January 2020

Characterizing Palmitoylation On The Sodium Hydrogen Exchanger Isoform 1 (NHE1)

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CHARACTERIZING PALMITOYLATION ON THE SODIUM HYDROGEN EXCHANGER ISOFORM 1 (NHE1)

By

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Bachelor of Arts, Minnesota State University Moorhead, 2014

A Dissertation

Submitted to the Graduate Faculty

of the

University of North Dakota

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Grand Forks, North Dakota

May

2020

This dissertation , submitted by Moriah Hovde in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Biomedical Sciences from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

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Department: Biomedical Sciences

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Moriah J. Hovde

March, 2020

Table of Contents

List of Figures

List of Tables

ACKNOWLEDGEMENTS

I would like to start by thanking Dr. James Foster for the opportunity to study in his laboratory and for funding this research. Dr. Foster has been an amazing mentor allowing me to pursue my ideas and interest with constant support and guidance and for enhancing my education greatly by supporting my attendance at many conferences. I would also like to thank Dr. Foster for always taking the time to talk with and advise me he has been a truly great mentor.

Thank you, Dr. Roxanne Vaughan, Dr. Keith Henry, Dr. Mark Wallert, and Dr. Tristan Darland, for being a part of my graduate committee. They have all provided advice and direction improving the quality of this research. Dr. Vaughan and Dr. Henry have pushed me to be the best scientist possible and done everything necessary to help me get there. A special thank you to Dr. Mark Wallert and Dr. Joseph Provost who have been valuable collaborators and mentors. They both have been instrumental in choice to attend graduate school and in my success throughout graduate school, always providing me with their honest opinions and advice. Thank you to the many faculty members that have helped and supported me along the way by attending seminars, asking thought provoking questions, and always having their door open for anything I needed.

Thank you to my peers in the Biomedical Sciences program that encouraged and supported me. Specifically thank you to Dr. Josh Kulas for all of the advice,

encouragement, and confidence in me as a scientist. I am grateful for Dr. Brett McGregor for his friendship and support throughout this experience. I am thankful for the advice and guidance from senior students Dr. Anne Schaar and Dr. Peter Knopic and for both of them always being wonderful and supportive friends.

Last but not least I would like to thank all of my friends and family. A huge thank you to my boyfriend Jason Gibbons for the constant support, love, and understanding. Thank you to my best friend Caitlin Lawrence who has been continually supportive and encouraging. Thank you to my mom, (Teresa Pettit) my grandparents, (Douglas and Joanne Pettit) my siblings, (Hannah and Oliver Hovde) my aunt, (Dawn Pettit) and my uncle and aunt, (Dean and Lori Pettit) for always encouraging me to pursue my passion and having faith that I would succeed. Thank you to my cousin Dr. Gary Solbrekken for the support and guidance. I would also like to thank the entire Gibbons and Rademacher families specifically, Sue, David, Trevor, Shane, and Megan, for their support as well. This has been a wonderful journey enhanced by all of amazing people in my life who have always supported me.

ABSTRACT

This dissertation focuses on the role of palmitoylation in regulating the sodium hydrogen exchanger isoform 1 (NHE1) and associated cellular functions as well as how various phosphorylation pathways alter NHE1 palmitoylation. Palmitoylation is the only known reversible lipid modification, allowing for the dynamic regulation of palmitoylated proteins not found with other lipid modifications. NHE1 regulates intracellular pH (pH_i) by exchanging an extracellular sodium for an intracellular hydrogen. Additionally, NHE1 is involved in regulating cell volume, cytoskeletal organization, protein anchoring, cell migration, and cell proliferation. NHE1 is highly regulated by protein interactions and modifications including phosphorylation. To begin, the first study shows NHE1 is palmitoylated in cells and rat tissues and can be inhibited by 2-bromopalmitate (2BP), a global inhibitor of palmitoylation. Furthermore, inhibition of palmitoylation with 2BP affects multiple NHE1 associated cell functions including pH_i, stress fiber formation, proliferation and migration.

Continuing to look NHE1 regulation, we used various agonists known to effect NHE1 phosphorylation followed by examination of NHE1 palmitoylation. Serum, lysophosphatidic acid (LPA) and phorbol 12-myristate (PMA) treatments all increased NHE1 palmitoylation while insulin decreased NHE1 palmitoylation. Multiple studies have shown that palmitoylation and phosphorylation within the same protein can impact each other in various ways and thereby work together to regulate protein function. As NHE1 is highly phosphorylated, we also utilized specific kinase inhibitors and measured the effect

on NHE1 palmitoylation. We found inhibition of PI3K, AKT, Rsk, and ROCK increased NHE1 palmitoylation while MEK inhibition decreased NHE1 palmitoylation. Together these studies establish a role for palmitoylation in the regulation of NHE1 activity and associated cellular functions and demonstrate palmitoylation and phosphorylation events may work in a coordinated manner to regulate NHE1.

CHAPTER I

BACKGROUND

Post-Translational Modifications

Proteins are the basis of cellular and physiological functions and their chemical and physical properties regulate activity and function within the cell[1,2]. Cellular proteins are regulated by a diverse range of chemical modifications on their amino acid side chains. These various modifying groups can affect charge, hydrophobicity, and other aspects of protein chemistry, resulting in marked changes in the behavior of protein molecules and hence in the control of physiological mechanisms[3]. Protein posttranslational modifications (PTMs) alter the physiological properties of proteins to change their behavior. PTMs are found in both eukaryotes and prokaryotes, being more common in eukaryotes where about 5% of the genome is dedicated to enzymes that carry out more than 200 types of PTMs[45]. PTMs are the attachment of specific chemical groups to amino acid side chains that occur both enzymatically or nonenzymatically[2,5]. Examples include phosphorylation, glycosylation, acetylation, methylation, sumoylation, palmitoylation, biotinylation, ubiquitination, nitration, chlorination, glycation, nitrosylation, acetylation,

and succination[2,6]. PTMs are important regulatory processes endowing proteins with the functional complexity that is essential for the maintenance of protein homeostasis within the cell.

Depending on the biological purpose of a modification on a specific protein reversibility may or may not be beneficial. Reversible PTMs allow for control of intensity and duration of the effect without the need to degrade the protein to end the desired effect. Important functions of reversible posttranslational modifications include maintenance of protein homeostasis as well as the ability of proteins to rapidly respond to changing extracellular and intracellular signaling[7]. Phosphorylation, acetylation, ubiquitylation, and S-palmitoylation are forms of covalent modifications that are readily reversed[5]. Reversible PTMs have dedicated enzymes, often large families of enzymes, that catalyze the addition and/or removal of covalent modifications[5]. This allows for protein regulation to occurring in both a spatial and temporal manner, which is vital for proper regulation of signaling. Phosphorylation is the quintessential reversible modification providing a good example of the importance and complexity of this type of dynamic regulation.

Phosphorylation is the reversible addition of a phosphoryl group from adenosine triphosphate (ATP) to primarily serine, threonine, or tyrosine residue and is broadly considered the most common posttranslational modification[2,8]. Phosphorylation introduces a charged phosphate group which induces an altered conformational change that can affect catalytic activity of proteins, the tendency of proteins to aggregate, and the ability to recruit binding partners, all of which result in altered protein function and

cell signaling[2,5]. This conversion of a neutral OH side chain to dianionic phosphate provides a useful conformation switch in protein-domain restructuring, as such it has evolved into the major recurring theme in eukaryotic proteome diversity[5].

Protein kinases, which add the phosphate to a protein, remain in a basal "off" state in the absence of a specific stimuli, once a signal is propagated, the kinases become active and are able to modify their target proteins. Controlling the duration and intensity of signal transduction is vital for proper protein signaling, as such the signals need to be turned off and, in this case, phosphatases hydrolytically remove the PO_3^{2-} group[5]. There are approximately 500 kinases and 150 phosphatases encoded by the human genome resulting in an almost infinite number of combinations creating amazing complexity[9,10].

Lipidation is a general term encompassing the attachment of different lipids and lipid-like groups onto proteins[11]. Protein lipidation is unique as it is structurally versatile and directly controls the attachment of soluble proteins to cell membranes without the requirement of other mediators[4]. Lipid modifications function to increase protein hydrophobicity can impact protein structure, localization, spatial distribution, membrane affinity, protein-protein interactions, and protein dynamics[12]. Covalent protein lipidation can occur enzymatically or nonenzymatically. Among the many lipid classes enzymatic modifications include myristylation, farnesylation, geranylation, and palmitoylation shown in Table 1. Palmitoylation is unique as it is the only known reversible lipid modification[4]. As such palmitoylation is crucial for the regulation of cellular plasticity due to the effects on the physical properties of proteins which therefore alter protein activity, localization, and membrane association.

Additionally, lipid modifications can, and often do, work together to increase intensity and duration of soluble protein membrane association[5,13]. Classically a single lipid tether provides hydrophobicity for a strong but kinetically unstable association with the membrane. Once another lipid is added there is a noticeable decrease in membrane off-rates, from as fast as one minute with a single tether to as long as multiple hours once another lipid is added. This greatly stabilizes the association of the protein with the membrane and enables increase vesicular trafficking[14,15].

Palmitoylation

S-acylation is a highly conserved process occurring in all eukaryotic organisms that have been examined and regulated by enzyme families that are conserved from yeast to humans. No evidence exists to show s-acylation occurs in prokaryotes, but many viral proteins can undergo s-acylation by hijacking host cell machinery[3]. S-acylation is the addition of a fatty acid, typically a saturated 16-carbon palmitate due to a combination of availability and affinity, to cysteine residues via a thioester bond. Enzymatic palmitoylation is catalyzed by palmitoyl acyltransferases (PATs) first identified in yeast. Genetic and biochemical studies showed these PAT enzymes share a highly conserved Asp-His-His-Cys domain termed the DHHC motif, resulting in these palmitoylating enzymes often being referred to as DHHCs[16–18].

Table 1. Types of Lipid Modifications

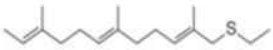
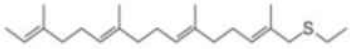
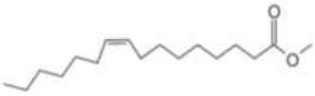
	linkage	amino acid	lipid species (major)	lipid structure	enzyme
Reversible	thioester	-Cys-	palmitate		zDHHC
Stable	amide	H_2N-Gly-	myristate		NMT
	amide	H_2N-Cys-	palmitate		Hhat
	thioether	-Cys-	farnesyl		FTase
	thioether	-Cys-	geranylgeranyl		GGTasel, GGTase II
	oxyester	-Ser-	palmitoleic		Porcn

Table 1. Types of Protein Lipid Modifications. This table demonstrates the various types of enzymatic lipid modifications, type of linkage, enzymes involved, and the lipid species. Important to note is the addition of a palmitate to a cysteine residue is the only known reversible lipid modification. Image copied with permission from Chamberlain and Shipston, 2015.

