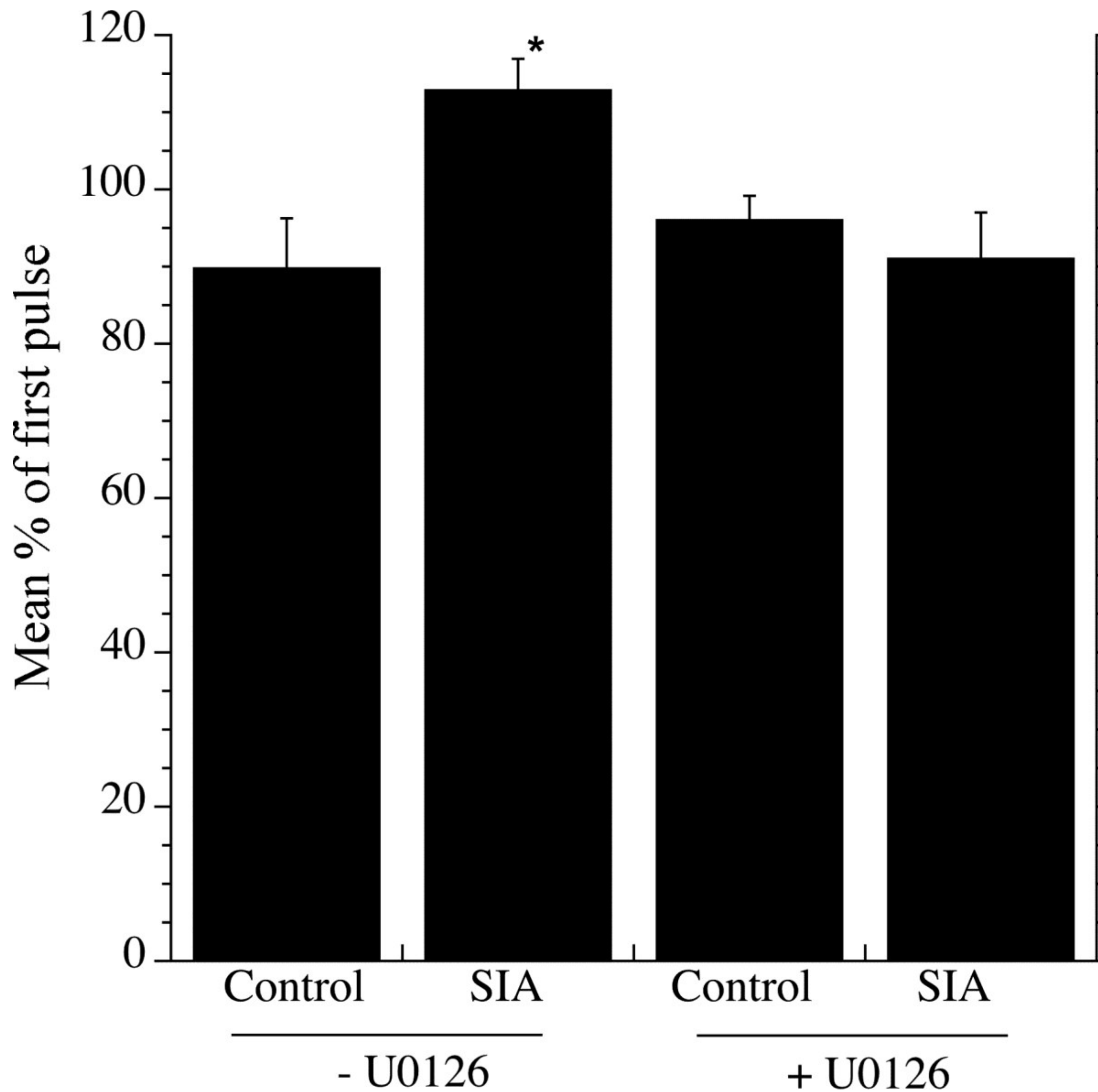


Figure 3.10: Effect of U0126 on NHE1 activity after SIA. MDCK cells overexpressing NHE1 were subjected to a two pulse Na^+/H^+ exchanger activity assays. Rate of recovery after the 2nd pulse was compared with that of the 1st in the presence or absence of sustained intracellular acidosis. S3226 was present in all assays (3 μM). U0126 was present (10 μM) where indicated. Values summarized are means ± SE of at least 10 experiments. $*P < 0.05$, significantly different from the control.

3.3 Discussion

Renal tissues are subject to both chronic metabolic or respiratory acidosis which result in a host of adaptive changes. While it has been demonstrated that chronic acidosis elevates renal NHE1 message levels (230,231), the acute effects on activity are not well characterized. We recently demonstrated that acute acidosis activates NHE1 via an ERK-dependent pathway in CHO cells (233)and in isolated cardiomyocytes. In these cell types, amino acids Ser770 and Ser771 were shown to be phosphorylated in response to sustained intracellular acidosis and were important in stimulation of NHE1 activity. While the effects of acute acidosis on the NHE3 isoform have been characterized in renal cell types (16), the NHE1 isoform has not been well studied. Whether it is activated by short-term acidosis and any putative molecular mechanism is unknown. We initially characterized two renal-derived cell lines HEK293 cells and MDCK cells. HEK293 cells were derived by transformation of primary cultures of human embryonic kidney cells with adenovirus DNA (213). MDCK cells are a well-characterized epithelial cell line derived from the kidney of a female adult cocker spaniel. MDCK are derived from the distal tubule/collecting duct and have retained morphological traits and enzymatic properties characteristic of their tissue of origin. They have been reported to express NHE1 at both apical and basolateral sides of these cells (221).

Initial experiments determined that both NHE1 and NHE3 protein and activity were present in HEK and MDCK cells, confirming previous observations. While it has been reported that endogenous NHE3 is not expressed in MDCK cells (239), other reports have shown that it can be induced in this cell type and a basal level of expression was shown (240). NHE1-dependent activity was shown by inhibition with EMD87580.

Additionally, we found that we were able to inhibit endogenous NHE3 with the compound S3226. S3226 is a specific inhibitor of NHE3 with very low efficacy of inhibition of NHE1 (235). We confirmed that S3226 was ineffective in inhibition of NHE1 by examining its effectiveness in AP-1 cells that expressed only NHE1 isoform (**Figure 3.7**). This experiment also confirmed that NHE1 with the IRM mutation was not sensitive to inhibition by S3226. We therefore included S3226 in assays of NHE1 activity so that we could examine effects on NHE1, free of any residual activity of NHE3.

When we treated either MDCK or HEK cells with sustained intracellular acidosis for a period of 3 min, both cell types had increased NHE1 activity upon recovery. EMD87580 attenuated NHE1 activity with or without SIA treatment (not shown). ERK activation occurred within 3 min in both cell types. We found more consistent activation of ERK in MDCK cells as opposed to HEK cells. Depletion of intracellular Na^+ could theoretically alter the cellular Na^+ gradient and augment NHE1 activity directly. We therefore induced SIA by use of NHE inhibitors, and this SIA induced in the presence of normal Na^+ still caused an increase in NHE1 activity. Other studies have also demonstrated that SIA stimulates NHE1 activity in the presence of normal external Na^+ (229,233). Prolonged depletion of extracellular sodium can deplete intracellular sodium; however, our assays only used 3 min of sodium depletion making it unlikely that intracellular sodium was depleted (241).

We made stable cell lines of MDCK and HEK cells expressing the NHE1 protein. The exogenous protein was both HA tagged and contained mutations that render it resistant to inhibition by EMD87580 (211). We were therefore able to measure

exogenous NHE1 activity by inhibiting endogenous NHE1 activity with EMD87580 and inhibiting any NHE3 activity with S3226. We examined the effect of SIA on the rate of NHE1 activity in both HEK and MDCK cells. For these experiments we examined activity of the wild-type NHE1 protein and of the NHE1 protein with the mutations S770A, S771A, S776/770/771A (IRM3), and T779A/S785A (IRM4). HEK cells gave a pattern of activation of NHE1 that was independent of the nature of the mutant protein. In contrast, mutation of Ser771 or the IRM3 and IRM4 group of mutations specifically eliminated activation of the NHE1 protein in MDCK cells while the wild-type NHE1 protein and the S770A mutant were activated by SIA. We examined basal phosphorylation levels of the exogenous wild-type NHE1 protein in comparison wild type. Mutation of either Ser770, Ser771, or the IRM4 site caused large decreases in the overall level of basal phosphorylation of the NHE1 protein. This indicated that a significant amount of the rapidly exchangeable phosphate incorporation into the NHE1 protein was in these amino acids. There was an indication that mutation of one site might affect phosphorylation levels at other sites, since both the S770A and S771A appeared to cause large reductions in the level of NHE1 basal phosphorylation. This may be through a change in the protein kinase consensus site or by a change in the conformation of the protein in this region, which affects phosphorylation of the adjacent site.

To gain insights into the mechanism by which SIA stimulated NHE1 activity, we examined the effect of SIA on the phosphorylation levels of the wild-type and mutant NHE1 proteins in MDCK cells. We found that our results mirrored the effects on NHE1 activity. Mutation of amino acids S771A or the IRM4 site prevented increases in the phosphorylation level of NHE1 in response to SIA. However, the mutation of S770A did

not prevent an increase in NHE1 phosphorylation in response to SIA. These results suggest that amino acid Ser771 and the phosphorylation sites Thr779/Ser785, in the IRM4 site, are important targets of NHE1 when stimulated by SIA.

These results have both similarities and differences from those that were reported earlier. In the present study we showed that Ser771 was critical in activation of NHE1 by SIA and that when it was mutated there was no enhancement of NHE1 phosphorylation levels by SIA. In contrast Ser770 was not critical for either activation of NHE1 activity or for elevated phosphorylation in response to SIA. However, previously we demonstrated that in CHO cells and in isolated cardiomyocytes both Ser770 and Ser771 were important in activation of NHE1 by SIA (96,97). Ser771 precedes Pro772 and forms a consensus sequence for ERK (242). We had earlier suggested that Ser770 may form part of the kinase binding site (97). Differences in conformation of this part of the tail region, perhaps induced by binding of tissue-specific regulatory proteins, might account for the differences shown in this study.

The IRM4 region of NHE1 contains Thr779 and Ser785. In the present study, we found that this region was important in activation of NHE1 activity by SIA. Mutation of these two amino acids to Ala prevented increased phosphorylation of NHE1 by SIA. Previously, in CHO cells, we showed that mutation of this site also prevented increases in NHE1 activity in response to SIA and there was an intermediate effect on phosphorylation levels (96). However, in isolated cardiomyocytes, mutation of this region did not prevent activation of the NHE1 protein and only had a partial effect on elevation of phosphorylation levels. It appears as though the relative importance of this

region varies, depending on the cell type. Tissue-specific regulation of NHE1 has been reported earlier. In the myocardium for example, the Hill coefficient of activation is near 3, steeper than in some other cell types indicating a different regulatory mechanism (243).

An interesting observation of the present study was that the total level of basal NHE1 phosphorylation was greatly reduced by mutation of Ser770 and Ser771. Additionally, mutation of Thr779 and Ser785 also reduced the total level of basal NHE1 phosphorylation. We earlier reported that in cardiomyocytes mutation of Ser770 and Ser771 reduced the basal level of phosphorylation of NHE1 greatly, while mutation of Thr779 and Ser785 did not have this effect in these cells. Our results in the present study with MDCK cells followed the same pattern, although we found mutation of Thr779 and Ser785 had a more significant effect, which correlated with their functional role. Why mutation of either Ser770 or Ser771 would reduce most of the basal level of phosphorylation may be because mutation of one of the sites affects the others accessibility to regulation.

A critical role of ERK-dependent pathways in SIA was initially described by Haworth *et al.*(229). We also demonstrated that ERK is critical in SIA activation of NHE1 in CHO cells and in cardiomyocytes (96,97). The studies demonstrated that ERK and $p90^{RSK}$ can be activated by sustained acidosis in several cell type (96,97,229). Our results in MDCK cells confirm the importance of this pathway. U0126, the MEK inhibitor, prevented SIA activation of NHE1. It also blocked activation of the ERK-dependent pathway by SIA. The effect of U0126 was more pronounced on ERK phosphorylation levels than it was with $p90^{RSK}$. We have earlier demonstrated that ERK

can directly phosphorylate the NHE1 cytosolic domain *in vitro* at multiple regions including regions encompassing Ser770, Ser771, Thr779, and Ser785 (244). The present results demonstrate that this pathway of activation of ERK and $p90^{RSK}$ is also active in MDCK cells. While phosphorylation of Ser703 by $P90^{RSK}$ has been demonstrated to be important in some pathological circumstances (244), we demonstrated that it was not important in activation of the Na^+/H^+ exchanger by SIA in both CHO cells and cardiomyocytes (233). The present results are consistent with these observations and suggest that phosphorylation sites that are further downstream are more critical in activation of NHE1 by SIA.

While our experiments demonstrated that SIA activated NHE1 in HEK cells, we did not demonstrate that mutation of any of the residues Ser770, Ser771, Thr779, and Ser785 played a significant role in this activation in this cell type. This contrasted with results in MDCK cells and with our results in CHO cells and isolated cardiomyocytes. In HEK293 cells, it seems clear that activation of NHE1 by SIA occurs by another mechanism, which has yet to be elucidated. While this result appears surprising, recently results have suggested that HEK293 cells have an unexpected close relationship to neuronal cells and are not typical kidney cells. They have a pattern of intermediate filament expression similar to that of early differentiating neurons, and despite their wide use as kidney cells, they have properties of neuronal lineage cells and not more typical of kidney cells (245). This may explain their different regulation of NHE1 when compared with MDCK cells.

Overall, our study demonstrates for the first time that SIA acutely activates NHE1 activity in kidney cells. We have defined specific residues that are involved in this process and the pathway of activation involved.

Chapter 4

Protein-mediated regulation of renal NHE1

4.1 Introduction

The regulation of NHE1 shows tissue specificity even when the same isoform is expressed and this may be a reflection of the presence of various receptors, different regulatory kinases, phosphatases or other regulatory proteins present in the various tissues in which NHE1 is found (246).

NHE1 is ubiquitous in mammalian tissues. One tissue in which it is present is the kidney. There it has been identified by western blotting, measurement of its activity, or identification of its mRNA. The different renal cell lines and tissues in which it has been identified include human embryonic kidney cells (222) renal tubules (223), in inner medullary collecting duct (224), in whole renal cortex (247), Madin-Darby canine kidney (MDCK) cells (221), rat proximal tubule cells (205), and in M-1 cortical collecting duct cells (194). Reports show that it is present on both the apical and basolateral membrane of polarized renal cells MDCK cells (239) though it is usually regarded as being present on the basolateral membrane (248). In the kidney, NHE1 is involved in NaCl absorption, $NaHCO_3$ absorption, pH regulation and maintenance of cell volume. We recently demonstrated that NHE1 in the kidney is regulated by Erk-dependent phosphorylation of the regulatory cytosolic tail (94). While this undoubtedly is an important part of NHE1 regulation, regulation of NHE1 is thought to be through a combination of phosphorylation of the distal tail, and through protein-protein mediated interactions which can occur in either more proximal or more distal regions of the regulatory tail (67,246). Regulation of the NHE1 protein by protein-protein interactions has been documented in number of tissues where NHE1 has been shown to bind and be regulated by calmodulin (249), calcineurin homologous protein (117), B-Raf (250), heat shock

proteins (126), carbonic anhydrase (114), phosphatases (106,209) and other proteins (reviewed in (246)).

In this study, we examine protein mediated regulation of the NHE1 isoform of the Na^+/H^+ exchanger in renal cells. Using affinity chromatography and mass spectometry, we identified a profile of proteins that potentially bind to the cytoplasmic tail of NHE1 in the kidney. We also confirmed the interaction of 14-3-3 and heat shock proteins (Hsps), Hsp70 and Hsp90 by co-immunoprecipitation and immunoblotting.

Hsps are known as 'molecular chaperones' that play a vital role in protein biogenesis by aiding protein folding and translocation (251). The most abundant hsps are Hsp 60, Hsp 70 and Hsp90 which are of particular importance under conditions of cellular stress (251). Our lab had previously shown that Hsp70 interacts with NHE1 in an ATP-dependent manner in fibroblasts (126). This interaction has been suggested to be involved in lipopolysaccharide-induced inflammatory response in the liver and microglia (127,252). Our results confirm the interaction of NHE1 with Hsp70 in renal cells and the possible involvement of this association in hypoxia/reoxygenation.

A unique characteristic of Hsp90 that differentiates it from other chaperones such as Hsp 60 and Hsp70 is its substrate specificity (253). Most of these substrates are signal transduction molecules involved in cell growth and proliferation (254). Akt, a serine/threonine kinase and a signaling mediator, forms a chaperone-substrate complex with Hsp90, and this complex is required for Akt activation (253). Akt has also been shown to directly phosphorylate and activate NHE1 in fibroblasts (83). We show for the first time that Hsp90 interacts directly with NHE1 and our results indicate that this interaction may be involved in the Akt-mediated regulation of NHE1. Some of

experiments involved the use of specific inhibitors against Hsp90 and Akt. To inhibit Hsp90, we employed the use of 17-allyamino-17-demethoxygeldamycin (17-AAG), a derivative of one of the first Hsp90 inhibitors discovered, geldamycin. 17-AAG has been shown to produce less toxic side effects than geldamycin in clinical trials (255). MK2206,8-[4-(1-aminocyclobutyl)phenyl]-9-phenyl-1,2,4-triazolo[3,4-*f*] [1,6]naphthyridin-3(2*H*)-one hydrochloride [1:1], is an allosteric Akt inhibitor (256) which we used in inhibiting Akt in some of our experiment. Our results are the first demonstration of the mechanisms involved in Hsp90-mediated regulation of NHE1 in the mammalian kidney.

4.2 Results

4.2.1 Affinity chromatography of NHE1-associated proteins in kidney

To identify proteins that directly associate with NHE1 in the kidney, the C-terminal domain of rabbit NHE1 (PCRB) was tagged with GST and cross-linked to an Affi-Gel 10 matrix. Affi-Gel 10 matrices are made up of N-hydroxysuccinimide esters crosslinked to agarose gel bead support which can be used to couple various ligands in affinity chromatography techniques. Coupling can occur in both aqueous and non-aqueous solutions. Ligands with free alkyl group displace the N-hyroxysuccinimide group of the Affi-Gel matrix to form a stable amide bond with the matrix (Figure 4.1). The immobilized reactive esters are highly selective for primary amino groups, hence, non-specific reactions with the ligands are avoided using this technique.

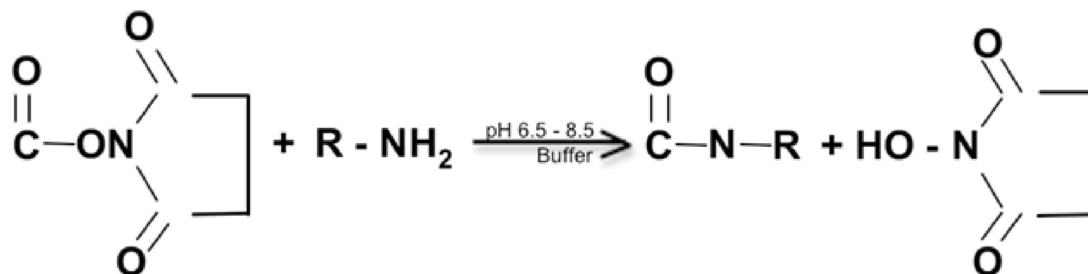


Figure 4.1: Coupling reaction of ligands with Affi-Gel 10. With suitable buffers at pH between 6.5 – 8.5, ligands displace the N-hydroxysuccinimide group to form a stable amide bond.

As a control, GST protein was also cross-linked to the Affi-Gel 10 matrix and this allowed us to identify those proteins that are specifically associated with the cytoplasmic domain of NHE1. The Affi-Gel 10 matrices were incubated overnight with solubilized kidney extracts and washed extensively afterwards. Bound proteins were then eluted with 1% SDS, precipitated with trichloroacetic acid and analysed by SDS-PAGE and mass spectrometry.

Figure 4.2 illustrates NHE-associated proteins analysed by SDS-PAGE. Many of the bands are clearly specific for GST-NHE1 column compared to the control (GST) column. Table 1 summarizes some of the interacting proteins that were unique to the NHE1-GST and not present in the proteins eluted from GST affinity chromatography. Four of these, HSP70, HSP90, 14-3-3 and Na^{+}/K^{+} -ATPase have been suggested to be in association with the NHE1 protein earlier (126,131,257).

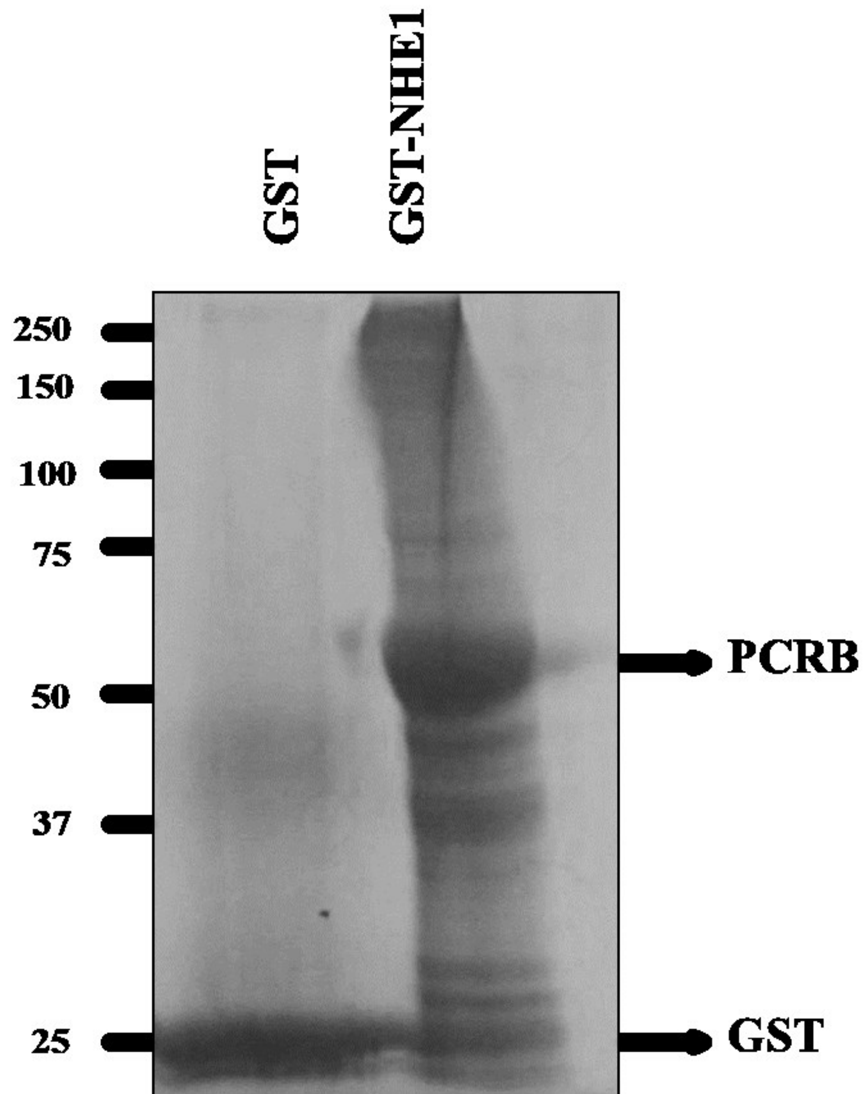


Figure 4.2: NHE1-associated kidney proteins eluted from GST-PCRB and GST columns. Eluted proteins from Affi-Gel-GST or Affi-Gel-GST/PCRB matrices were separated by SDS-PAGE and stained with Coomassie Blue G-250. The bands were trypsin-digested and analysed by mass spectrometry.
Mass spectrometry analysis are outlined in Table 1 below.

Table 2: List of NHE1-interacting peptides unique to PCRB-GST versus GST (control)

Accession #	Protein	PSM	Function
P63017	Heat Shock cognate 71 kDa protein	68	Signal transduction
A2ARV4	Low-density lipoprotein receptor-related protein 2	28	Molecular Chaperone
Q9D0K2	Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial	11	Metabolism
Q8VDN2	Sodium/potassium-transporting ATPase subunit α-1	10	Ion transport
Q61696	Heat shock 70kDa protein 1A	8	Signal transduction
P60710	Actin,cytoplasmic 1	7	Cell Motility
P17751	Triosephosphate isomerase	7	Metabolism
P16858	Glyceraldehyde-3-phosphate dehydrogenase	6	Metabolism
P16125	L-lactate dehydrogenase B chain	6	Metabolism
O88338	Cadherin-16	5	Cell Adhesion
P26443	Glutamate dehydrogenase 1, mitochondrial	5	Metabolism
P11499	Heat shock protein HSP 90-β	5	Signal transduction
P63101	14-3-3 protein zeta/delta	4	Signal transduction
Q9CQV8	14-3-3 protein β/α	3	Signal transduction
Q99KI0	Aconitate hydratase, mitochondrial	3	Metabolism
Q9CPY7	Cytosol aminopeptidase	3	Housekeeping
Q61425	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	3	Metabolism
P14152	Malate dehydrogenase, cytoplasmic	3	Metabolism
P09405	Nucleolin	3	Biosynthesis
Q9D051	Pyruvate dehydrogenase E1 component subunit beta	3	Metabolism
Q99L13	3-hydroxyisobutyrate dehydrogenase, mitochondrial	2	Metabolism
Q8VCR7	α/β hydrolase domain-containing protein 14B	2	Transcription
Q8K0L3	Acyl-coenzyme A synthetase ACSM2, mitochondrial	2	Metabolism
P45376	Aldose reductase	2	Metabolism
Q9CZ13	Cytochrome b-c1 complex subunit 1, mitochondrial	2	Metabolism
P08113	Endoplasmin	2	Biosynthesis
Q99LC5	Electron transfer flavoprotein subunit alpha, mitochondria	2	Metabolism
Q64467	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	2	Metabolism
P17156	Heat shock 70 kDa protein 2	2	Signal transduction
Q61847	Meprin A β-subunit	2	Inflammation
Q01853	Transitional endoplasmic reticulum ATPase	2	Protein degradation

4.2.2 Immunoblot analysis of NHE1-associated kidney proteins

To examine if some of the proteins identified by mass spectrometry were in association with NHE1 in live cells, we used co-immunoprecipitation experiments. The NHE1 protein was immunoprecipitated from MDCK cells using anti- HA tag antibody. Immunoprecipitated proteins were run on a SDS-PAGE gel, transferred to a nitrocellulose membrane and immunoblotted with available antibodies.