Palmitoylating Enzymes

The family of genes that encode the DHHCs are found in all eukaryotes with numbers ranging from five in yeast to 23 in humans[19]. DHHC proteins are transmembrane proteins with four to six transmembrane domains. The conserved DHHC motif located on the cytoplasmic face between the second and third transmembrane domain and is essential for enzymatic activity as it provides the necessary cysteine for the two-step kinetic mechanism. To begin, the DHHC protein autoacylates using an acyl-CoA donor forming a transient acyl-enzyme intermediate. Then the fatty acid, typically palmitate, is transferred to the protein substrate[20]. The C- and N-terminal tails are highly varied and typically contain domains involved in protein-protein interactions including, ankyrin repeats, SH3 domains, and PDZ-binding motifs.

This variation likely contributes to the substrate specificity seen with DHHC enzymes[21,22]. Some DHHC proteins appear to have a broad substrate specificity such as DHHC3 which has been show to palmitoylate both soluble and transmembrane proteins and is localized in the Golgi which is thought of as the hub of palmitoylation for soluble proteins. DHHCs also display specific subcellular localization (Table 2) with the majority of DHHCs residing in the ER and Golgi and a few found at the plasma membrane or in vesicles[23–25]. Additionally, some DHHCs such as DHHC2 cycle between the endosomes and plasma membrane[26]. This specific subcellular localization regulates access to substrates and contributes to selectivity. More recently is has been found that certain DHHCs are more common in specific tissues and others are expressed in only select tissues as can be seen in Table 3[27]. While the discovery of DHHCs as palmitoylating enzymes occurred in

2002[16,17] many of the enzymes were already known to be involved in various physiologies and diseases, as such they were given different names which are still often used. Some of the most common examples include DHHC3, named GODZ, important for the palmitoylation of AMPA and NMDA receptors thus playing a role in regulating the excitation and inhibition balance in the brain which when abnormal could lead to various neurological disorders[25,28,29]. DHHC17 was first identified as HIP14 (huntingtin-interacting protein) and palmitoylates huntingtin, the major protein involved in Huntingtin Disease, as well as other select neuronal proteins such as SNAP-25 and PSD-95. DHHC17/HIP14 plays an important role in regulating huntingtin and is implicated in the pathophysiology of Huntingtin Disease[25,30,31]. Other DHHCs that are related to specific disease include, DHHCs 6,9, and 21 implicated in Alzheimer's Disease, DHHC8 in schizophrenia and bipolar disorder, and DHHCs 9 and 15 in X-linked intellectual disability[25]. Consequently, understanding the regulation of palmitoylation via DHHCs is vital for understanding both healthy physiology and disease.

Depalmitoylating Enzymes

In contrast to the more detailed study of DHHCs, much less is understood regarding depalmitoylating enzymes. Palmitoyl protein thioesterase 1 (PPT1) is a member of the α/β serine hydrolase family and was characterized as a thioesterase through observations that it depalmitoylates H-Ras[32]. PPT1 localizes exclusively to lysosomes and late endosomes which separates PPT1 from substrate targets in the cytosol or at the plasma membrane[33].

Figure 1 DHHC Mechanism

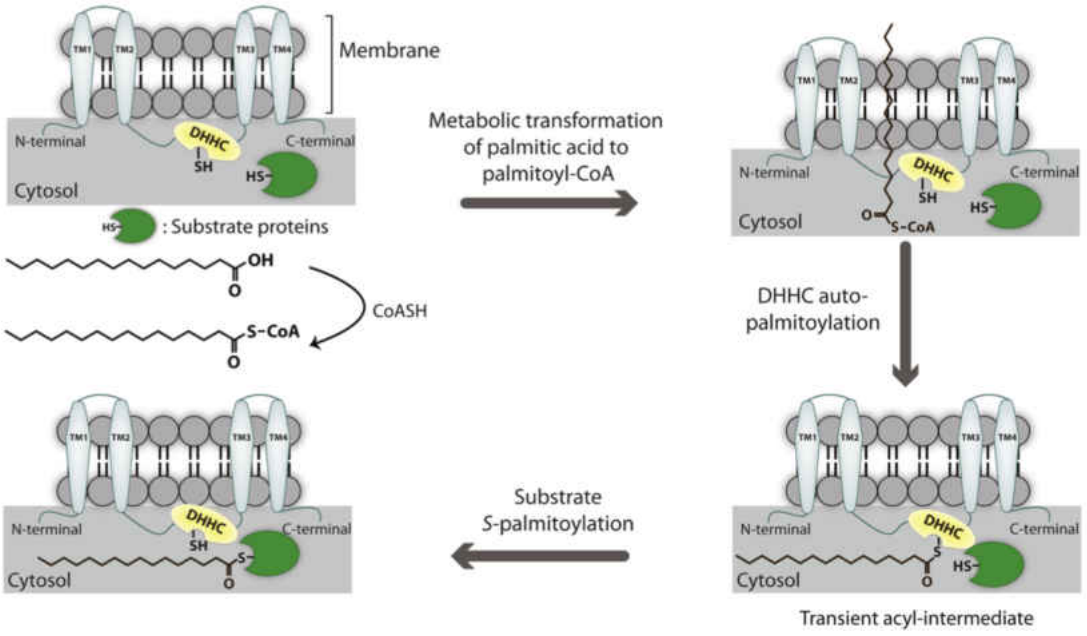


Figure 1. DHHC Mechanism. For S-palmitoylation palmitic acid is transformed to palmitoyl-CoA. The palmitoylating enzymes then use a ping-pong mechanism where the palmitate group is transferred from the CoA to the open thiol in the DHHC region of the palmitoylating enzyme creating a transient acyl-intermediate. The palmitate is then transferred to the free thiol on the substrate resulting in a palmitoylated protein. Image copied with permission from De and Sadhukhan, 2018.

Figure 2 Schematic of DHHC Enzymes

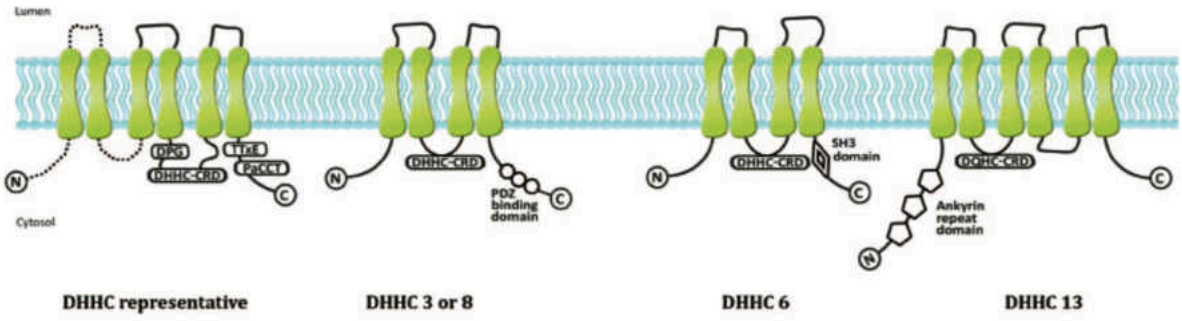


Figure 2. Schematic of DHHC Enzymes. This figure shows representative isforms of DHHCs showing their diversity and similarities. DHHCs all have the conserved DHHC active site but can vary in the number of transmembrane domains, regulatory domain, and binding sites. Image copied with permission from Tabaczar et al., 2017

Table 2. DHHC Subcellular Localization

zDHHC	Alternative names	Intracellular localization
1	DHHC1	ER EE
2	DHHC2	ER/Golgi Dendritic vesicles in neuron PM, recycling endosome
3	DHHC3, GODZ, Erf2	Golgi
4	DHHC4	ER Golgi
5	DHHC5	PM Endosomes in dendritic shafts
6	DHHC6	ER
7	DHHC7	Golgi
8	DHHC8	Golgi Dendritic vesicles Spines in neuronal cells
9	DHHC9	ER/Golgi
11	DHHC10	ER EE
12	DHHC12, AID	ER/Golgi
13	DHHC22, HIP14L	ER/Golgi
14	DHHC14	ER
15	DHHC15	Golgi
16	DHHC16	ER
17	DHHC17, HIP14, Akr1p	Golgi Intracellular vesicles Presynaptic terminals
18	DHHC18	Golgi
19	DHHC19	ER
20	DHHC20	PM
21	DHHC21	PM Golgi
22		Unknown
23	DHHC11, NIDD	ER PM (synaptic)
24	DHHC13	ER

ER, endoplasmic reticulum; EE, early endosome; PM, plasma membrane.

Table 2. DHHC Subcellular Localization. This table shows the known cellular localization of the different DHHC isoforms. While most DHHC reside in the ER and Golgi some are plasma membrane specific, with specific localizations such as neuronal spines and presynaptic terminals. This localization compartmentalizes the DHHCs which likely plays a role in regulating access to specific substrates. Image copied with permission from Cho and Park, 2016.