Figure 4.3 illustrates typical results confirming the identification of Hsp 70, Hsp90 and 14-3-3 by mass spectrometry. A mock immunoprecipitation was done from MDCK cells that did not express the tagged NHE1 protein. The Co-IP complex was solubilized with SDS-PAGE sample buffer for 3 min at 95-100^{0}C. After SDS-PAGE analysis proteins were transferred onto nitrocellulose for immunoblotting (IB) was done with anti-HA tag antibody or with antibody indicated.

Fig. 4.3A shows that Hsp70 immunoprecipitates with the NHE1 protein. There was evidence of Hsp70 in the control, however it was greatly increased in the immunoprecipitation from MDCK cells transfected with HA tagged NHE1. Immunoblotting with anti HA antibody confirmed that the immunoprecipitation was successful. Fig. 4.3B illustrates a similar experiment with 14-3-3 protein. There was a clear association of 14-3-3 with the immunoprecipated NHE1 protein. Despite repeated attempts, under varying conditions, we were unable to co-immunoprecipitate Na^+/K^+ -ATPase with NHE1 (Fig. 4.3C). Using anti Hsp90β antibody, we co-immunoprecipitated Hsp90 with NHE1 (Fig. 4.3D).

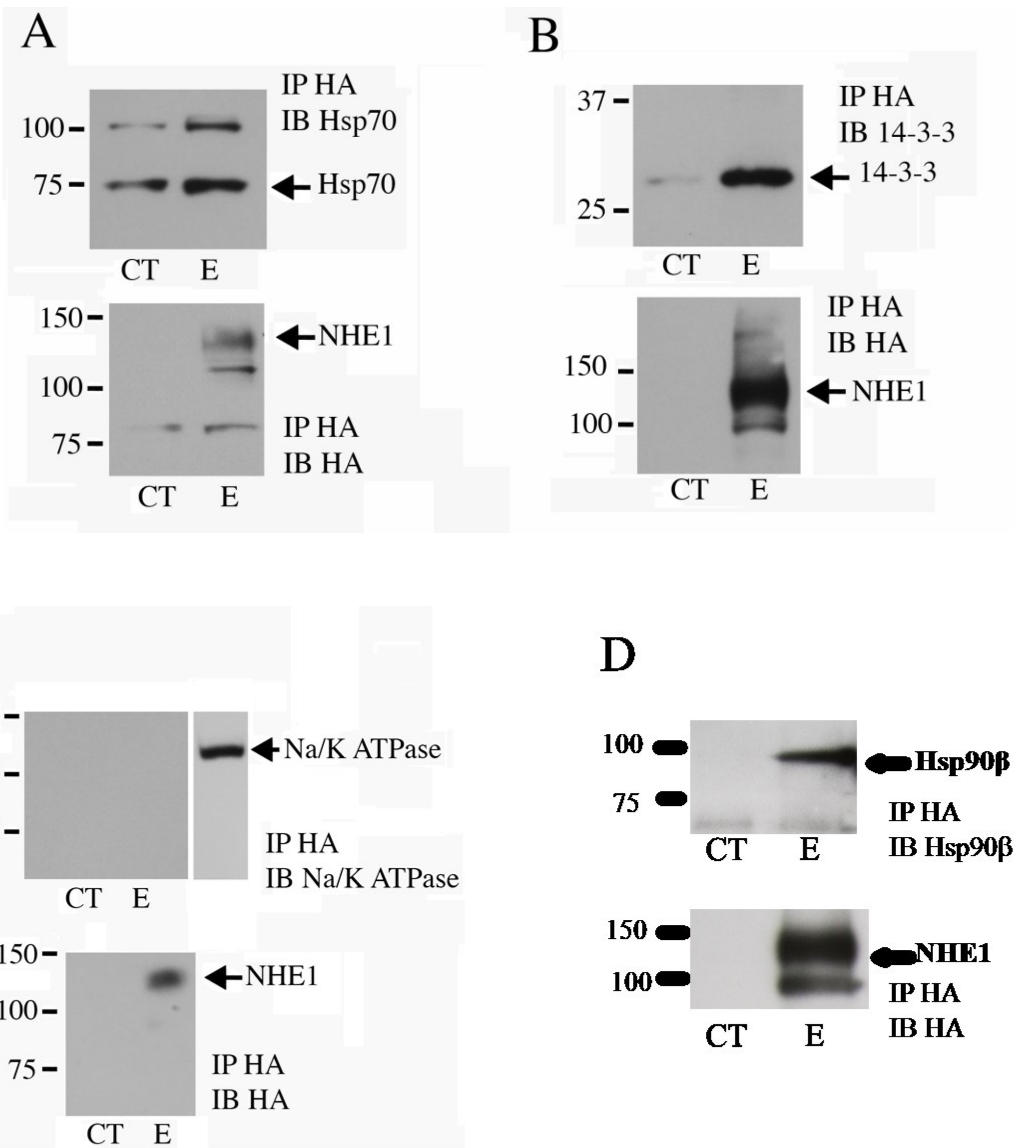


Figure 4.3: Co-immunoprecipitation of HA-NHE1 and associated proteins.

NHE1 was immunoprecipitated from MDCK cells with transfected with HA-tagged NHE1 protein or from MDCK cells without the HA-NHE1 protein (CT). After immunoprecipitation with rabbit polyclonal antibody, samples of the control (CT) and experimentals (E) were immunoblotted with antibodies against HSP70, 14-3-3, Na^{+}/K^{+} -ATPase and HSP90 (Fig. A-D respectively). Upper panels, immunoblots using antibody against putative co-immunoprecipitating proteins indicated. Lower panel, immunoblot against HA tag using monoclonal anti-HA antibody to confirm the immunoprecipitation of the HA-NHE1 protein. Adjacent lanes at right are cell lysates (WCL) used as a positive control where necessary. Arrow denotes location of NHE1 protein or other proteins indicated. Results are typical of at least 3 experiments.

4.2.3 Effects of sustained intracellular acidosis on NHE1 association with 14-3-3

To examine if 14-3-3 binding to the NHE1 protein varied with the level of NHE1 activity we treated cells with sustained intracellular acidosis, which we have earlier shown activates NHE1 activity in these cells through phosphorylation dependent mechanisms (94). 14-3-3 binding to NHE1 has been shown to affect activity of the protein and has also been reported to increase following ischemia and reperfusion. We therefore examined the amount of 14-3-3 binding to NHE1 under conditions of stimulation of activity of the protein by sustained intracellular acidosis vs. control. SIA was induced in MDCK cells stably expressing HA-NHE1(IRM) by incubating the cells in a Na^+-free buffer for 3 min at 37ºC (as described in Section 2.6). NHE1 was then immunoprecipitated from cell lysate via the HA tag, transferred to a nitrocellulose and immunoblotted for 14-3-3 co-immunoprecipitation. The results showed that there was no change in the level of 14-3-3 binding with sustained intracellular acidosis (Figure 4.4).

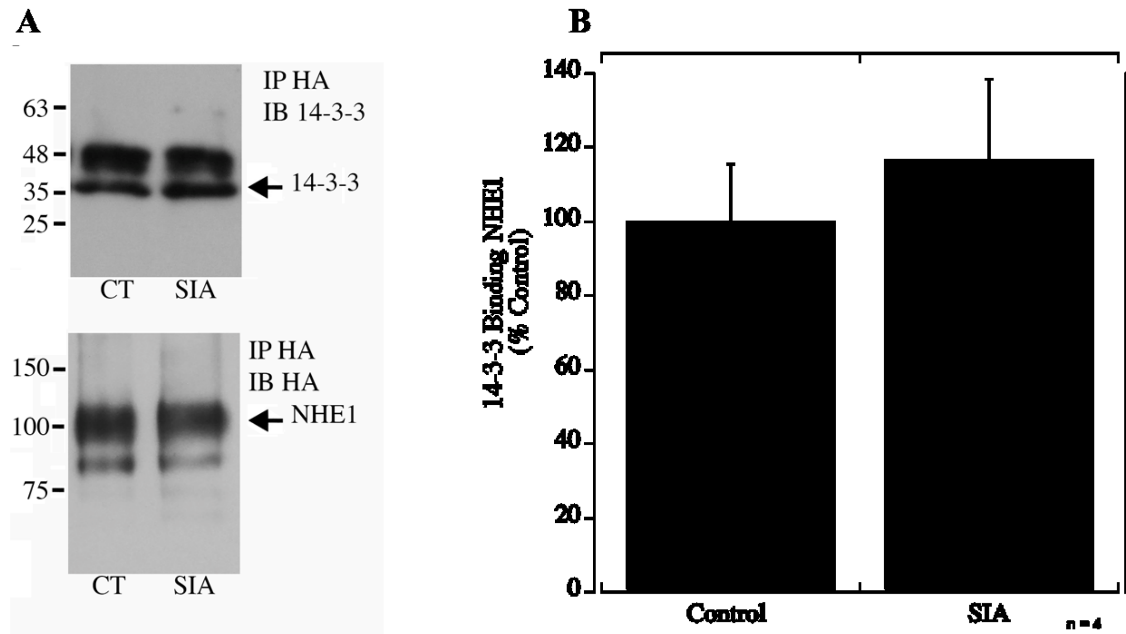


Figure 4.4: Association of NHE1 with 14-3-3 following SIA. MDCK cells were subjected to SIA as described in Section 2.6. A, *Top panel;* NHE1 was immunoprecipitated via the HA-tag and immunoblotted for 14-3-3 binding. *Bottom panel;* immunoblot for total NHE1 precipitated. B, summary of co-immunoprecipitation experiment. N = 3

4.2.4 Regulation of NHE1 activity by heat shock proteins following cellular stress

Heat shock proteins are upregulated during cellular stress such as heat, hypoxia and ischemia (258). Our laboratory has also shown that increased expression of NHE1 predisposes cardiomyocytes to apoptotic damage induced by hypoxia/reoxygenation (259). We therefore examined the effect of hypoxia on NHE1 activity in kidney cells. MDCK cells overexpressing NHE1 were assayed for NHE1 activity after being subjected to the stress of hypoxia followed by reoxygenation. Fig. 4.5A, B shows that hypoxia caused a decrease in NHE1 activity after treatment. The presence of the specific NHE1 inhibitor EMD87580 in the NHE1 assay prevented NHE1 activity in either the normoxia or hypoxia treated cells (Figure 4.5B).

To understand the role of heat shock proteins in regulating NHE1 activity, we examined the association of Hsp70 and Hsp90 to NHE1 under normoxic and hypoxic conditions. Following hypoxia/reoxygenation, the association of Hsp70 and Hsp90β with NHE1 in MDCK cells was examined by co-immunoprecipitation and immunoblotting. The results are illustrated in Figure 4.6 and 4.7 and show about 90% and 50% increase in the association of Hsp70 and Hsp90 with NHE1 following hypoxia compared to normoxia respectively.

To further explore the role of Hsp90 in NHE1 activity, we also used the compound, 17-AAG, which is an inhibitor of Hsp90. Fig. 4.8A shows the effect of 17-AAG on NHE1 activity in MDCK cells. Treatment of cells with 17-AAG resulted in approximately a 40% decrease in NHE1 activity.

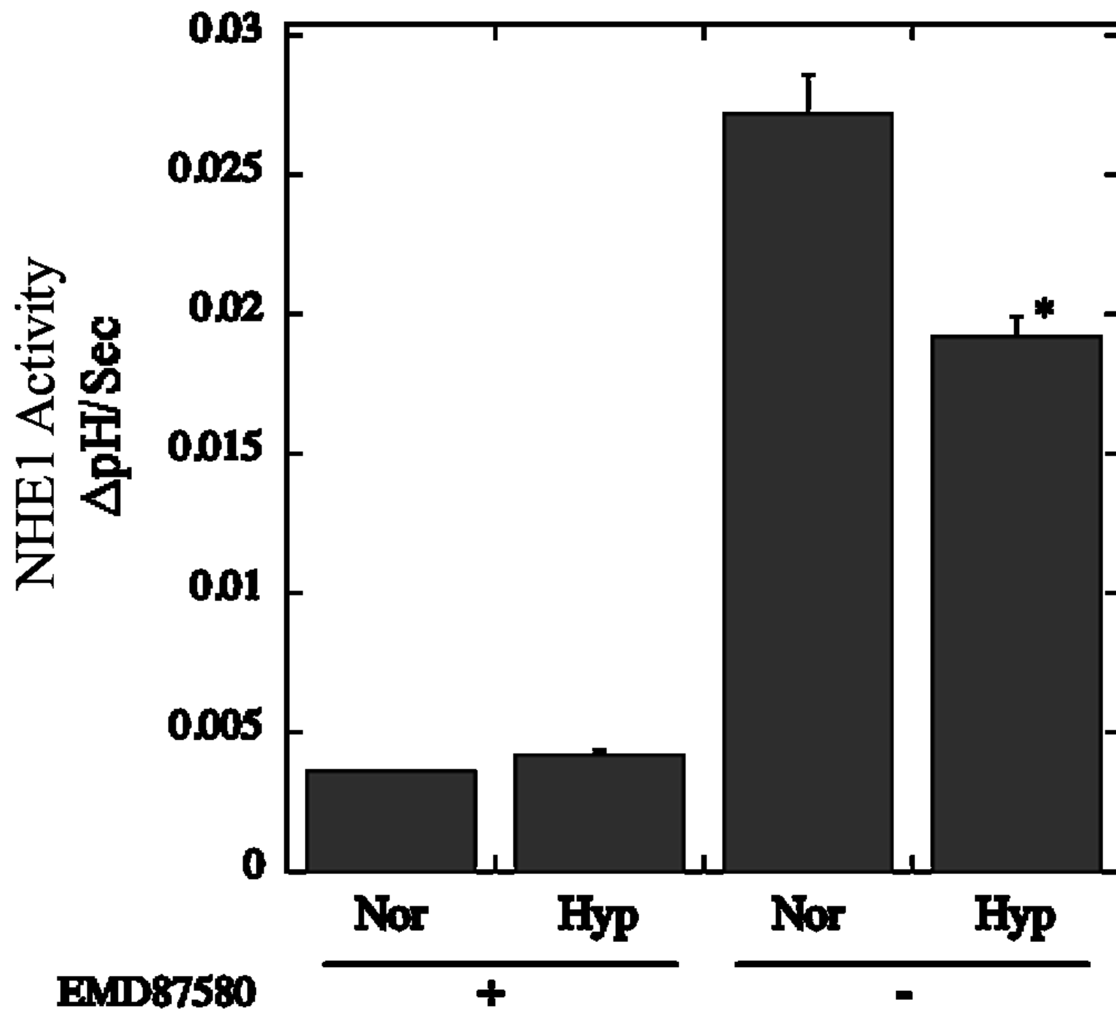
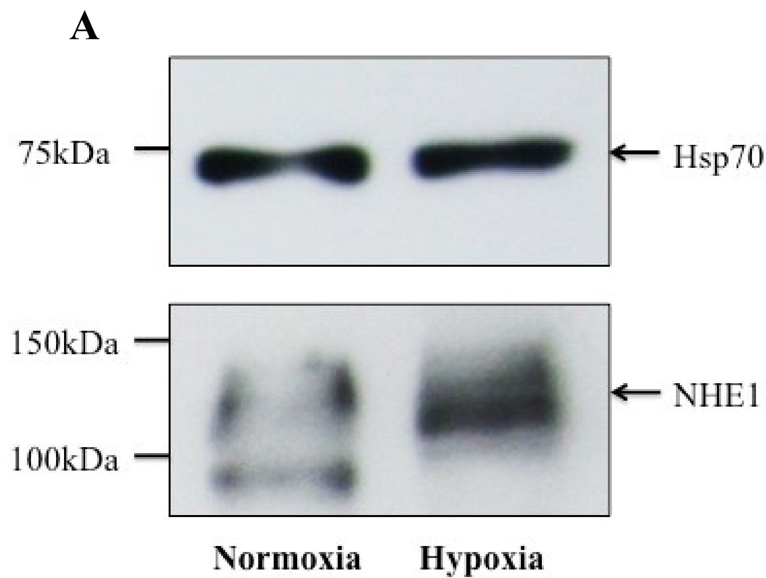


Figure 4.5: Effect of hypoxia on Na+/H+ exchanger activity of MDCK cells. MDCK cells overexpressing NHE1 were subjected to hypoxia/reoxygenation (hypoxia 18 h followed by reperfusion 2 hr) and assayed for NHE1 activity. Summary of the initial rate of recovery of NHE1 activity. Results are the mean ± SE of 10 experiments. Na^+/H^+ exchanger activity was determined as measured in the "Materials and Methods". * significantly decreased from control value at $P < 0.001$. Where indicated, NHE1 activity was assayed in the presence of 10 μM EMD87580.



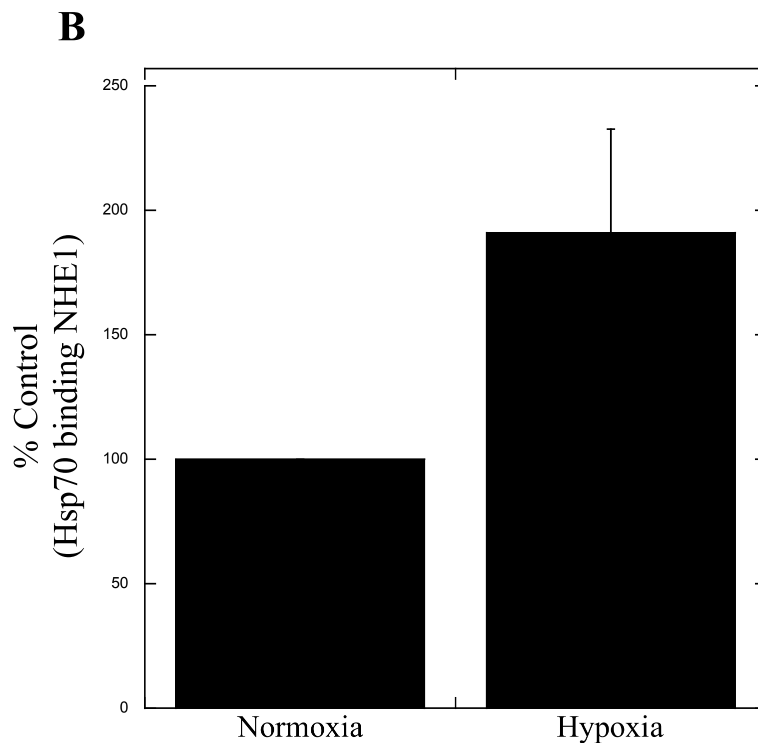


Figure 4.6: Association of NHE1 with Hsp70 following hypoxia/reoxygenation. MDCK cells were subjected to hypoxia/reoxygenation. A, Top panel; NHE1 was immunoprecipitated via the HA-tag and immunoblotted for Hsp70 B, summary of co-immunoprecipitation experiment. Results were normalized to the total amount of immunoprecipitated NHE1. n=3

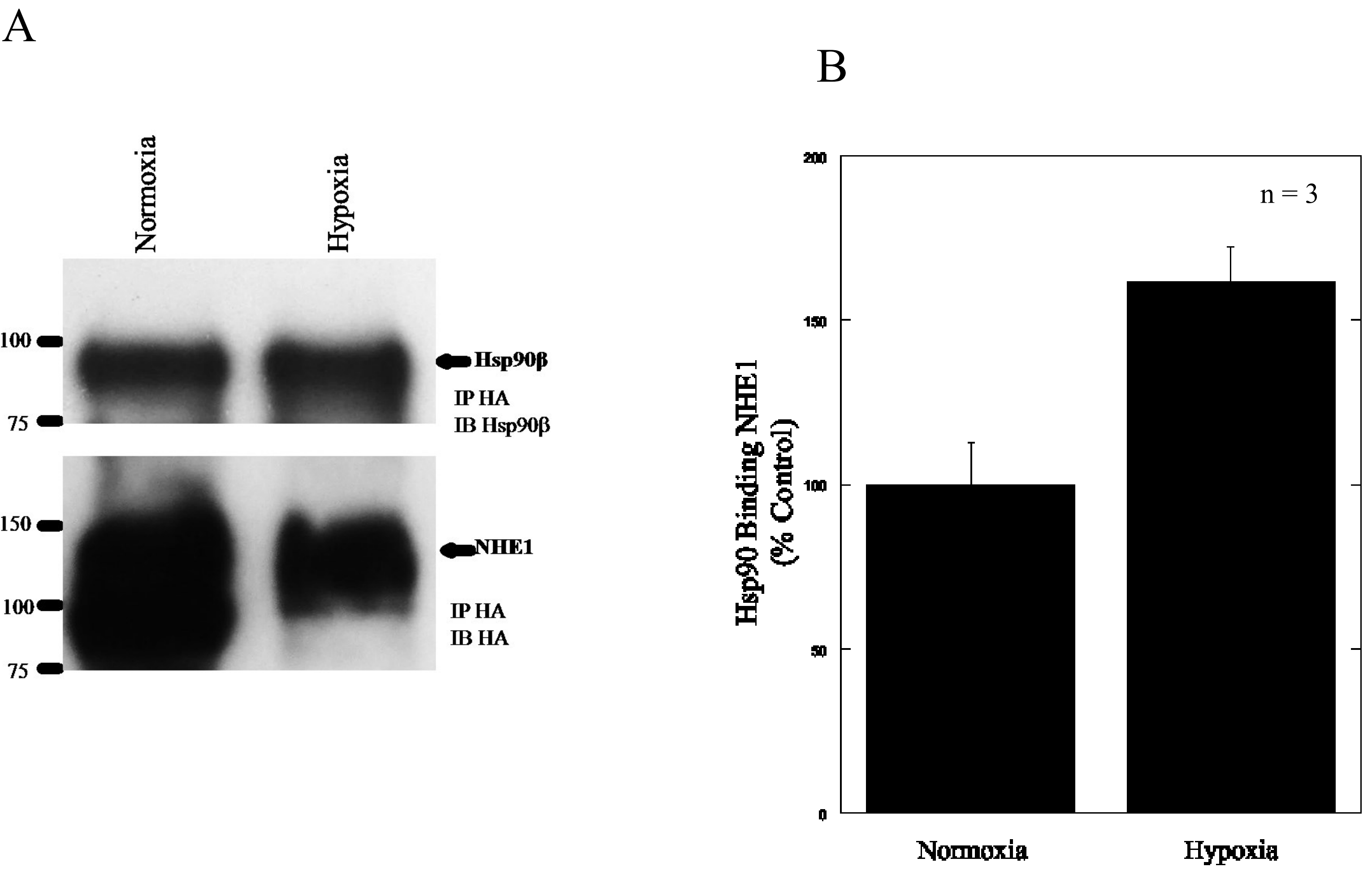


Figure 4.7: Association of NHE1 with Hsp90 following hyoxia/reoxygenation. MDCK cells were subjected to hypoxia/reoxygenation. A, Top panel; NHE1 was immunoprecipitated via the HA-tag and immunoblotted for Hsp90 binding. Bottom panel: nitrocellulose membrane was stripped and immunoblotted for the total amount of NHE1 immuprecipitated. B, summary of coimmunoprecipitation experiment. Results were normalized to the total amount of immunoprecipitated NHE1