Table 3. DHHC Tissue Distribution

	Accession number	Alternative names	Chromosomal location of gene	Number of spliced variants	Number of SNPs	Size of gene [kb]	Number of exons	Intracellular localization of protein	Tissue-specific distribution
ZDHHC 2	NM_016353.4	ZNF372, DHHC2	8p21.3-p22	5	798	69.89	15	ER/Golgi	Brain, kidney, pancreas, testis, lung, eye
ZDHHC 3	NM_016598.2	GODZ, ZNF373	3p21.31	10	459	60.93	9	Golgi	Liver, spleen, lung, brain, prostate, colon, placenta, eye
ZDHHC 7	NM_017740.2	SERZ1; SERZ-B, ZNF370, FLJ10792, FLJ20279	16q24.1	7	552	37.55	9	Golgi	Lung, colon, brain, liver, prostate, skin, kidney
ZDHHC 8	NM_013373.2	ZNF378, ZDHHCL1	22q11.21	7	147	18.56	11	Golgi	Brain, lung, ovary, eye, pancreas, kidney
ZDHHC 9	NM_001008222.1	CGI-89, ZNF379, ZNF380, CXorf11 or ZDHHC10, LOC51114	Xq26.1	12	189	40.86	13	ER/Golgi	Brain, prostate, lung, kidney, thalamus
ZDHHC 12	NM_032799.4	ZNF400, FLJ14524, MGC13153, MGC54050	9q34.11	7	55	3.26	4	ER/Golgi	Stomach, ascites, skin, lung, prostate, brain
ZDHHC 13	NM_001001483.1	HIP14L, HIP3RP, FLJ10852, FLJ10941, MGC64994	11p15.1	10	640	59.50	15	ER	Uterus, brain, stomach, placenta, testis, colon
ZDHHC 17	NM_015336.2	HIP3, HYPH, HIP14, HSPC294, KIAA0946	12q21.2	15	875	90.36	18	Golgi	Brain, uterus, eye, lung, thalamus
ZDHHC 21	NM_178566.4	DNZ1, HSPC097, 9130404H11Rik	9p22.3	10	2599	147.33	10	Plasma membrane	Brain, testis, uterus, eye, liver

Table 3. DHHC Tissue Distribution. This table highlights that specific DHHC enzymes are found in specific tissues. The same DHHC isoform may be found in multiple tissues however not every DHHC is found in every tissue. This distribution may play an important role in substrate access or change the available DHHC binding partners regulating DHHC activity. Image copied with permission from Korycka et al., 2012.

PPT1 dysfunction has been implicated in infantile neuronal ceroid lipofuscinosis, a neurodegenerative disorder where lipid thioesters from acylated proteins accumulate in the lysosome causing devastating decreases in motor skills and speech with the development of seizures and death between age 9-11[34]. Palmitoyl protein thioesterase 2 (PPT2) is lysosomal specific and shares roughly 18% homology with PPT1. PPT2 is not able to depalmitoylate the same proteins as PPT1 demonstrating that PPTs also display substrate specify like PATs. Mutation of PPT2 also appears to cause a milder form of infantile neuronal ceroid lipofuscinosis[35]. Due to the localization of PPT1 and PPT2, neither are likely to contribute to depalmitoylation at the plasma membrane, instead contributing to lysosomal degradation of palmitoylated proteins and potentially playing a role in vesicular depalmitoylation.

Acyl protein thioesterases (APTs) are also members of the α/β serine hydrolase family and were originally purified from rat liver as lysophospholipases (lypla). APTs can hydrolyze multiple lysophospholipids and long-chain mono-acyl glycerol esters, at a much lower catalytic efficiency than palmitoylated substrates[36]. Three isoforms have been identified APT1, APT2 and APTL1 all of which are cytosolic proteins with the ability to interact with internal membranes and the plasma membrane. APT1 and APT2 share 81% homology and were originally thought to be redundant. Study of these enzymes have been limited due to the lack of antibodies, leading to most studies being done with GFP or HA tagged proteins. Overexpression of APT1 leads to decreased palmitoylation of small GTPase, endothelial nitric oxide synthase, and other soluble proteins in cells[37]. Recent studies have shown APT1 and APT2 are not redundant and do display substrate

specificity[38,39]. One interesting detail is that both APT1 and APT2 are palmitoylated, with APT1 being able to depalmitoylate both isoforms and APT2 been shown to only depalmitoylate itself but not APT1, adding complexity to the regulation of palmitoylation.[37,40]. Use of inhibitors has aided in the study of endogenous APTs, specifically the recent development of isoform specific inhibitors ML348 (APT1) and ML349 (APT2)[41]. APTL1 is a homologue of APT1 with 31% similarity, however structurally APTL1 is more similar to PPT2 with a narrow substrate binding pocket which prefers short-chain substrates. Currently, no protein substrates have been identified for APTL1[25].

While the study of APTs is ongoing and improving with the development of specific antibodies, many researchers found that APTs and PPTs alone could not explain all depalmitoylation. This reinvigorated the search for new depalmitoylating enzymes, specifically within the α/β serine hydrolase family and resulted in the discovery that ABHD enzymes can depalmitoylate proteins as well. Lin and Conibear found that ABHD17 depalmitoylates N-Ras, but not APT1 or APT2[42]. Three isoforms, ABHD17A, 17B, and 17C are also able to depalmitoylate PDS-95[43]. This indicates there may be more depalmitoylating enzymes

Cellular and Physiological Roles of Palmitoylation

Palmitoylation is the only known reversible lipid modification, as such palmitoylation plays an important role in the dynamic regulation of cellular proteins. Palmitoylation of a protein can tether it to membranes as well as direct proteins to specific membranes or membrane domains rich in cholesterol referred to as lipid rafts. Soluble proteins such as the Ras family provide a classic example of proteins whose

localization is dynamically regulated by palmitoylation. H-Ras and N-Ras are palmitoylated in the Golgi which then stabilizes their association with the membrane to facilitate vesicular trafficking to the plasma membrane where they function as important signaling molecules. Depalmitoylation at the plasma membrane then releases H/N-Ras to the cytoplasm, ending the signaling in which they are involved and allows its return to the Golgi, where the cycle can begin again[15,44,45]. Similar palmitoylation driven protein cycling has been documented for multiple other signaling molecules include G-proteins, kinases, synaptic proteins, and even depalmitoylating enzymes[14,46–49]. The rapid and dynamic nature of palmitoylation is akin to phosphorylation. However, it should be noted that rapid cycling of acyl chains on palmitoylated is not universal and low turnover rates can occur[3] adding to the complexity.

Palmitoylation of soluble membrane proteins promotes stable membrane binding, which as mentioned above is important even for dual lipidated proteins as a single myristoyl or prenyl group is not sufficient to provide a strong membrane interaction. As the enzymes that mediate palmitoylation are exclusively transmembrane proteins (see above) the weak association provided by a single irreversible lipid group is likely important for mediating the interaction between soluble proteins and the membrane to facilitate palmitoylation. Again, the Ras protein family provides an example of this. When the palmitoylation sites are mutated Ras proteins still show a weak association with the membrane, however when the farnesylation site is mutated loss of both palmitoylation and membrane association is seen[50]. Combining palmitoylation, a reversible lipid modification, with another irreversible lipid modification such as

prenylation or myristylation is a powerful way to regulate soluble protein membrane association as well as providing dynamic regulation of signaling proteins[5,15,45,51]. Of note some soluble proteins are exclusively palmitoylated with no obvious primary membrane targeting signals, such as SNAP25 an important component of the SNARE complex; it is suggested that such proteins rely on weak intrinsic membrane affinity to undergo palmitoylation[52]. Additionally two important neuronal proteins PSD-95, which functions to scaffold glutamate receptors at the post-synaptic membrane, and GAP43, which is an important component of the axonal growth cone rely solely on palmitoylation at multiple cysteines for their membrane tethering with the combination of palmitoylation sites being important for regulation of these factors[14,15].

Lipids within cell membranes are diverse and can form microdomains rich with cholesterol and saturated phospholipids, typically termed lipid rafts. Multiple studies have been done to understand the physiologic importance of these lipid enriched areas, with recent studies revealing palmitoylated proteins largely copurify with cholesterol-enriched detergent-insoluble membranes.[53] Further study showed palmitoylation plays an important role as a regulator of lateral distribution of proteins within the membrane and that palmitoylation is an important signal for sequestrating proteins to lipid rafts[54]. This palmitoylation dependent association of proteins to lipid rafts regulates important cellular pathways such as actin cytoskeleton remodeling via Rac1[55] and SNARE mediated exocytosis via SNAP25[56,57] demonstrating an important physiologic role for lipid rafts and palmitoylation.

Palmitoylation is also known to increase protein stability by preventing

ubiquitination thus decreasing protein degradation[4,21]. The interplay between palmitoylation and ubiquitination was reported for Tlg1, a SNARE protein, where palmitoylation alters the position of the transmembrane domain preventing acidic residues from associating with the membrane which is a signal for Tul1-mediated ubiquitination[58]. Additionally, proteomic analysis has been performed on mice with reduced expression of palmitoylating enzymes noting that decreased palmitoylation of proteins often correlated with an overall loss of expression[3].

Palmitoylation effects the localization of soluble membrane proteins by promoting accumulation of palmitoylated proteins on membranes containing the specific palmitoylation and depalmitoylation enzymes that act on that protein. Palmitoylation can also promote clustering of proteins within various membrane compartment as is the case with Ras trafficking. This allows for Ras signaling to be compartmentalized, with different Ras isoforms distributed within the Golgi stack based on di- and mono- palmitoylation events which aid in the theory that Ras proteins signal from the Golgi[4,59].

Since transmembrane proteins do not require palmitoylation for membrane localization the role of palmitoylation in trafficking is more prevalent and potentially easier to study than in soluble proteins. Trafficking from the ER after translation is dependent on proper topological arrangement of the protein. Various chaperons and enzymes in the ER lumen facilitate protein folding and catalyze modifications such as glycosylation which are important for the assembly of proteins by facilitating folding, subunit assembly and dimerization necessary for proper trafficking[60]. A large number of palmitoylating enzymes are found in the ER indicating palmitoylation plays an important

role in proper protein assembly[61]. LRP6 is a single transmembrane protein that is palmitoylated near the cytoplasmic side of the transmembrane region. When LRP6 palmitoylation sites are mutated the protein is retained in the ER, likely due to hydrophobic mismatching that occurs between the transmembrane domain and the ER bilayer. Palmitoylation reduces this mismatch by titling the transmembrane domain and promoting trafficking of LRP6 to the plasma membrane[62]. Trafficking of membrane proteins between the plasma membrane and endosomal system is also a common role of palmitoylation, where the ability of a protein to be internalized can be altered by the palmitoylation status as seen with AMPA and NMDA receptors. This is important to consider as many proteins only function at the plasma membrane, thus studying expression levels alone may not be sufficient to draw certain conclusions. In addition to altering surface expression, palmitoylation can also affect endosomal accumulation of proteins, such as MUC1, and impact protein cycling between endosomes and the Golgi as seen with Sortillin[3]. Palmitoylation plays a clear role in protein trafficking of both soluble and transmembrane proteins, leading to an important role for palmitoylation in regulating protein expression, signaling, and function.