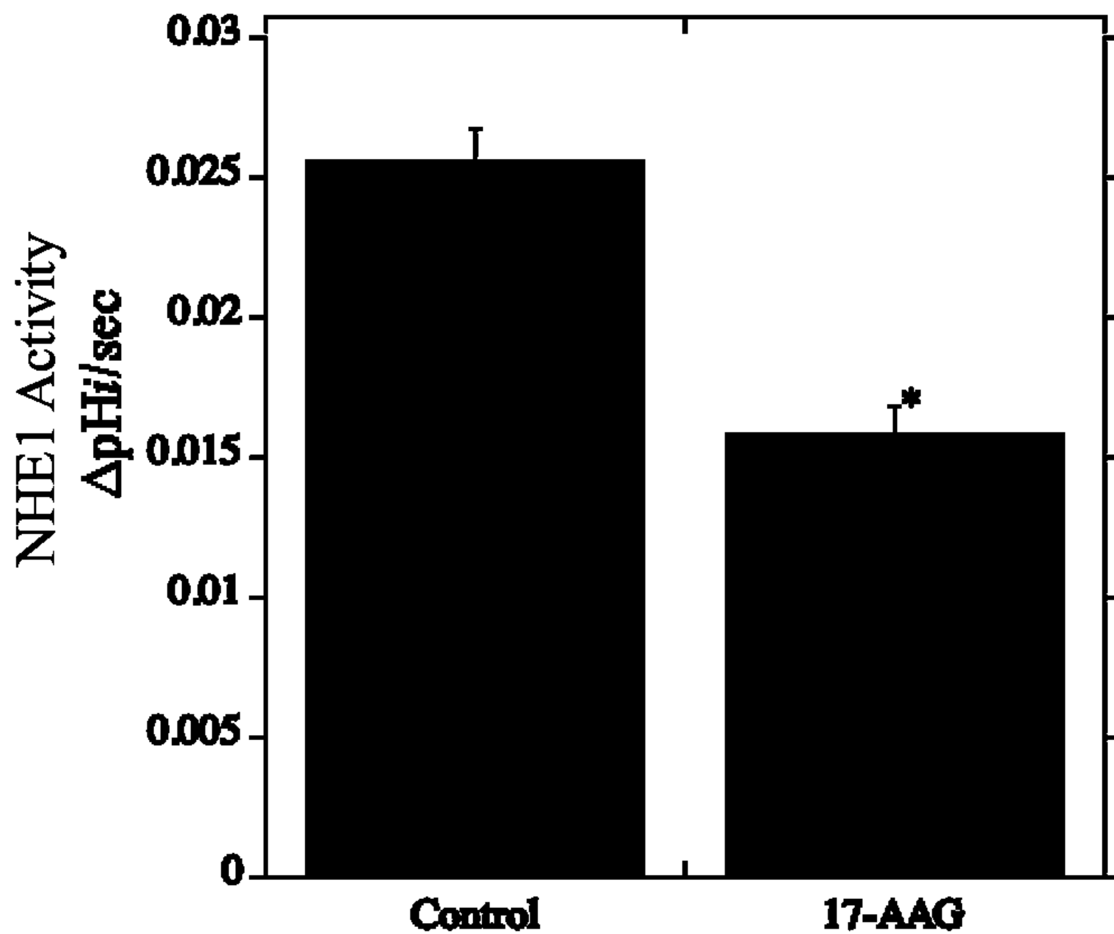


Figure 4.8: Effect of 17-AAG on Na^+/H^+ exchanger activity of MDCK cells. Na^+/H^+ exchanger activity was assayed in MDCK cells grown on coverslips as described in the 'Materials and Methods". MDCK cells were treated with 1 μM 17-AAG for 18 hrs which was maintained throughout the assay. Summary of the initial rate of recovery of NHE1 activity. Results are the mean ± SE of 10 experiments. Na^+/H^+ exchanger activity was determined as measured in the "Materials and Methods". * significantly decreased from control value at $P < 0.001$.

4.2.5 Cell viability following Hsp90 and/or NHE1 inhibition

We examined if treatment of the cells with 17-AAG affected their viability under normoxia or hypoxia, and if this was influenced by NHE1 inhibition (Fig. 4.9). Cells were grown on 384-well plates for 24 h and subjected to hypoxia/reoxygenation in the presence of 17-AAG and/or EMD87580. Cell viability was determined by measuring fluorescent signal generated by a fluorogenic, cell permeant substrate (glycyl-phenylalanyl-aminofluorocoumarin; GF-AFC) cleaved by live cell proteases.

With 18 h of hypoxia followed by 2 h of normoxia, there was not a significant decline in cells viability in comparison to controls. However, interestingly, 17-AAG treatment decreased viability of cells under normoxic conditions but not when cells were treated with hypoxia followed by normoxia. This is consistent with similar observations reported in mouse models of renal IRI (258). EMD87580 treatment of control cells or hypoxia treated cells did not affect their viability while the combination of EMD87580 and 17-AAG treatment was not different from 17-AAG treatment alone.

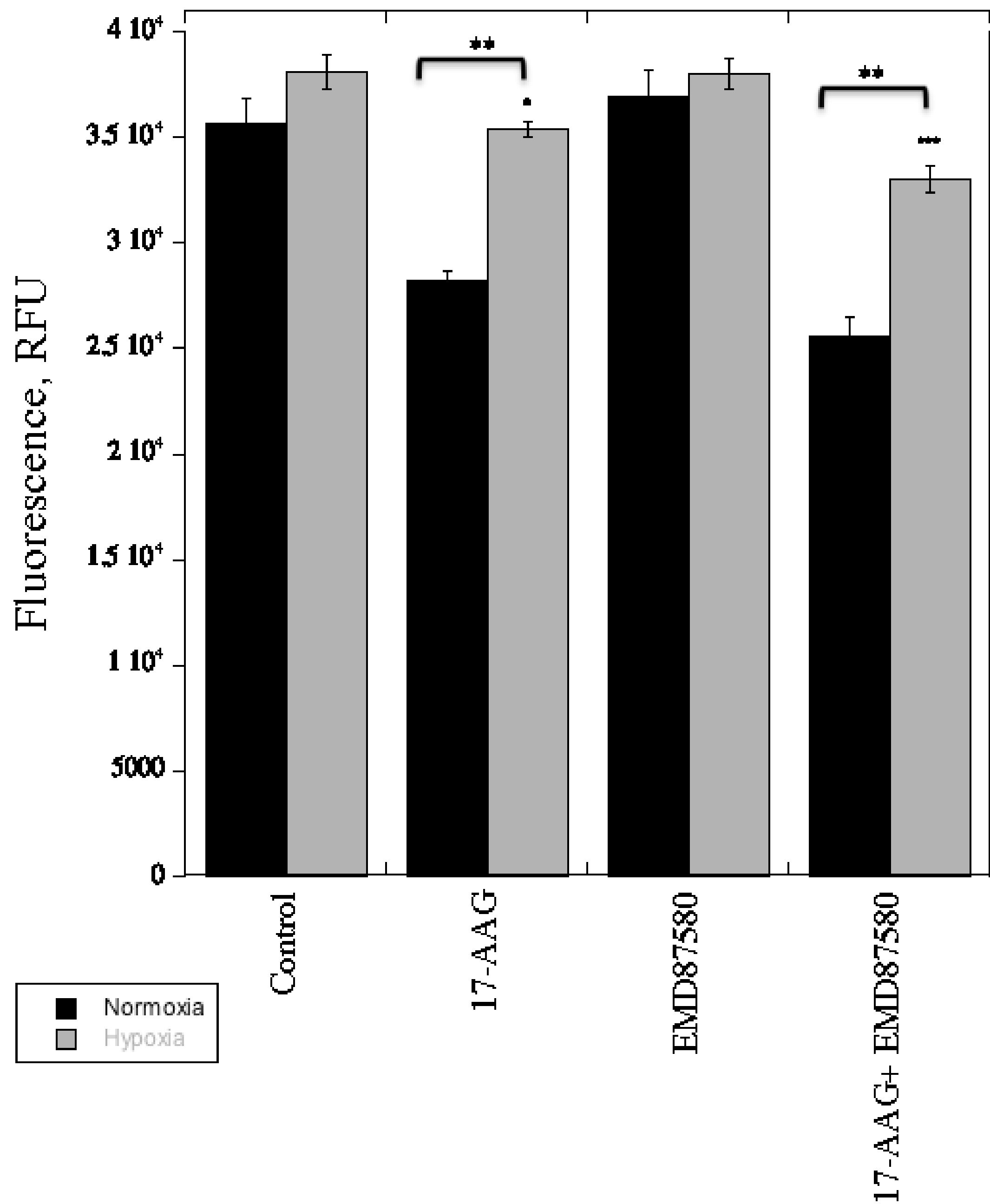


Figure 4.9: Effect of 17-AAG treatment on cell viability of MDCK cells. MDCK cells were subjected to hypoxia/reoxygenation in the presence of the following as indicated – 1 µM 17-AAG and/or 10 µM EMD87580. The CellTiter-Fluor™ Reagent (GF-AFC + Buffer) was added to wells and viability measured after incubation for 30 mins at 37°C. ** significantly decreased from normoxia value at $P < 0.01$, n=18.

4.2.6 Effect of 17-AAG on NHE1 phosphorylation.

To determine the mechanism by which 17-AAG decreased NHE1 activity we examined the level of phosphorylation of the NHE1 protein in MDCK cells. Cells were incubated with [^{32}P] containing medium following treatment with 17-AAG. MDCK cells had the NHE1 protein with a HA tag and this was used to immunoprecipitate out NHE1 and examine the level of phosphate incorporation into the protein. The results (Figure 4.10A and B) showed that phosphorylation levels of NHE1 decreased dramatically with 17-AAG treatment. We confirmed (Figure 4.10A) that the NHE1 protein was immunoprecipitated from treated cells in comparable amounts to untreated cells.

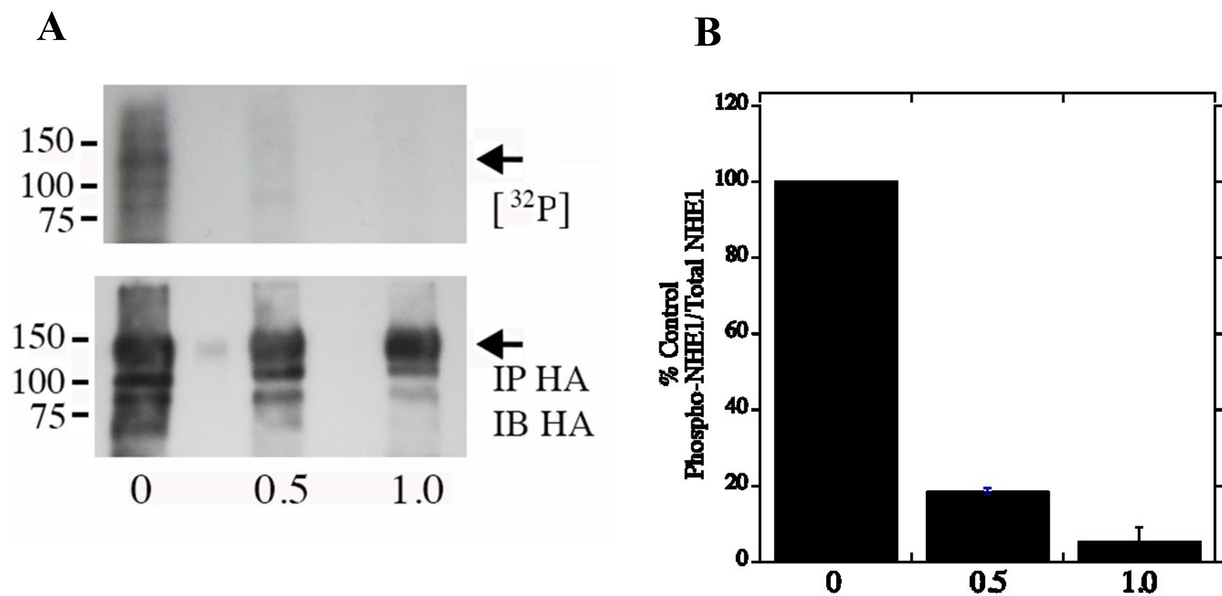


Figure 4.10: Effect of 17-AAG on NHE1 phosphorylation in MDCK cells. Cells were incubated in the presence of 0, 0.5 or 1 μM 17-AAG for 18 h prior to examination of phosphorylation levels of the NHE1 protein. **A**, example of effect of 17-AAG on NHE1 phosphorylation, **B**, summary of results of A, n=4.

4.2.7 Akt mediates the regulation of NHE1 by Hsp90

Hsp90 has been shown to complex with the kinase AKT and modulate its phosphorylation (253,260). Akt has also been shown to directly phosphorylate NHE1 (83). We therefore reasoned that treatment with 17-AAG might have an affect on activation of AKT which is mediated by its phosphorylation. Figure 4.11 A and B show an example and a summary of the effects of 17-AAG on phospho-AKT levels. Treatment with 17-AAG resulted in a decrease in the phospho-AKT levels at higher concentrations. We also confirmed that 17-AAG did not affect NHE1 protein expression by examining the level of the NHE1 protein using western blotting against the HA tag on the protein (Fig. 4.11A and C). The results showed that 17-AAG did not decrease the level of NHE1 protein expression.

To examine the role of AKT more directly, we used the highly selective AKT inhibitor MK2206 (256). Initially we characterized the effect of MK2206 on NHE1 expression and on phospho-AKT levels in MDCK cells. The results showed that there was no significant effect on levels of the NHE1 protein (Fig. 4.12A,B). β-tubulin served as a control for protein loading. However, when phospho-AKT levels were examined, we found that they were greatly reduced by this treatment (Fig. 4.12A,C).

We also observed a significant decrease in the activity of the NHE1 protein with treatment with MK-2206. To examine if there is an additive effect of the addition of 17AAG and MK2206, we treated cells simultaneously with the two compounds and measured NHE1 activity. The results showed that there was no additional inhibition by addition of the two compounds in comparison with addition of either compound individually (Fig. 4.13).