Palmitoylation plays an important role in protein localization and thus can facilitate or inhibit protein-protein interactions. Palmitoylation can affect this interaction through steric hindrance, conformational changes, and/or interaction of cytoplasmic protein segments to the membrane interface. The generation of protein platforms through palmitoylation creates dynamic multiprotein complexes which can contain both palmitoylated and non-palmitoylated proteins. Oligomerization of proteins can also be

promoted by palmitoylation, which can alter protein functionality[63,64]. Direct proof that palmitoylation is required for the function of a given transmembrane protein can be difficult to prove as function is often dependent on localization and protein interactions. However, this does demonstrate the importance of palmitoylation in regulating both duration and intensity of protein activity.

Typically, when a palmitoylation site is found in close proximity to a transmembrane region the conformation of the domain and thus the function of the protein can be altered. Palmitoylation can increase the hydrophobic length of the domain and/or the orientation of the domain with respect to the bilayer. Tilting of the transmembrane domains are important, especially when considering the thickness of the membrane, as mentioned above with LRP6. Membrane thickness typically increases from the ER to the Golgi to the plasma membrane; additionally, the curvature of the membrane and cholesterol content can affect membrane thickness. If the transmembrane domain is too long or too short, it can lead to a hydrophobic mismatch which can have detrimental effects on the protein. Palmitoylation is typically used to reduce this mismatch by titling the membrane and allows for proper processing especially from the ER to the Golgi.

Palmitoylation, like phosphorylation, has been implicated in a diverse range of physiological processes. These processes include but are not limited to synaptic plasticity, host-pathogen interactions, innate immunity, cell death, metabolism, cell polarity and migration, and membrane trafficking. As palmitoylation affects so many vital cellular processes it can be implicated in almost any disease either directly or indirectly, making understanding the regulation of proteins by palmitoylation important for numerous

pathologies.

Sodium Hydrogen Exchanger Isoform 1 (NHE1)

Similar to how palmitoylation can be implicated in a broad range of diseases ubiquitously expressed proteins, especially those that regulate basic cellular functions, are also implicated either directly or indirectly in a broad range of diseases. Intracellular pH (pH_i) is one of these basic systems that is regulated by the sodium hydrogen exchanger isoform 1 (NHE1). NHE1 is a ubiquitously expressed transmembrane protein that exchanges an intracellular hydrogen for an extracellular sodium to regulate pH_i and control cell volume[65]. The study of NHE1 was greatly enhanced when Pouyssegur et al used a highly specific proton suicide technique on Chinese hamster lung fibroblast (CCL39), which express only isoform 1 of NHE, to create mutant cells lacking NHE1 expression (PS120)[66]. Later human NHE1 was transfected into these cells resulting in the PSN cell line which now expresses human NHE1 as the main regulator of pH_i, making these cell lines the gold standard to study NHE1 activity via pH_i measurement.

NHE1 Physiology

Regulation of pH and control of cell volume are the most basic roles of NHE1 these factors also have wide ranging implications on many cell processes such as cell migration, cell proliferation, vesicle trafficking, apoptosis, cell survial[67] and cell metabolism[68,69]. In fibroblast type cells NHE1 preferentially localizes to the leading edge of lamellipodia, where ion transport creates an alkaline intracellular environment paired with an acidic extracellular environment. This allows for actin stress fibers to form at the membrane[70] while simultaneously aiding in the degradation of the extracellular matrix allowing for

increased cell migration and invasion. Multiple studies have shown that the presence of NHE1 is essential for proper directional cell migration[71,72]. Recently, NHE1 has been shown to play a role in lysosome trafficking to the cell periphery in the prostate[73]. Additionally, NHE1 acts as a protein scaffold, binding many partners including PIP_2, ERM, calmodulin and CHP1/2 and various posttranslational modifications[74,75]. Through this scaffolding, NHE1 contributes to various cell signaling processes in ion independent and dependent manners with the binding of proteins and addition of modifications having been shown to alter transport activity[76–78].

NHE1 has also been well researched in specific organ systems, especially the cardiovascular system where NHE1 is a central regulator of cardiomyocyte pH_i and contractility[79] and is localized to the plasmalemma, intercalated discs, and transverse tubules. Cardiac pH_i changes during increased cardiac workload and heart rate with increased NHE1 activity contributing to cardiac ischemia-reperfusion damage and hypertrophy[80]. NHE1 is also found in the vasculature in both smooth muscle and endothelial cells. Based on several studies in mice and cells, NHE1 is thought to play a role in pulmonary artery remodeling, angiogenesis, volume control and fibronectin production[76,81]. NHE1 is also essential for the central nervous system, where studies using NHE1 knockout mice show abnormal neuronal excitability, epilepsy from Na^+ permeability, and death before weaning. Other studies show NHE1 playing a role in several neurological diseases including dementia, specifically Alzheimer's Disease, neuropathic pain, and cerebral ischemia[82]. In the central nervous system (CNS) NHE1 regulates pH_i in neurons, glial cells, and microvascular endothelial cells. The mechanisms

surrounding the role of NHE1 in the CNS are largely unknown. Most studies have focused on the role NHE1 plays in glioblastoma multiform, an aggressive cancer of the glial cells, as the role of NHE1 in cancer has been clearly established[83–85]. A number of mitogenic factors have been shown to activate NHE1 resulting in increased cell growth, proliferation, migration, and invasion, all processes important for the neoplastic transformation and maintenance of malignant cells. The pH_i of malignant cells is more basic than that of the corresponding nontransformed cells, thus disturbance of pH homeostasis has been thought to correspond to an increased cancerous state[86]. NHE1 has been implicated in a number of cancers apart from glioblastoma, including but not limited to breast[87,88], prostate[89], ovarian, and lung[90,91]. Recent interest has surrounded the role NHE1 plays in activation of brain microglial cells and how this may contribute to brain focal ischemia[92]. In addition, activation of brain microglia and increased inflammation has been shown to play a role in neurodegenerative disease, specifically Alzheimer's Disease, indicating NHE1 could play a bigger role in neurological diseases than originally thought. Furthermore NHE1-mediated pH-regulation has been shown to be important for IgE-induced macrophages and correctly functioning neutrophils[76,93,94].

NHE1 Structure

Human NHE1 (hNHE1) is an 815 amino acid protein with the first 500 residues making up 12 transmembrane spanning segments with interconnecting loops and the last 315 comprising an intracellular tail. Due to the difficulties associated with crystallizing membrane proteins, relatively little is known about the structure of NHE1. Some insight has been provided by the crystallization of the *E. Coli* Na^+/H^+ antiporter (NhaA) by Hunte

et al (2005) which shows structural similarity to hNHE1[95,96]. Additional various molecular biology techniques have been employed to gain a general understanding of NHE1 structure. Substituted-cysteine-accessibility analysis was used to confirm the 12 TM domain topology and that the N- and C-termini were cytosolic. It also identified three membrane associated regions, intracellular loop 2 (IL2), intracellular loop 4 (IL4) and extracellular loop 5 (EL5), which may be important in for NHE1 function. The transport function of NHE1 occurs in the N-terminal half of the protein (first 500 residues) with TM IV, TM VII, TM IX, and TM XI containing residues that are important for ion binding and transport[97]. The C-terminal region (315 amino acids) is completely intracellular and considered the regulatory domain of NHE1. The membrane proximal region (503-685) termed the predicted folded domain is 50% random coil with 35% of that being α-helix and the membrane distal domain (686-815) being intrinsically disordered[98,99]. The proximal region contains multiple protein binding sites while the distal region has multiple phosphorylation sites which operate to regulate NHE1 function and are described in greater detail below.

NHE1 Binding Partners

Phosphatidylinositol 4,5-bisphosphate (PIP_2) is a signaling phospholipid at the plasma membrane that can be converted into other signaling molecules that are important 2nd messengers. NHE1 has two PIP_2 binding sites (513-520 and 556-564) and studies show loss of PIP_2 binding inhibits NHE1 function making the NHE1-PIP_2 interaction important for proper regulation[69,100,101]. Calcineurin homologous protein 1 (CHP1) also binds NHE1 in the proximal membrane region between residues 510-530. The association

between CHP1 and NHE1 is essential as interrupting this interaction results in a dramatic loss of NHE1 activity[102]. Overexpression of CHP1 in fibroblast cells leads to inhibition of serum stimulated proton transport, while mutations of the CHP1 binding site on NHE1 reduce proton sensitivity and decrease NHE1 activity[103,104]. It is thought that the CHP1-NHE1 interaction promotes an NHE1 structure with a more available proton sensing domain, preserving pH_i sensitivity of NHE1[101]. Additionally, CHP2, which is highly expressed in tumor cells, interacts with NHE1 and protects cells from serum deprivation induced cell death by increasing pH_i to maintain the malignant state of transformed cells[103].

Ezrin, radixin, and moesin (ERM) proteins form a complex at the plasma membrane that provides an important link between the plasma membrane and actin filaments that form the cytoskeleton. NHE1 contains two ERM binding domains on the C-terminal tail between residues 553-564 which directly mediates this interaction[105]. The cellular cytoskeleton is essential for maintaining cell shape and establishing mechanical and chemical properties within the plasma membrane. The ERM -NHE1 interaction directs NHE1 localization in the lamellipodia of migrating cells and if this interaction is disrupted, cells develop an irregular shape and have limited motility[71]. Additionally, the physical interaction of ERM proteins and NHE1 activates Akt, a pro-survival kinase which leads to stalled apoptosis[75,106]. Calmodulin (CaM) is a calcium binding protein that binds NHE1 to regulate pH_i in response to calcium signaling. Binding of calcium and calmodulin to the high affinity site on NHE1 (636-656) activates NHE1 by blocking the autoinhibitory interaction thus regulating intercellular pH[107]. NHE1 also has three consensus sites for N-

linked glycosylation, N75, N370, and N410. Interestingly mutation of all three sites, individually and in combination, produced functional transporters with similar transport rate and pharmacological profiles to the wild type exchanger, however cleavage using endoglycosidase does result in reduced apparent size of NHE1 from 110kDa to 90kDa[108,109].

NHE1 Phosphorylation

NHE1 regulation by phosphorylation has been widely studied. Bioinformatic and mass spectrometry analysis has predicted multiple phosphorylation sites on the C-terminal tail focused mostly on the distal end. Many of these phosphorylation sites have been experimentally validated along with the kinases involved and various stimuli including hormones, growth factors, and sustained intracellular acidosis[97]. Growth factor stimulation of fibroblast cells induces the MAPK (mitogen-activated protein kinase) cascade resulting activation of ERK1/2 (extracellular-signal-regulated kinase) leading to NHE1 activation. Inhibition of the ERK signaling cascade reduces growth factor stimulated NHE1 activation by 50-60%, a similar reduction is seen when ERK sites on NHE1 (S770/771) are removed[110]. ERK2 also stimulates phosphorylation on S693, S766, S770, and S785, however, the importance of these residues needs to be further explored. ERK is also an upstream regulator of Rsk (p90 ribosomal S6 kinase), which phosphorylates NHE1 at S703[111]. Both the ERK and Rsk sites are important for invasion and migration of breast cancer cells. Additionally, the Rsk site (S703) can act independently of the ERK sites to control movement of tumor cells into and out of the circulatory system which is important for cancer metastasis[112,113]. Interestingly, stimulation of fibroblast cells with

platelet derived growth factor (PDGF), which activates the ERK/Rsk pathway, results in NHE1 mediated cell migration and cytoskeletal formation but not alteration of NHE1 transport activity[114]. AKT phosphorylation is also required for NHE1 activation through PDGF stimulation. AKT phosphorylates NHE1 at S648 and activates NHE1 in fibroblasts, however, AKT phosphorylation of NHE1 in cardiomyocytes has an inhibitory effect[70].

ROCK (Rho-associated kinase $p160^{ROCK}$) is a downstream target of RhoA (GTPase) which stimulates NHE1 via direct phosphorylation at T653. Both the ROCK and ERK pathways are required for full activation of NHE1 through growth factor receptor stimulation[98]. Apoptotic signals stimulate MAPK p38 which induces intracellular alkalization activating NHE1 through phosphorylation at potential sites including T718, S723, S726, and S729[115,116]. Platelet derived growth factor activates NHE1 via NIK (Nck-interacting kinase) phosphorylation at S638[114,117]. NHE1 activation through phorbol ester stimulation has also been shown, however, no direct PKC phosphorylation sites on NHE1 have been found[118,119]. In addition, acidification stimulates CaMK II (Ca^{2+}/calmodulin-dependent kinase II) medicated NHE1 phosphorylation and contributes to increased NHE1 activity. Several other kinases including PKA, Raf, Pyk2 and Daxx have been found to bind and phosphorylate NHE1 but specific residues have not been found[120]. Figure 3 displays these phosphorylation and binding partner sites on NHE1. Overall NHE1 is a highly modified protein, which is important for regulating activity as well as localization, protein scaffolding, and protein-protein interactions. Proper regulation of NHE1 is vital for maintaining cellular homeostasis. The presence of multiple cysteine residues on the C-terminal tail of NHE1 combined with its highly modified nature lead us to explore NHE1

palmitoylation and the role the regulation plays physiologically.

Dissertation Research Objective

The objective of this dissertation is to demonstrate NHE1 is palmitoylated and inhibition of palmitoylation alters NHE1 associated physiology. As well as show preliminary evidence that multiple phosphorylation pathways that act to alter NHE1 palmitoylation indicating palmitoylation and phosphorylation work in a coordinated manner to regulate NHE1.

Figure 3 Schematic Model of NHE1

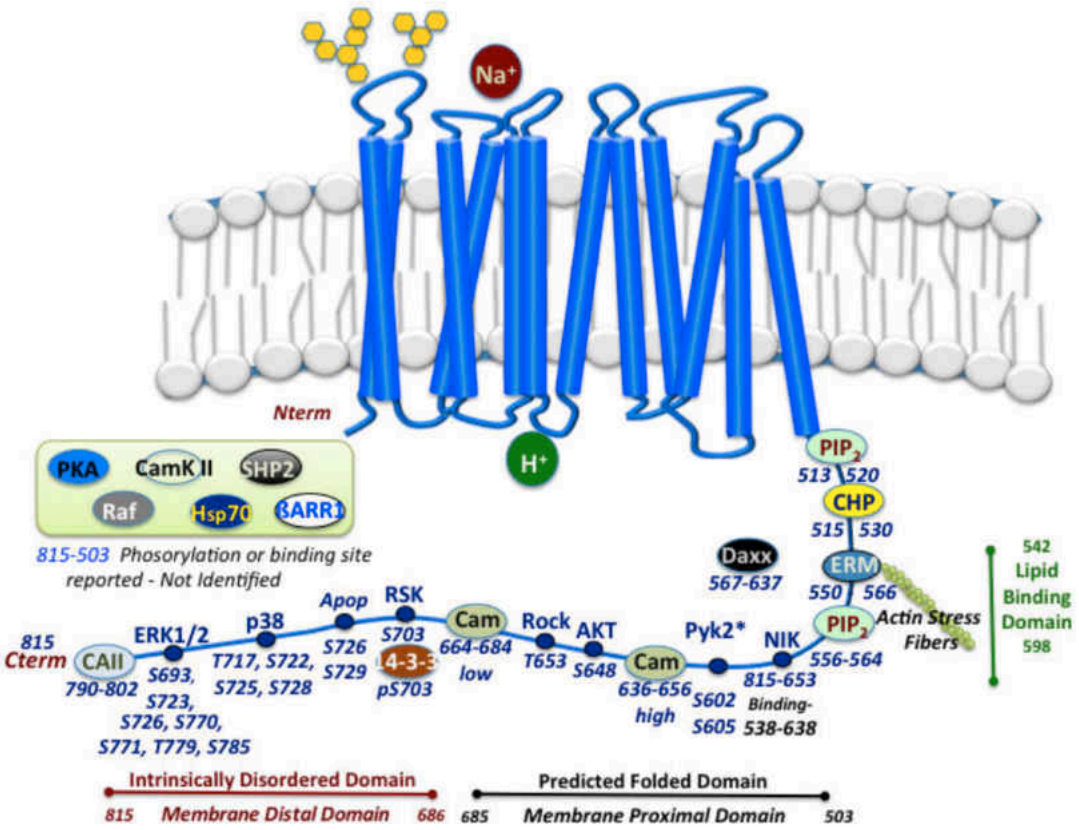


Figure 3. Schematic Model of NHE1. This NHE1 diagram highlights the C-terminal tail showing the large number of phosphorylation sites and kinases involved. Also shown are various biding partners and their subsequent binding sites. Copied with permission from Wallert et al., 2016.

Chapter II

FUNCTIONAL CONSEQUENCES OF INHIBITING SODIUM HYDROGEN EXCHANGER 1 (NHE1) PALMITOYLATION

Abstract

Palmitoylation is a reversible lipid modification that regulates an array of protein functions including activity, trafficking, membrane microlocalization and protein-protein interactions. Identification of over 5000 proteins with this modification suggests that palmitoylation plays a critical role in regulation of many cellular processes. In this study we demonstrate for the first time a member of the SLC9 family, the sodium hydrogen exchanger isoform 1 (NHE1), is palmitoylated in both cells and rat tissue. In addition, using the irreversible PAT inhibitor 2-bromopalmitate (2BP) we have identified several cellular functions commonly associated with NHE1 are affected by decreased NHE1 palmitoylation. Treatment of Chinese hamster lung fibroblast cells expressing human NHE1 with 2BP results in decreased palmitoylation. This treatment also resulted in decreased NHE1 activity, detected by measuring the change in intracellular pH. Additionally, PSN cell migration and proliferation was inhibited by 2BP in a manner consistent with the degree of inhibition of NHE1 palmitoylation. These results suggest that palmitoylation plays a major role in NHE1 regulation that could significantly impact multiple critical cellular functions.

Introduction

The SLC9 transporter family is made up of transmembrane sodium hydrogen exchangers which function to regulate intracellular pH (pH_i) by exchanging an intracellular proton for an extracellular sodium ion in various tissues[75]. Sodium hydrogen exchanger isoform 1 (NHE1) functions at the plasma membrane and is ubiquitously expressed throughout tissues[121,122]. Multiple cellular processes are associated with NHE1 activity including coordinated cell migration, cellular proliferation, and control of cell volume. NHE1 also functions as a membrane anchoring and scaffolding protein resulting in the formation of different protein complexes that participate in regulating signaling pathways within the cell[123,124]. Regulation of NHE1 activity and function occurs within the regulatory domain found on the large intracellular C-terminal tail. Many binding partners and modifications occur within this regulatory domain and influence exchanger activity and trafficking. Major binding partners include PIP_2[100] and ERM[71] which are important for association of the tail to the inner membrane leaflet and the formation of stress fibers, respectively[123]. Ca^{2+}/calmodulin binding sites also on the C-terminal tail have been shown to play an important role in proton affinity and thus regulate NHE1 activity[107]. Additionally, multiple phosphorylation sites are also present on the C-terminus which have varying effects on NHE1 activity and the multiple cellular processes in which NHE1 plays a role. Together these many regulatory factors control NHE1 activity, localization, and expression, all of which are important for regulation of critical cellular processes.

S-Palmitoylation is the addition of a 16-carbon fatty acid to a cysteine residue via a thioester bond. Palmitoylation is a unique lipid modification because it is reversible, with

palmitoyl acyltransferases (PATs) adding the palmitate while acyl protein thioesterases (APTs) and palmitoyl-protein thioesterases (PPTs) remove the palmitate. The most well studied function of palmitoylation is trafficking of proteins between the organelles and increasing membrane association of cytosolic proteins. Recent studies have demonstrated that palmitoylation has multiple other effects on proteins, particularly transmembrane proteins where effects include regulation of protein activity, membrane microlocalization and protein-protein interactions[125,126]. Dysregulation of protein palmitoylation has been implicated in neurological dysfunction including Huntington and Alzheimer's disease, multiple forms of cancer and many other diseases[26,127–130].

Due to the highly modified nature of the NHE1 C-terminal tail and the presence of multiple cysteine residues which are potential sites of palmitoylation we hypothesized that palmitoylation could be present on NHE1 playing a role in regulating activity and associated cellular functions. Using acyl biotinyl exchange (ABE) and metabolic incorporation of [^{3}H]palmitate we showed NHE1 is palmitoylated in both cells and various rat tissues. Additionally, we used 2-bromopalmitate (2BP), an irreversible inhibitor of palmitoylating enzymes (PATs)[131], to decrease palmitoylation of NHE1 in a time dependent manner. Using 2BP to decrease palmitoylation we also showed a decrease in pH_i indicating palmitoylation plays a role in regulation of NHE1 activity. Additionally, stress fiber formation, cell migration, and cell proliferation were decreased upon 2BP treatment, all critical cellular functions regulated by NHE1. Together these data establish a role for the reversible lipid modification, palmitoylation, in the regulation of NHE1 activity and associated cellular functions.