To further confirm the role of Akt in NHE1 phosphorylation in renal cells, we performed *in vivo* phosphorylation assays with MK2206. MDCK cells were incubated in [^{32}P] for 3 h as described in Section 2.5.3. However, 5 min before the end of the incubation, 10% serum was added along with MK2206 (data not shown). Preliminary results indicate that MK2206 appears to cause a decrease in NHE1 phosphorylation. However, more experiments need to be carried out to confirm our observations. We also confirmed that 5 min incubation with MK2206 was sufficient for inhibiting Akt phosphorylation as illustrated in Figure 4.14.

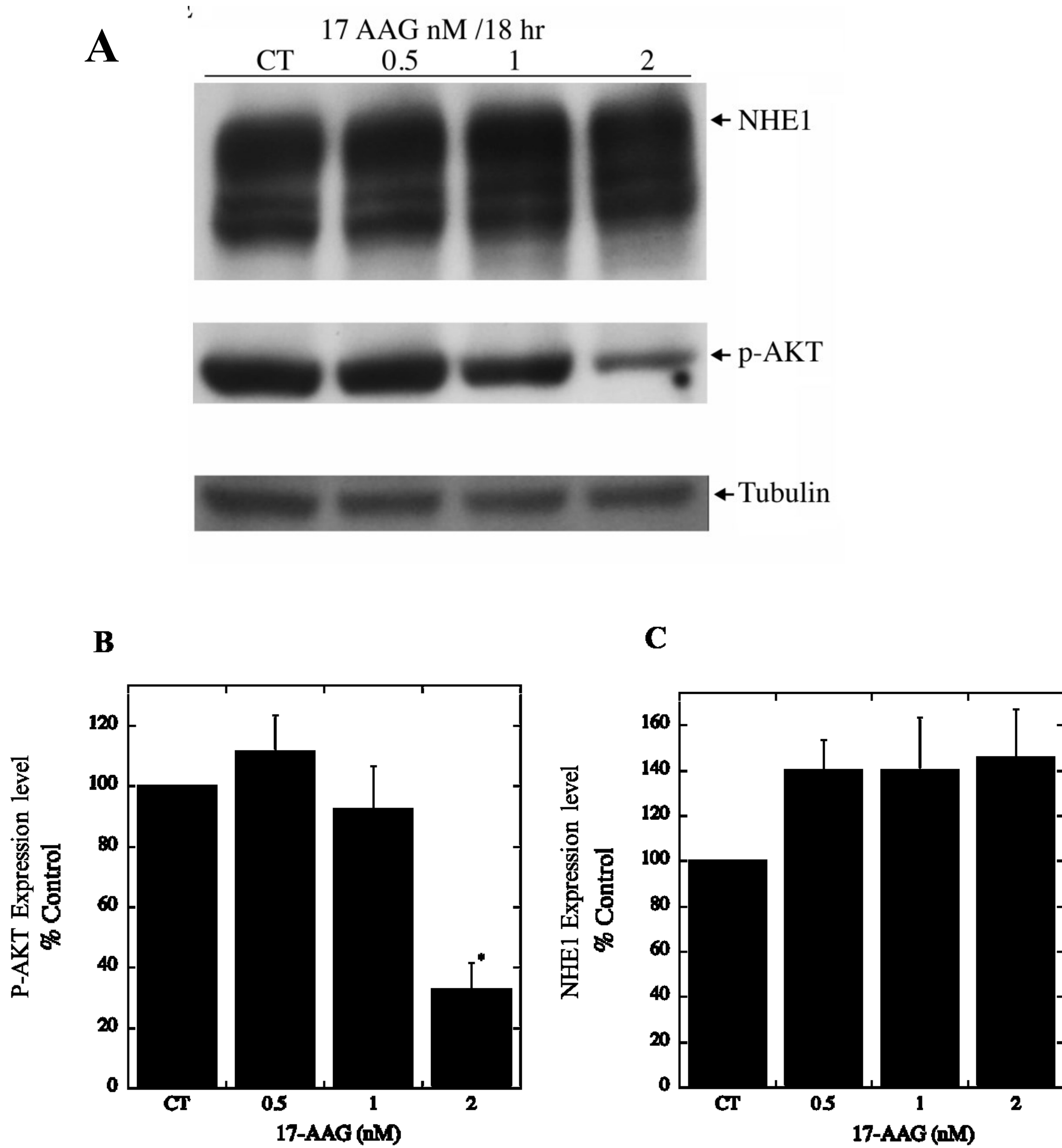


Figure 4.11: Effect of 17-AAG on NHE1 protein and phospho AKT levels. Cells were incubated in the presence of 0 (CT), 0.5, 1 or 2 μM 17-AAG for 18 hrs prior to examination of expression levels of the NHE1 and p-Akt. **A**, example of effect of 17-AAG on protein expression levels, **B** and **C**, summary of results of A, n=4.

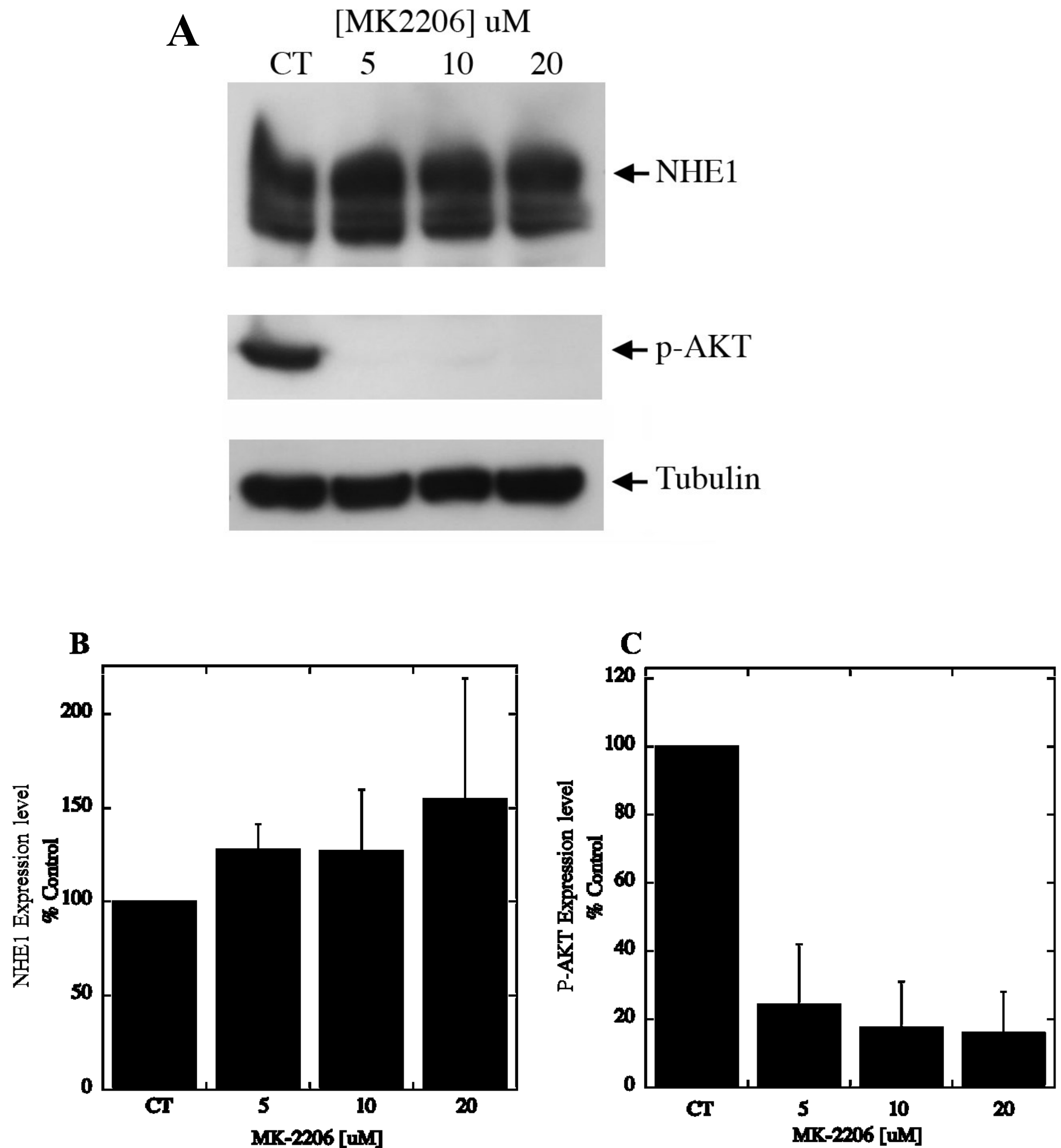


Figure 4.12: Effect of MK-2206 treatment on MDCK cells. **A**, Cells were treated with either 0 µM (CT) or MK-2206 for 18 h at the indicated concentrations. *Upper panel*, immunoblot for NHE1 protein using anti- HA (tag) antibodies. *Middle panel*, immunoblot against phospho-AKT protein. *Lower panel*, immunoblot against tubulin, used as a loading control. **B,** Summary of effect of MK-2206 on NHE1 protein levels. **C**, Summary of effect of MK-2206 on phospho- AKT levels. N=3

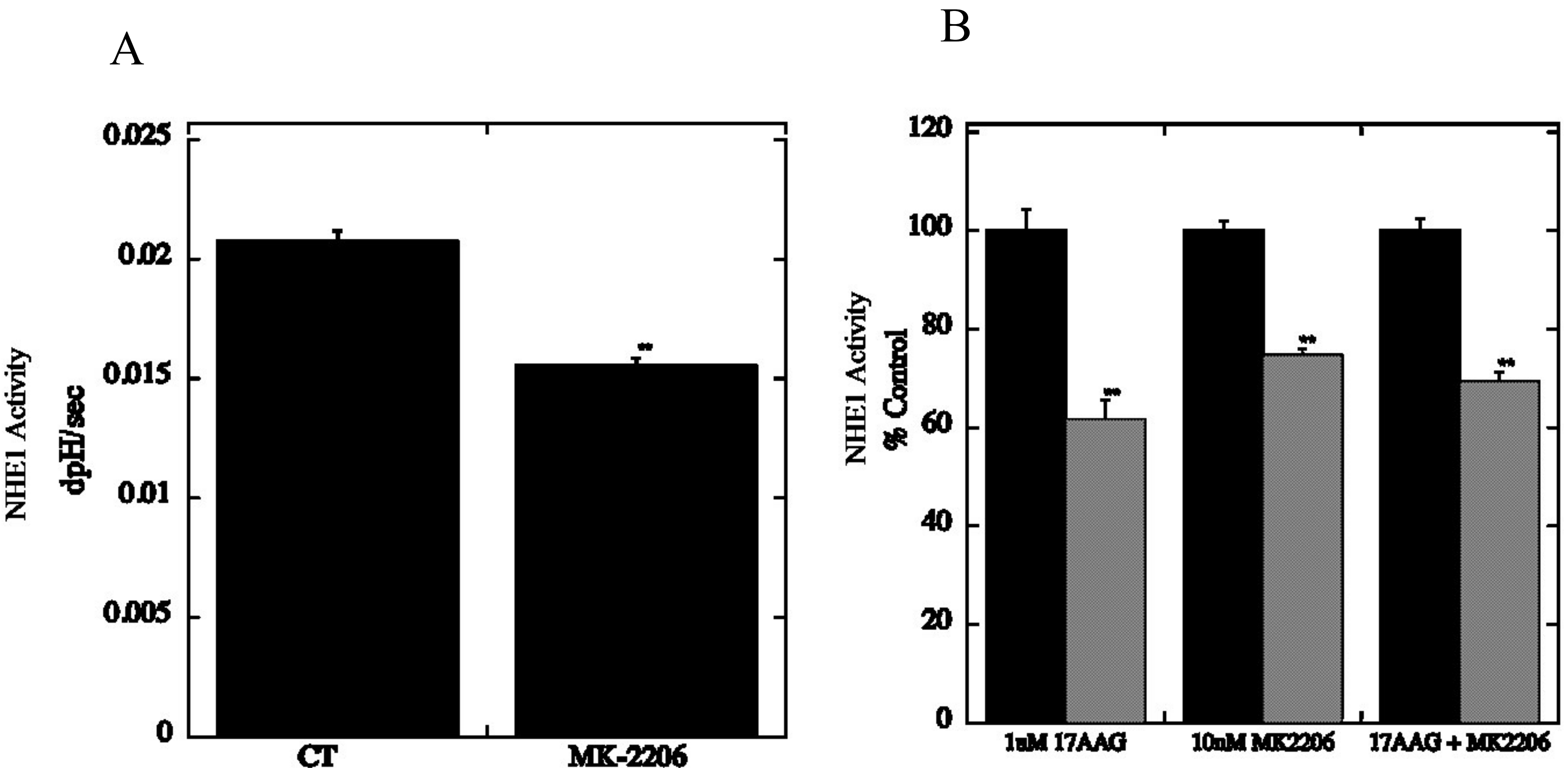


Figure 4.13: Effect of MK-2206 treatment on NHE1 activity of MDCK cells. *A,* effect of 10µM MK2206 on NHE1 activity *B,* effect of combinatorial treatment of 1 µM 17AAG and 10µM MK-2206 on NHE1 activity, in comparison with the effect of individual treatments (results from Fig 4.5 and 4.10A). Results are the mean ± SE of 10 experiments. ** significantly different at P < 0.001.