Materials

Colorburst molecular mass standard, 2- bromopalmitate (2BP), and mouse anti-flag tag antibody were from Sigma Millapore and anti-NHE1$_{(54)}$ mouse monoclonal antibody (sc-136239) was from Santa Cruz Biotechnology. MMTS (*N*-(6-(biotinamido)hexyl)-3-(2-pyridyldi-thio)-propionamide), high capacity NeutrAvidin ®-agarose resin, and bicinchoninic acid protein assay reagent was from Thermo Scientific. HPDP-biotin was from APExBio, BCECF-AM 2',7'-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein was from Molecular Probes and Nigericin was from Tocris Bioscience. LPA in the form of 14:0 1-myristoyl-2-hydroxy-sn-glycero-3phosphate (sodium salt) was from Avanti Polar Lipids and XTT(2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxyanalide) reagent from Trevigen.

Methods

Cell Culture

NHE1-null cells (PS120 cells, a gift from D. Barber, University of California, San Francisco) derived from Chinese hamster lung fibroblasts CCL339 were used to generate a stably expressing human NHE1 with HA DDK epitope tag on the C terminus labeled PSN cells. These cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 20% fetal bovine serum, 2 mM L-glutamine, and 200 µg/ml G418 at 37^{o}C in a humidified 5% CO_2 incubator after clonal isolation [98].

Cell Treatments and Membrane Preparation for Palmitoylation Detection

PSN cells were subcultured, seeded on 150 mm culture dishes, and grown to confluency and treated with experimental treatment conditions as described. After

treatment, cells were washed twice with 3 mL of ice-cold buffer B (0.25 M sucrose, 10 mM triethanolamine, 10 mM acetic acid, pH 7.8) and then scraped to one side of the plate using 0.5 mL of buffer B and transferred to a 2 mL microcentrifuge tube on ice. Cells were then pelleted by centrifugation at 3,000 x g for 5 min at 4°C. The supernatant fraction was removed and 1 mL of ice-cold buffer C (0.25 M sucrose, 10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, pH 7.8) containing protease inhibitor was added to break the pellet loose. The solution containing the loose pellet was then homogenized in a Dounce homogenizer with 30 up and down strokes on ice. The resulting homogenate was centrifuged at 800 x g for 10 min at 4°C to pellet and remove nuclei and cell debris. The supernatant fraction was transferred to a new tube which was centrifuged at 16,000 x g for 12 min at 4°C to precipitate cell membranes. The supernatant fraction was removed and the pellet containing cell membranes was resuspended in 1 mL sucrose-phosphate (SP) buffer (10 mM sodium phosphate, 0.32M sucrose, pH 7.4) and assayed for protein content.

Metabolic Labeling of NHE1 with [^{3}H]Palmitate

PSN cells were metabolically labeled with [9,10-^{3}H]Palmitic acid (0.5 mCi/ml) for indicated times at 37 °C in α-MEM, containing 1 mM sodium pyruvate to inhibit palmitate metabolism through fatty acid -oxidation[132,133]. After labeling, cells were washed with SP buffer and lysed in radioimmunoprecipitation assay buffer (RIPA; 10 mM sodium phosphate, 150 mM NaCl, 2 mM EDTA, 50 mM sodium fluoride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, pH 7.2). Lysates were then immunoblotted with anti-DDK to determine NHE1 levels and volumes containing equal amounts of NHE1 (verified

by a second immunoblot) were immunoprecipitated with anti-DDK protein A beads. Precipitated NHE1s were resolved on 4-20% SDS-polyacrylamide gels, treated with Fluoro-Hance (Research Products International) fluorographic reagent for 30 min, dried, and exposed to x-ray film for ~ 60 days.

Palmitoylation Detection using Acyl-Biotinyl Exchange (ABE)

Adapted from Wan et al[134] palmitoylated proteins are detected using three steps. (i) Free cysteine thiols are blocked; membranes isolated from PSN cells were solubilized in lysis buffer (50 mM HEPES pH 7.0, 2% SDS (w/v), 1 mM EDTA) containing protease inhibitors and 20 mM methyl methanethiosulfonate (MMTS) and incubated for 20 minutes at 37^{o}C, followed by acetone precipitation. Precipitated proteins were again solubilized in lysis buffer containing protease inhibitors and MMTS and incubated at room temperature with end over end mixing overnight. MMTS was removed by three consecutive acetone precipitations in which the protein pellet was resuspended in 4SB buffer (4% SDS, 50 mM Tris, 5 mM EDTA, pH 7.4) followed by acetone precipitation. (ii) Thioester linked palmitoyl groups are specifically removed by hydroxylamine (HAM); The resuspended protein sample is split equally into negative control (50 mM Tris-treated) and HAM-treated (0.7 mM) halves and incubated at room temperature for 15 min with end-over-end mixing. (iii) The previously palmitoylated and now free sulfhydryl groups are then biotinylated by the addition of sulfhydryl specific HPDP-biotin (0.4 mM, 50 mM Tris-HCl, pH 7.4) for 1 h at room temperature with end over end mixing, HAM and unbound biotin was removed with three sequential acetone precipitations; the biotinylated proteins were then affinity purified using NeutrAvidin® resin. Bound proteins

were eluted, subjected to SDS-PAGE, and immunoblotted with mouse anti-Flag M2 primary antibody to detect tagged NHE1.

Live/Dead Cell Assay

PSN and PS120 cells were grown in media with the indicated 2BP concentrations and cell viability was were assessed using the viability/cytotoxicity assay from ThermoFisher. Live cells were counted after staining with calcein-AM while dead cells were determined by uptake and staining with ethidium homodimer-1.

Cell Proliferation Assay

Cell proliferation assay was performed by the reduction of XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxyanalide). Cells were plated at 2,500 cells/well in a 96-well plate and incubated for 4 h to allow for attachment. Then the cells were incubated in DMEM media containing 0.5% FBS with the indicated agonist or inhibitors. After 48 h, activated XTT reagent was added to each well to attain a final concentration of 0.3 mg/mL and absorbance was read at 450 nm and 690 nm.

Intracellular pH Determination

Steady-state intracellular pH (pH_i) was measured using 2',7'-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF), a pH-sensitive fluorescent dye. Cell monolayers were cultured in multi-well dishes in either 20% or 0.5% serum and treated with experimental treatment conditions as described. Prior to pH_i determination, cells were incubated with 10 µM BCECF in PBS for 10 min at 37^{o}C and 5% CO_2. BCECF solution was replaced with treatment condition media to remove free dye and incubated at 37^{o}C and 5% $CO_2$for 10 min at before imaging. Fluorescence was measured using a fluorescent plate reader

(ThermoFisher Scientific Fluoroskan Ascent Fluorescence 96/384 Well Plate Reader) at 440:527 and 485:527 nm excitation: emission corresponding with pH independent and pH dependent regimes of BCECF excitation respectively. pH_i was calculated by taking the fluorescence intensity ratio of pH independent over pH dependent fluorescence compared to an internal K^+-Nigericin calibration curve from each well. Curves were generated by incubating cells in buffered 50 µM Nigericin at pH 6.5 and pH 7.5 for 5 min followed by the measurement of BCECF fluorescence.

Stress Fiber Formation

Cells were grown on quartz coverslips and following the indicated treatment were fixed with 3% paraformaldehyde at 4°C for 30 min and permeabilized with a 0.4% (v/v) solution of Triton-X100 in water for 10 min. Actin stress fibers were stained with 0.5 µg/mL phalloidin-Alexa Fluor 488 for 60 min in a humidified container. Prior to mounting, prolong anti-fade reagent was applied. Cells displaying significant and strong stress fibers[98] were counted in five random fields for each slide.

Electric Cell-substrate Impedance Sensing (ECIS) Migration Assay

Using the ECIS Z system (ECIS® Z, Applied Biophysics Inc.), impedance was measured to characterize migration rates in cultured cells. Cells were seeded at 100,000 cells/well in 8-well arrays (8W1E-PET) in DMEM medium containing 0.5% FBS and allowed to attach for approximately 21 h. Cells were then treated with 15 µM 2BP for 18 h after which a 20 sec. 16,000 Hz shock was sent to the 250 µm diameter electrode located in the middle of each well. Cell death occurs only to the cells attached to the electrode and then impedance was measured in real-time as cells migrated onto the electrode.

Wound Healing Assay

Cells were seeded to each well of culture inserts (Ibidi) and incubated for attachment and until complete confluence at 37^{o}C in a humidified 5% CO_2 incubator. Twelve h prior to removal of the culture insert, cells were treated with the indicated concentrations of either DMSO (control) or inhibitor before removal of insert. Images of each wound was collected at the indicated time point and determined at four sites. Results are presented as an average of four trials repeated 2-3 times. The percent wound remaining was calculated as compared to each initial wound.

Results

NHE1 is Palmitoylated in PSN Cells and Rat Tissues.

Palmitoylation occurs on intracellular cysteine residues and regulates many functions including protein trafficking, membrane microdomain localization, and protein activity. NHE1 is a versatile protein that requires specific membrane microdomain localization and trafficking and is also highly modified by phosphorylation. Upon examination of the human NHE1 sequence we found five intracellular cysteines, with three residing on the highly modified C-terminal tail. Taken together, this information led us to speculate that NHE1 may be a palmitoylated protein.

To test this hypothesis, we used PSN cells which stably express human NHE1 and various rat tissues including brain, lung, liver, and heart to explore NHE1 palmitoylation utilizing an acyl biotinyl exchange (ABE) method. The ABE process is an in vitro assay for post-hoc detection of endogenous palmitoylation. In short, the ABE is done by removing endogenous acyl thioesters using hydroxylamine (HAM) and replacing them with a

sulfhydryl-specific biotinylating reagent (HPDP biotin). Control samples treated with Tris in place of HAM do not incorporate the sulfhydryl reagent, demonstrating the specificity of labeling. Figure 4 is a representative western blot of NHE1 demonstrating the ABE palmitoylation assay. The presence of NHE1 in the in the HAM fraction (*Palm NHE1*, Fig 4A) is representative of palmitoylated NHE1, while the absence of NHE1 in the negative control Tris fraction (*Tris Control*, Fig 4A) shows there was no non-specific incorporation of HPDP-biotin nor nonspecific binding to the NeutrAvidin™ resin. Additionally, immunoblotting samples of the HAM and Tris treated fractions for NHE1 prior to NeutrAvidin™ chromatography shows total NHE1 levels were not changed by the HAM exposure. To confirm this finding, we used metabolic labeling of PSN cells with radiolabeled palmitic acid[132]. After labeling with 10 µM [^{3}H]palmitic acid for 1-6 h, NHE1s were extracted by immunoprecipitation and subjected to SDS-PAGE followed by fluorography (Fig. 4B). A [^{3}H]palmitate-labeled band appeared at a mass of ˜90kDa, the monomeric mass of NHE1. An additional band was present slightly below 90kDa representing the immature non glycosylated form of NHE1. Time course studies show [^{3}H]palmitate labeling of NHE1 was scarcely detectable within 1 h and increased in intensity after 3 and 6 h of metabolic labeling indicative of rapid palmitate turnover.