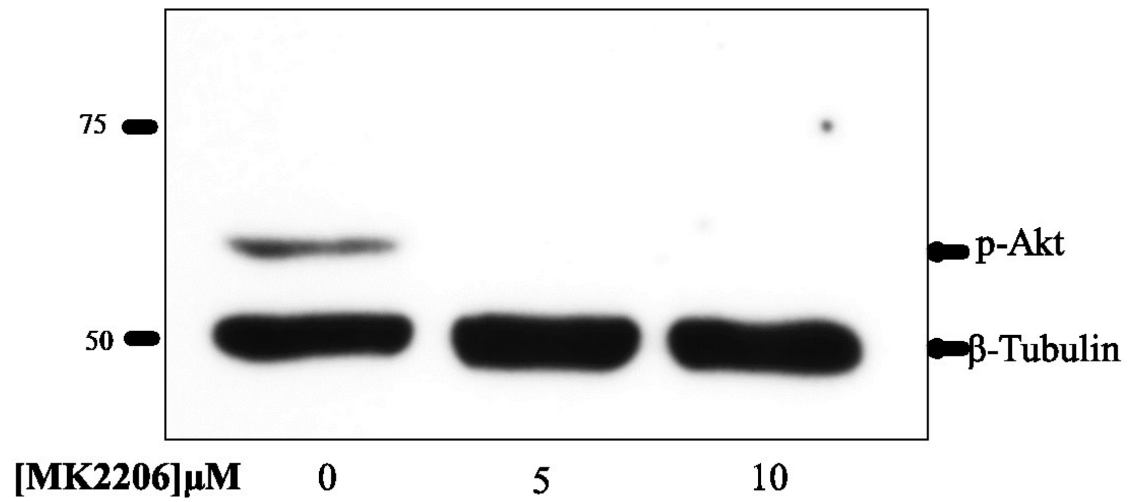


Figure 4.14: Effect of 5 min incubation with MK2206 on phospho-AKT levels. Cells were treated with MK2206 for 5 min at the indicated concentrations. Immunoblot against phospho-AKT and β-tubulin (loading control). N=3

4.3 Discussion

NHE1 is a critical pH regulatory protein in many tissues. In the kidney, NHE1 expression and activity has been shown to be upregulated in response to acidosis and is therefore thought to be important in pH regulation in these cells (230,231). We recently demonstrated that in renal cells, a cluster of phosphorylatable amino acids in the regulatory tail are important in mediating NHE1 activation though Erk-dependent pathways (94). However, many regulatory proteins of NHE1 have been shown to bind to the NHE1 C-terminus and activation of the protein is thought to be mediated through a combination of both phosphorylation and protein mediated interactions on the regulatory C-terminus (246). We therefore examined proteins interacting with NHE1 in the mammalian kidney. Our initial screen used the cytoplasmic regulatory domain of NHE1 to identify NHE1 binding proteins from kidney extracts. We identified a number of proteins from kidney extracts (Table 1) that bound to the cytosolic domain. Among them, some with higher scores were heat shock proteins, 14-3-3 proteins, and Na^+/K^+ ATPase.

We chose several of these to investigate further, based on their higher score and on previously publications. It has earlier been suggested that NHE1 and Na^+/K^+ ATPase interact in a complex (257). For this reason, we attempted to confirm the interaction demonstrated in our screen of proteins bound to the NHE1 tail. Nevertheless, despite repeated attempts including cross-linking of proteins, we were unable to immunoprecipitate Na^+/K^+ ATPase with NHE1. The reason for this is not yet apparent but could be due to a transient interaction of the two proteins or perhaps an interaction that is regulated, and was not apparent under the conditions of the assay we used.

Nevertheless, our demonstration of an association using the cytosolic domain of NHE1, when taken together with the previous published report suggests that there is an interaction, perhaps transient in nature (257).

The 14-3-3 proteins have been demonstrated to interact with the NHE1 tail. They regulate phosphorylation of serine 703 of NHE1 in the myocardium (131,261). However, while 14-3-3 regulation of NHE1 has been demonstrated in the myocardium there has been little study of it in other tissues. We found that 14-3-3 both immunoprecipitated with NHE1 from renal cells, and also bound to the NHE1 C-terminal protein *in vitro*. We had previously demonstrated that sustained intracellular acidosis stimulated NHE1 activity (94). We therefore examined if 14-3-3 might be involved in this phenomenon. However, there was no change in the level of 14-3-3 binding with sustained intracellular acidosis. 14-3-3 binds to and regulates phosphorylation of Ser703. These results are therefore in agreement with our previous results which suggested that sustained acidosis activates NHE1 independent of Ser703 (233). The role of 14-3-3 in regulation of NHE1 in the kidney is unresolved at this time. Experiments with treatment of MDCK cells with aldosterone, failed to show and change in the level of 14-3-3 association with NHE1 (not shown). Future experiments may address this phenomenon.

The heat shock proteins Hsp70 and Hsp90 of kidney cell extracts were found to associate with NHE1 *in vitro* (Table 1). Initial attempts at immunoprecipitation of these two proteins with NHE1 were successful for Hsp70 and for Hsp90. There have been earlier reports of an association of heat shock proteins with NHE1. An association was demonstrated earlier by both affinity chromatography and by immunoprecipitation from PS127A cells (126). More recently, Hsp70 was shown to interact with NHE1

dynamically in macrophages (127). Hsp70 is known to be inducible by stress such as hypoxia (262-264). Hsp90 levels can also increase up to 10 folds in response to physiological stress including hypoxia and acidosis (255). We therefore examined the effect of hypoxia on NHE1 activity and the association of Hsp70 and Hsp90 with NHE1. Treatment of MDCK cells with hypoxia resulted in decreased NHE1 activity while the binding of Hsp70 and Hsp90 was also upregulated following hypoxia/reoxygenation.

Immunoprecipitation experiments showed that Hsp90 interacted with NHE1. To investigate if there was a potential role for Hsp90 in regulation of NHE1, we treated cells with 17-AAG which is a Hsp90 inhibitor (255). Treatment with 17-AAG resulted in a decrease in NHE1 activity and there was also a significant decrease in the level of NHE1 phosphorylation (Fig. 4.5 and 4.7). NHE1 contains phosphorylation sites for a number of protein kinases (97,246). So the almost total disappearance of phosphorylation of NHE1 by treatment with 17-AAG was somewhat surprising, and could suggest that the activity of one or more protein kinases was affected. Indeed, kinases are one of the main groups of Hsp90-dependent substrates which include AKT (265). AKT signalling pathway has also been suggested to be one of mechanisms of cell survival under hypoxic conditions (266). Hence, AKT phosphorylation may represent much of the phosphorylation present in our MDCK cells. In addition to 17-AAG, the selective AKT inhibitor MK-2206 decreased NHE1 activity and Phospho-AKT levels, supporting a role for AKT regulation mediated through Hsp90. It was noteworthy that addition of 17-AAG and MK-2206 together, did not result in an additive inhibitory effect on NHE1 activity, suggesting they may act through the same mechanism. We also have preliminary data indicating that treatment with MK2206 similarly caused a decrease in NHE1 phosphorylation.

It was therefore apparent that in our system, Hsp90 can act to regulate NHE1 activity. Inhibition of Hsp90 and AKT resulted in a decrease in phosphorylation of NHE1 and decreased activity. This would suggest that AKT has a stimulatory role on NHE1 in kidney cells. In previous reports AKT phosphorylation of NHE1 has been shown to be either stimulatory or inhibitory. In cardiac tissue, the activity of the NHE1 protein was inhibited by AKT mediated phosphorylation of Ser648 (102,267). However, another report studied fibroblasts and showed that AKT phosphorylation of NHE1 is stimulatory and is necessary for activation of NHE1 by insulin and platelet-derived growth factor (PDGF) (83). It has been suggested that the exact role of AKT mediated phosphorylation of NHE1 may depend on cell type (246). AKT phosphorylation of NHE1 occurs at Ser648, which overlaps with the first calmodulin binding site of the autoinhibitory domain. Snabaitis et al. (102) demonstrated that phosphorylation of Ser648 inhibits calmodulin binding to this domain. We have demonstrated that calmodulin interaction with NHE1 is dynamic *in vivo* in fibroblasts, increasing in response to calcium and hormonal stimulation (268). In the myocardium, calcium levels are key to contractility and rise to relatively high levels with contraction. It may be that in myocardial cells, the elevated level of calcium interacts with calmodulin and that this, leads to a more chronic activation. AKT-mediated phosphorylation in this circumstance may be inhibitory. In other tissues, with lower calcium levels, the role of calmodulin association at the site may be reduced. Phosphorylation by AKT could be stimulatory by mediating reduced inhibition of the auto-inhibitory domain in the absence of calcium/calmodulin complex. Further studies are necessary to examine this phenomenon.

The association of Hsp90 with Hsp70 has also been indicated to be required for its chaperone functions (269). In multiple myeloma cells, inhibiting Hsp70 resulted in a concomitant decrease in the protein levels of Hsp90 client proteins. Interestingly, inhibiting the PI3K/Akt pathway downregulated Hsp70 expression in these cells (270). There is therefore a possibility that Hsp70, Hsp90 and AKT form a complex that regulate NHE1 activity and this can be examined in future studies.

NHE1 is phosphorylated by various kinases, many of which are not Hsp90 client proteins. Hence, the conspicuous decrease in NHE1 phosphorylation following treatment with 17-AAG also suggests the possibility of increased phosphatase activity. Protein phosphatase 5 (PP5) is a serine/threonine phosphatase that binds to both Hsp70 and Hsp90. However, PP5 binding to Hsp70 produces a much higher phosphatase activity than its interaction with Hsp90 (271). Also, Hsp70 was upregulated in the kidneys, livers, lungs, and hearts of mice treated with Hsp90 inhibitors (258). It is therefore possible that the upregulation of Hsp70 as a result of Hsp90 inhibition may result in increased activity of phosphatases such as PP5, which may be responsible for NHE1 dephosphorylation as observed in our studies. Another phosphatase that may be involved is PP2A. PP2A can inhibit Akt kinase activity by dephosphorylation and the interaction between Akt and Hsp90 protects Akt from dephosphorylation by PP2A (253). PP2A has also been shown to regulate sarcolemmal NHE1 activity following adenosine A_1 receptor stimulation and this is thought to be due to enhanced proximity of PP2A to NHE1. (107). It is therefore possible, that just as with Akt, disrupting the Hsp90/NHE1 interaction makes NHE1 susceptible to dephosphorylation by PP2A. Further investigation

is however required to confirm the possible role and identity of phosphatases that may be involved in NHE1 dephosphorylation following the inhibition of Hsp90.

We also observed that inhibiting Hsp90 appears to improve cell viability following hypoxia/reoxygenation compared to normoxic cells. This is consistent with previous studies that show that Hsp90 inhibition is associated with cellular protection *in vitro* and *in vivo* following simulated ischemia and IRI (258). However, the inhibition of NHE1 did not improve cell viability and may be as a result of this experiment being carried out in MDCK cells overexpressing NHE1. Previous studies from our laboratory reported that isolated cardiomyocytes were more susceptible to apoptotic damage induced by hypoxia/reoxygenation but the susceptibility of these cells to apoptotic damage was not significantly reduced by NHE1 inhibition by EMD87580 (259). However, inhibition of endogenous NHE1 activity (in the absence of exogenous NHE1) reduced the sensitivity of cardiomyocytes to hypoxia/reoxygenation damage and we are inclined to think that this is what is happening also in MDCK cells.

Our study is the first that demonstrates the interaction of Hsp90 with NHE1 and that this association plays a role in the regulation of NHE1 phosphorylation and activity. Our report also indicates that Hsp90-NHE1 association is mediated by AKT-dependent NHE1 phosphorylation although further studies need to be carried out to confirm this. Our laboratory has also previously demonstrated that overexpression of NHE1 in cardiomyocytes predisposes them to apoptotic damage induced by hypoxia/reoxygenation (259). Based on our results so far, we propose that AKT plays a stimulatory role in NHE1 activity in renal cells, which is mediated by the association of Hsp90 with NHE1, and this may play a role in the induction of apoptotic damage by hypoxia/reoxygenation.