Figure 4 S-Palmitoylation of NHE1

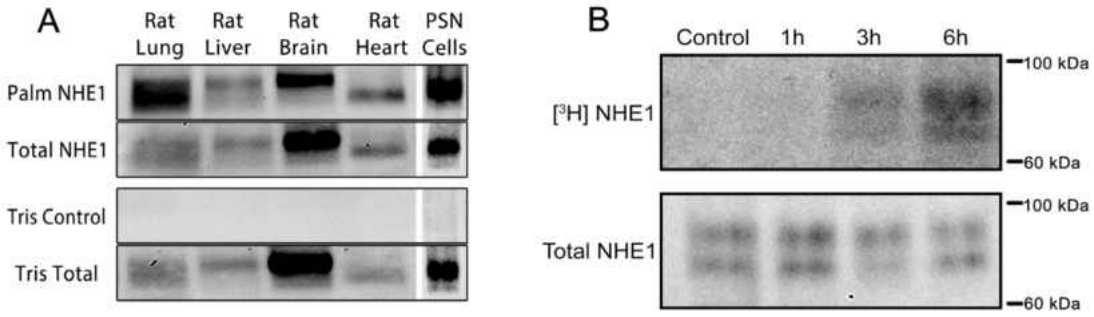


Figure 4. S-Palmitoylation of NHE1. Determination of NHE1 palmitoylation was confirmed by acyl biotin exchange (ABE) in lysates from various rat tissues and PSN cells (A) as well as by [^{3}H]palmitic acid labeling in PSN cells (B). A, Lysates were treated with Tris or hydroxylamine (HAM) and immunoblotted for NHE1. NHE1 in the total portion of the immunoblot shows NHE1 present in the lysate. The presence of NHE1 in the HAM fraction (top panel) shows NHE1 is modified by acylation. The lack of NHE1 in the negative tris control fraction indicates only NHE1 modified by an acyl thioester was detected by the assay. (B) PSN cells were metabolically labeled [^{3}H] palmitic acid for indicated times. Cells were lysed and immunoprecipitated with anti-NHE1 followed by SDS-PAGE/fluorography. The top panel shows [^{3}H]palmitic acid incorporated into NHE1 showing NHE1 is palmitoylated consistent with the ABE. Results are show as representative western blots of three independent experiments.

Inhibition of NHE1 palmitoylation

To further evaluate NHE1 palmitoylation we utilized 2-bromopalmitate (2BP), a well-known irreversible inhibitor of palmitoyl-acyl transferase enzymes (PATs), which catalyze the addition of palmitate to proteins. To begin, we assessed the potential toxicity of 2BP on lung fibroblast cells (PSN). These cells, derived from NHE null PS120 fibroblasts, are stable expressers of epitope tagged WT human NHE1[135,136]. This allows us to identify specific NHE1 related functions as no other NHE isoforms are expressed in the parental cell line[98]. Figure 5 shows that after treatment with either 20 μM or 50 μM 2BP, cells showed a minimal decrease in cell viability (5A and B) and the PSN cells began to lose viability only after 70 h of treatment (5A). A similar effect was observed in non NHE1 expressing cell lines (5B). Incubation of both PSN and PS120 cells with the corresponding concentrations of palmitate had no effect on cell viability as expected, clearly indicating that potential changes in cell physiology after 2BP treatment would not be due to acute toxic effects. As PSN cell viability was not affected by 2BP treatment during the 18 h time frame, we began to assess NHE1 palmitoylation by treating PSN cells with a low concentration of 2BP (1 μM) for various time points. Here we saw a stepwise decrease in NHE1 palmitoylation from 1 h to 18 h (All $p<0.05$ vs. NT; 0.5 h $p<0.05$ vs. 9 h and 18 h) without the loss of total NHE1 levels (Fig. 6A). For palmitoylation analysis via ABE, control samples treated with Tris in place of HAM did not incorporate the sulfhydryl reagent, demonstrating the specificity of palmitoylation site labeling. Additionally, immunoblotting showed that total NHE1 levels were not changed by the 2BP incubation or HAM exposure ($p>0.05$). These results demonstrate that 2BP suppresses

palmitoylation of NHE1 at low concentrations, which are not toxic to the cells, and this suppression occurs in a time dependent manner consistent with the enzymatic nature of palmitoylation. To determine if increased concentrations of 2BP would further reduce NHE1 palmitoylation without reducing total NHE1 expression or affecting cell viability we treated cells with a range of 2BP concentrations for 18 h (6B). We found 2BP equally decreased NHE1 palmitoylation at 1 µM, 5 µM, 10 µM and 15 µM without reduction of total NHE1 levels (Fig. 6B). This established effective concentrations and incubation times in which NHE1 palmitoylation is reduced by 2BP without the reduction of total NHE1 protein or cell toxicity, allowing us to move forward confidently using 2BP within these ranges and time frames to treat PSN cells and monitor the effects of 2BP on NHE1 mediated cellular processes.

Figure 5 2BP Reduces Cell Viability Only at High Concentrations for Extended Periods of Time

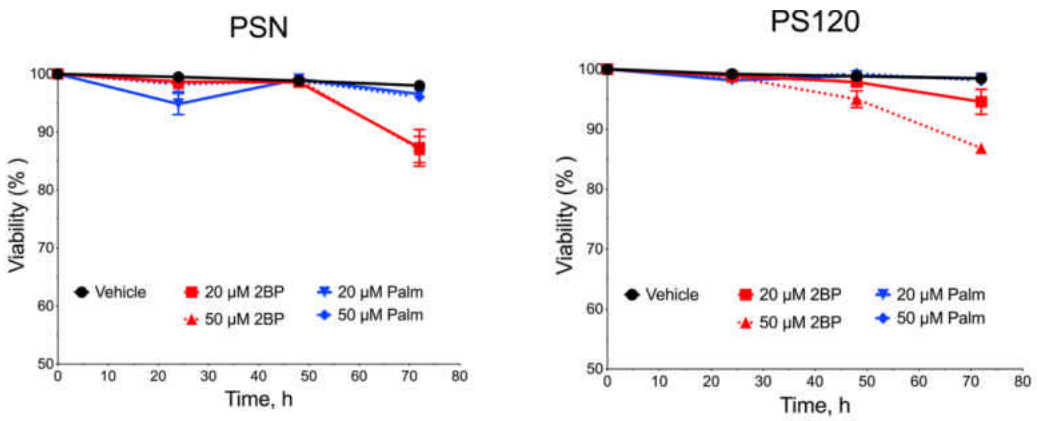


Figure 5. 2BP Reduces Cell Viability Only at High Concentrations for Extended Periods of Time. Viability of PSN and PS120 cells was assessed after incubation with 20 µM or 50 µM 2BP. A, PSN cell viability decreased only after over 70 h of 2BP while incubation with palmitate had no effect on cell viability. B, 2BP treatment of PS120 cells which do not express any isoforms of NHE only slightly decreased cell viability after 70 h, with palmitate having no effect.

Figure 6 Inhibition of NHE1 Palmitoylation

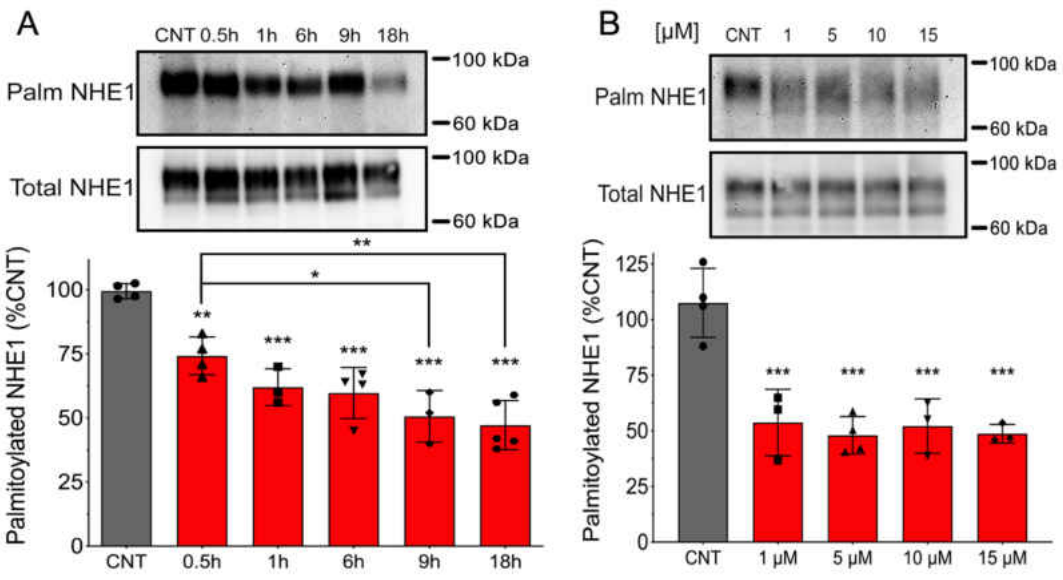


Figure 6. Inhibition of NHE1 Palmitoylation. PSN cells were treated with 1 μM 2BP for the indicated time (A) and indicated concentration for 18 h (B) to show inhibition of NHE1 palmitoylation. The western blots representative at least three independent experiments performed to analyze acyl thioester content. The top panel of the western blot represents acyl modified NHE1, measured by ABE assay. The lower panel of the western blots shows total NHE1 in the samples after having undergone ABE which is used to normalize palmitoylated NHE1. The decrease in NHE1 palmitoylation begins within 30 min with a 25% decrease and continues in a stepwise manner to a 50% decrease at 18 h. When the concentration of 2BP is increased up to 15μM NHE1 palmitoylation remains decreased by approximately 50%. Quantification of NHE1 palmitoylation mean ± SD of at least three independent experiments performed in duplicate relative to control (CNT), * < p 0.05, ** < p 0.01 *** < p 0.001 versus NT (one-way ANOVA with Tukey's post hoc test).