Chapter 5

Conclusions and Future Directions

5.1 Conclusions

The mammalian Na^+/H^+ exchanger isoform 1 (NHE1) is a ubiquitously expressed plasma membrane protein that protects cells from intracellular acidosis by through an electroneutral exchange of one intracellular proton for an extracellular sodium ion. NHE1 has two major domains – the N-terminal membrane domain that transports ions and a cytosolic domain which regulates the membrane domain (65). NHE1 is distributed in virtually all segments of the nephron excluding the macula densa and intercalated cells of the distal nephron. In renal epithelial cells, NHE1 is expressed on the basolateral membrane (18). NHE1 is regulated primarily by phosphorylation and protein interactions (reviewed in (65,246)). While regulatory stimuli such as acidosis and MAPKs have been shown to regulate NHE1 in the kidney, the exact mechanisms involved were not well known. In addition, while many proteins interacting with NHE1 have been identified, this has not been well studied in kidney cells. NHE1 has also been demonstrated to play an important role in apoptosis in renal cells under various disease conditions (134,146,147,187). The main objective of this thesis was therefore to examine the regulation of NHE1 in kidney cells.

The aim was to elucidate the mechanism of acidosis-mediated regulation of NHE1 in kidney cells by identifying the phosphorylation sites and protein kinases involved. I also wanted to identify novel binding partners of NHE1 in kidney cells and determine their role in regulating NHE1 activity. Our hypotheses were as follows:

- NHE1 is activated by acidosis in mammalian kidney cells and this is an ERK-dependent pathway.

- Novel protein interactions with NHE1 exist in kidney cells which regulate its activity.

Characterization of NHE1 regulation was done in MDCK cells which is an epithelial cell line derived from the distal tubule/collecting duct of a female adult cocker spaniel. To determine the regulatory sites of phosphorylation on the cytosolic domain of NHE1, MDCK cells were transfected with cDNA of wild type NHE1 or NHE1 proteins with mutations to the ERK phosphorylation sites. All the cDNAs had a L163F/G174S mutation which confers a 100-fold resistance to EMD87580, an NHE1-specific inhibitor. We also demonstrated this mutation is insensitive to S3226, an NHE3-specific inhibitor. ERK has been shown to phosphorylate the cytoplasmic domain of NHE1 at one or more of the following amino acids *in vitro* – Ser693, Ser766, Ser770, Ser771, Thr779, and Ser785 (95). Ser770 and Ser771 have also been shown to be critical for NHE1 activity and phosphorylation in CHO cells and cardiomyocytes (96,97). Two regions of the cytoplasmic domain, which contains phosphorylatable amino acids have been shown to be involved in acidosis-mediated stimulation of NHE1 activity. These are region 3, which contains Ser766, Ser770 and Ser771, and region 4 which contains Thr779 and Ser785.

Acidosis has been demonstrated to activate NHE1 in both renal and non-renal cell lines (94). This mediated by ERK pathway-dependent phosphorylation of the C-terminal tail of NHE1 in cardiomyocytes (97,229). We initially confirmed that sustained intracellular acidosis (SIA) increased NHE activity in MDCK cells. We therefore set to characterize the mechanism of NHE1 activation by acidosis in kidney cells. Cells stably

expressing wild type and mutant NHE1 proteins were subjected to SIA and our results revealed that mutations to Ser771, Ser776, Thr779 and Ser785 prevented the activation of NHE1 by SIA in MDCK cells. These mutations also resulted in decreased NHE1 phoshphorylation following SIA, in contrast to wild type NHE1 and Ser770 mutants. However, all the mutations studied caused large decreases in the basal phosphorylation levels of NHE1 compared to wild type. We therefore concluded that amino acids Ser771, Ser776, Thr779, Ser785 are important for NHE1 activation and phosphorylation following SIA.

The activation of NHE1 by SIA has been determined to be mediated by the ERK pathway in cardiomyocytes (93,97,229). The ERK pathway has also been implicated in hormonal regulation of NHE1 in MDCK cells (232). We therefore investigated if the pathway was involved in the SIA-induced stimulation of NHE1 activity. We observed an increase in phospho-ERK and phospho-p90^{RSK} levels following SIA for 3 mins. This increase was blocked by U0126 particularly for ERK phosphorylation. U0126 also prevented the stimulation of NHE1 activity by SIA. Taken together, these results suggest that phosphorylation and activation of NHE1 in MDCK cells occur through the ERK pathway.

NHE1 is regulated both by phosphorylation and phosphorylation-independent mechanisms such as the binding of various regulatory proteins to the cytoplasmic domain. Having characterized one mechanism of phosphorylation dependent activation of NHE1, I wanted to examine the role of protein-protein interaction in the regulation of NHE1 in kidney cells. By using affinity chromatography, we pulled down NHE1-interacting proteins from mouse kidney cell extracts. Specifically, GST tagged C-

terminal NHE1 protein (PCRB) was covalently cross-linked to Affi-Gel 10 matrices and incubated with mouse kidney cell extracts. Mass spectrometry analysis showed that a variety of proteins interacted with NHE1 in the kidney including Na^+/K^+/ATPase, 14-3-3 and heat shock proteins, Hsp70 and Hsp90 (Table 1). Co-immunoprecipitation confirmed the association of 14-3-3, Hsp70 and Hsp90 with NHE1 in MDCK cells. 14-3-3 and Hsp70 have also been shown to associate with NHE1 in other cell lines (126,131). However, our results are the first to demonstrate a direct interaction between NHE1 and Hsp90. We were unable to co-immunoprecipitate Na^+/K^+/ATPase with NHE1 despite reports of their interaction in human kidney cells (257).

We further investigated how these interacting proteins regulate NHE1 activity in kidney cells. Heat shock proteins are also known as stress proteins as they are upregulated under conditions of cellular stress such as hypoxia, heat shock and ischemia. Increased NHE1 expression has also been implicated in hypoxia-induced apoptosis in cardiomyocytes, a condition which is reversed when NHE1 is inhibited (259,272). We therefore examined the association of Hsp70 and Hsp90 with NHE1 under hypoxic conditions. Our results show that hypoxia increased the association of heat shock proteins with NHE1 compared to control. We also observed a decrease in NHE1 activity and phosphorylation when Hsp90 was inhibited with 17-AAG, a specific inhibitor of Hsp90.

A unique feature of Hsp90 is its substrate specificity and one of its substrates is Akt (253). The interaction between Hsp90 and Akt has also been demonstrated to be crucial for Akt activation (260). Having observed a decrease in NHE1 phosphorylation following treatment with 17-AAG, we hypothesized that this decrease could be, at least,

in part due to a consequent decrease in Akt activation. Treating MDCK cells with MK-2206, an allosteric Akt inhibitor resulted in a decrease in NHE1 activity. In addition, the combination of 17-AAG and MK-2206 did not have an additive effect on NHE1 activity when compared to single treatment with the inhibitors, suggesting that the effects of each inhibitor on NHE1 activity was mediated through a similar mechanism. Another interesting observation was that inhibition of Hsp90 resulted in better cell viability following hypoxia/reoxygenation.

In conclusion, we demonstrated that hypoxia increases the association of Hsp90 and Hsp70 to NHE1. My results also show that the inhibition of Hsp90 or an Hsp90 substrate, Akt, resulted in decreased NHE1 activation and phosphorylation suggesting that these proteins work in tandem to stimulate NHE1 activity.

5.2 Future directions

This study has examined some of the mechanisms involved in the regulation of NHE1 by phosphorylation and protein-protein interactions. However, future studies are required to further understand the mechanisms of NHE1 regulation in the kidney.

5.2.1 Characterization of single mutations of amino acids in Region 4 phosphorylation site

Our study showed that single mutation to Ser771 of Region 3 of phosphorylatable amino acids in the cytoplasmic domain (IRM3) caused a decrease in NHE1 activity and phosphorylation. We also observed similar results for mutations to Region 4 of the

phosphorylatable amino acids (IRM4). However, the exact amino acids in the region 4 involved in the regulation of NHE1 by SIA need to be identified. To do this, single mutations to Thr779 and Ser785 will be made to Alanine in the cytoplasmic domain of NHE1 with the IRM mutation expressed in plasmid pYN4^{+}. The cDNAs of these NHE1 mutants will then be transfected in MDCK cells and analysed for their effect on NHE1 activation and phosphorylation as described previously (Chapter 3).

5.2.2 *In vivo* models of phosphorylation mutants in the kidney

After determining the exact amino acid(s) in Region 4 involved in acidosis-mediated regulation of NHE1 as described above, an *in vivo* model would be necessary in further understanding the regulation of NHE1 during renal ischemia reperfusion. I therefore propose that NHE1 with mutations to these phosphorylation sites be expressed in the kidney of transgenic mice. The transgenic mice can then be assessed for their response to renal ischemia/reperfusion. It is believed that NHE1 is activated in response to cellular acidosis caused by renal ischemia reperfusion injury (IRI) (161). I therefore hypothesize that these mutations will cause a resistance to IRI in the kidney. I anticipate that these experiments will provide more insight into the role NHE1 plays in renal ischemia which induces acute kidney injury (47).

5.2.3 Characterization of Hsp90-mediated NHE1 regulation.

While our initial studies suggest that Hsp90 plays a role in NHE1 regulation, further experiments are required to elucidate the mechanisms of this regulation and what

physiological role this plays in the kidney. One of our observations was that inhibiting Hsp90 in MDCK cells under hypoxic conditions promoted cell viability compared to untreated cells. To further investigate if and how NHE1 may be involved in this response, NHE1 can be knocked down in kidney cells and assessed for cell viability following hypoxia in the presence of 17-AAG (and MK-2206). This would be compared to cells expressing NHE1 and a difference in cell viability can provide more insight into the role of NHE1.

Further investigation into the role of Akt phosphorylation with regards to the regulation of NHE1 activity by Hsp90 is also required. Akt phosphorylates NHE1 at Ser648 and stimulates NHE1 activation in fibroblasts (83). Our observations following treatment of MDCK cells with MK-2206 suggest that it also plays a stimulatory role in kidney cells. Akt is a bona fide substrate of Hsp90 and we think it mediates the regulation of NHE1 by Hsp90. To study this, MDCK cells can be transfected with NHE1 proteins with mutation to Ser648 and analysed for NHE1 activity and phosphorylation in the presence or absence of 17-AAG. If indeed this amino acid is involved in Hsp90-mediated regulation of NHE1, it is expected that this NHE1 mutant will be resistant to reduced activity and phosphorylation. Further experiments may also involve the use of transgenic mice expressing NHE1 with mutation to Ser648. These mice can be treated with 17-AAG and/or MK-2206 and subjected to ischemia/reperfusion, following which their kidneys are assessed for apoptotic damage.

Akt signalling pathway is stimulated by hormones and growth factors including insulin, PDGF, EGF, thrombin and NGF (273) which was not explored in this study. In adult rat ventricular myocytes, the inhibitory effect of insulin-like growth factor 1 (IGF-

1) on NHE1 activity following SIA was prevented in the presence of MK2206, (267) which is consistent with the inhibitory role Akt has been reported to play in these cells. Interestingly, IGF-1 had no effect on NHE1 following acute acidosis as induced in the activity assays we performed in this study. Hence, evaluating NHE1 activity following the inhibition of Hsp90 and AKT, in the presence of hormones (e.g. insulin) and/or SIA may help us further unravel the respective and collective roles of Hsp90 and Akt in regulating NHE1 in kidney cells.

5.2.4 Understanding the role of Hsp70 in NHE1 activity

Our results show that Hsp70 binds to NHE1 and this association is upregulated under conditions of hypoxia. Future experiments could further investigate what role Hsp70 may play in regulating NHE1 activity beyond being a molecular chaperone. Induction of Hsp70 before or after renal ischemia has been shown to protect mouse kidneys from renal injury (274). This study suggests that Hsp90 promotes renal epithelial cell survival by modulating Akt activation after ischemia. Inhibition of Hsp90 has also been demonstrated to upregulate Hsp70 *in vitro* and in mouse models of IRI (258). I therefore hypothesize that the cell viability observed when Hsp90 is inhibited is probably due to an increased association of Hsp70 which promotes renal cell survival. To confirm this, MDCK cells can be subjected to hypoxia in the presence or absence of 17-AAG and MK-2206, followed by co-immunoprecipitation and immunoblotting to determine the level of Hsp70 binding to NHE1 under these conditions. Hsp70 and/or NHE1 can also be genetically knocked down in cell culture which is then subjected to hypoxia in the presence or absence of 17-AAG and analysed for cell viability and apoptosis.

5.3 Summary

The regulation of intracellular pH and cell volume by NHE1 are of significant importance in renal physiology. Our present and future experiments are expected to provide deeper insight on the regulation of NHE1 in the kidney. We report new information on relevant protein interactions with NHE1 and how this affects its activity in kidney cell lines. The involvement of NHE1 in various renal pathologies such as acute renal failure, renal hypertrophy and chronic kidney diseases, indicates that it might be an important factor to consider in developing effective therapeutic strategies in treating such diseases. This experimental approach may also provide a working framework that can be adapted for the study of NHE1 in other tissues, such as cardiomyocytes, where NHE1 has also been shown to be of great importance.

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