NHE1 Transport Capacity is Decreased in Response to Palmitoylation Inhibition

To examine the effect of 2BP on NHE1 activity, changes in intracellular pH (pH_i) were measured using 2',7'-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) in PSN cells after treatment with increasing concentrations of 2BP for 18 h. pH_i significantly decrease by 0.13 ± 0.04 and 0.25 ± 0.04 pH units in the presence of 10 μM or 15 μM 2BP, respectively, compared to vehicle indicating acidification of the cells and decreased capacity of NHE1 in maintaining cellular pH since it is the primary proton transporter in these cells.

LPA is a well-known activator of NHE1 transport[98], as such, we examined the impact of reduced NHE1 palmitoylation in the presence of 2BP on LPA-stimulated NHE1 transport to provide initial insight on the role of lipid modifications on NHE1 regulation and function. As seen in previous work under serum starved conditions (0.5% FBS)[98,137], incubation with 10 μM LPA led to a 0.18 ± 0.03 increase in pH_i (Fig 7B). Under these same serum conditions, pretreatment of PSN cells with 5 or 15 μM 2BP for 18 h significantly muted the LPA stimulated NHE1 response (Fig 7B). While NHE1 palmitoylation is decreased with as low as 1 μM 2BP, change in pH_i occurs only in the presence of 10 μM or higher concentrations of 2BP (Fig. 7A). Taken together, Figure 7 shows 2BP treatment decreases pH_i and suppresses the ability of LPA to increase pH_i through NHE1 activity.

Inhibiting Palmitoylation Decreases the Formation of Actin Stress Fibers

The association of stress fibers with the plasma membrane provides critical structural support for this membrane and the cell as a whole[138]. Stress fibers are made up of actin filaments which bind ERM complex proteins (Ezrin, Radixin, Moesin) in order to interact

with the plasma membrane[139,140]. NHE1 directly binds Ezrin which also binds actin making NHE1 a critical anchoring protein for the ERM complex and creating stress fibers necessary for directed cell migration[71]. PSN cells display minimal stress fibers in a quiescent state which can be increased by stimulation of NHE1; specifically LPA has been shown to increase stress fiber formation through NHE1 activation[98]. To assess the effect of palmitoylation on stress fiber formation, cells were treated with vehicle or 15 µM 2BP for 18 h then stimulated with LPA after which the number of stress fibers present were counted. As shown in Figure 8, 2BP treatment significantly decreased stress fiber formation compared to LPA stimulated cells. Surprisingly, 2BP treatment alone significantly reduced the number of stress fibers compared to control cells. Additionally, when cells are treated with 2BP prior to stimulation with LPA, stress fiber formation remained at control levels again demonstrating the ability of 2BP to block LPA stimulation of NHE1 (Fig. 8) consistent with its ability to inhibit LPA stimulation of NHE1 activity.

Figure 7 2BP Treatment Decreases Intracellular pH

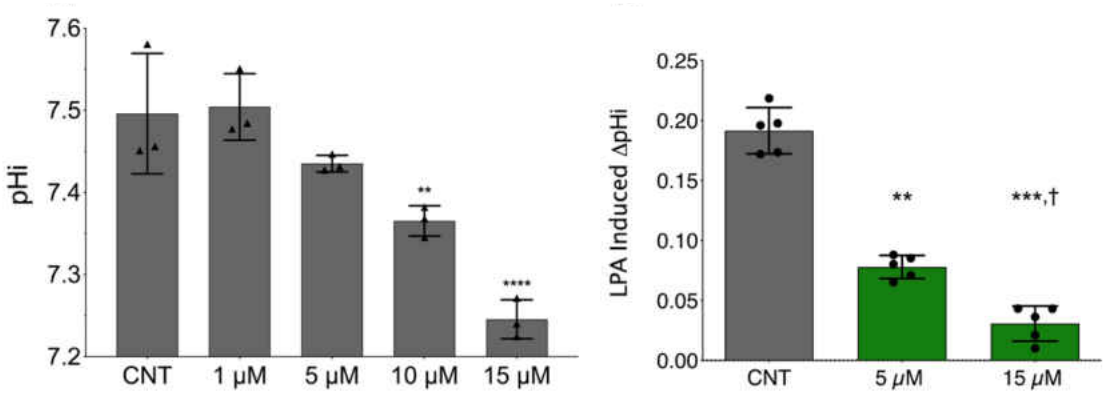


Figure 7. 2BP Treatment Decreases Intracellular pH. A, pH_i after 2BP showing 10 µM and 15 µM 2BP significantly decreased pH_i by 0.13 ± 0.04 and 0.25 ± 0.04 pH units respectively compared to CNT. Quantification performed on at least three independent experiments performed in duplicate relative to control (CNT) * < p 0.05, ** < p 0.01 **** < p 0.0001 versus CNT (one-way ANOVA Tukey's post hoc test, mean ± SD). B, quantification of the change in pH_i after LPA treatment (CNT) shows 2BP significantly abrogates the ability of LPA to activate NHE1. At least three independent experiments performed in duplicate relative to CNT ** < p 0.01 *** < p 0.001 versus CNT † < p 0.05 versus 5 µM (one-way ANOVA Tukey's post hoc test mean ± SD).

Figure 8 2BP Attenuates LPA Stimulated Stress Fiber Formation in PSN Cells

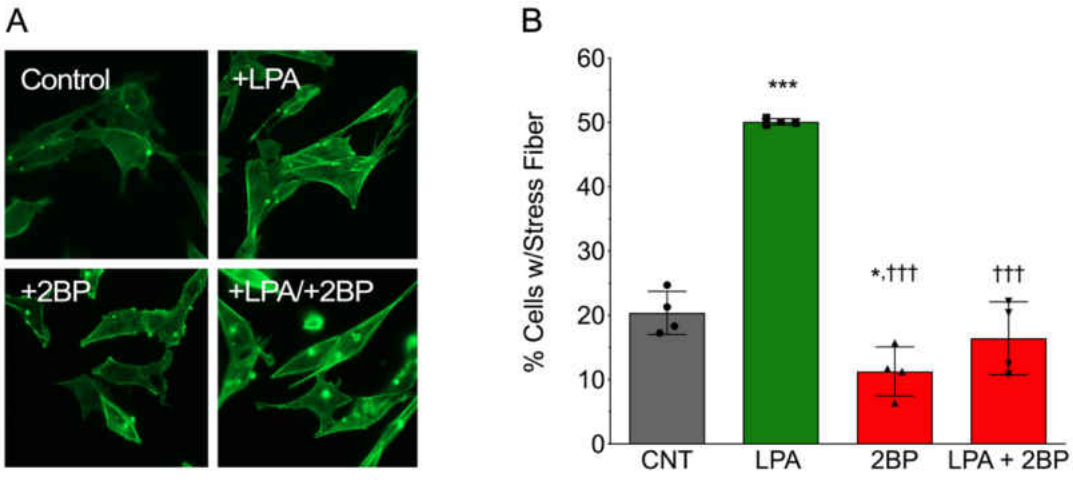


Figure 8. 2BP Attenuates LPA Stimulated Stress Fiber Formation in PSN Cells. A, PSN cells with fluorescently labeled actin stress fibers showing and increase number of stress fiber containing cells after LPA treatment (29.7 ± 2.7%) and a decreased number of cells containing stress fibers after 2BP treatment (9.1 ± 2.7%). Cells pretreated with 2BP then stimulated with LPA had significantly less cells containing stress fibers compared to LPA alone (33.6 ± 2.7%). B, quantification of the number of cells containing stress fibers as mean ± SD of four independent experiments performed in duplicate relative to control (CNT), * < p 0.05, *** < p 0.001 and ††† < p 0.001 versus LPA (one-way ANOVA with Tukey's post hoc test, Mean ± SD).

Inhibition of Palmitoylation Reduces Cell Migration and Proliferation

The role of ion translocation through NHE1 in cell migration is well documented[107,141–143]. NHE1 localizes to the leading edge of the cell resulting in the formation of a pH gradient within the cell. The alkalization of the leading edge of the cell is important for the formation of stress fibers which associate directly with NHE1 through ERM binding and are vital for directed cell migration[106,123]. Thus both proton transport and stress fiber formation mediated through NHE1 are critical for directed cell motility in healthy cells[144–146]. As such determining the effect of 2BP treatment on PSN cell migration provides valuable insight on potential mechanisms of NHE1 regulation by palmitoylation.

As shown in Figure 9, PSN cells were treated with increasing concentrations of 2BP for 18 h then wound healing assays were performed to measure cell migration and calculate cell velocity. In reference to the wound healing assay, we found a dose-response effect to the palmitoylation inhibitor where increasing concentrations of 2BP further reduced the migration of cells into a vacated wound. After 24 h, cells nearly filled the entirety of the wound with control cells and cells treated with lower concentrations of 2BP (1 μM and 5 μM). Whereas cells treated with higher concentrations of 2BP (10 μM and 15 μM) did not display closed wounds even after 28 h. The velocity (μm/min) of cell migration was examined while PSN cells were in the mid-stage of movement (between 6 h and 12 h, see Methods for calculation). Even the lowest concentration of 2BP (1 μM) inhibited the cellular rate of travel from 0.64 μm/min (control) to 0.48 μm/min (2BP). We continued to see decreased cell motility in a concentration-dependent manner with 2BP treatments as high at 15 μM. Electric cell impedance system (ECIS) is a biophysical

approach used to dynamically measure cell migration in real time. Using this method, PSN cells were seeded on the chamber and grown to confluence followed by treatment with 15 µM 2BP. After 18 h, an electric current was applied electroporating the cells resulting in death of cells only on the electrode thereby creating a defined wound. Cell impedance was then measured continuously for 7 h as cells migrated toward the electrode. Figure 9D shows that cell impedance over time is reduced when cells are treated with 2BP compared to control cells. Furthermore, we found 2BP significantly reduced PSN cell adhesion on collagen, poly L-lysine, or Matrigel™ treated surfaces which was not observed with PS120 (NHE null) cells (data not shown). This indicates that global inhibition of palmitoylation by 2BP led to decreased cell attachment, most likely due to loss of NHE1 palmitoylation and associated cellular functions. This could in part, explain the impact of 2BP treatment on cell migration and velocity. Figure 9E shows treatment with 15 µM 2BP for 18 h significantly decreased PSN cell proliferation stimulated in the presence of lysophosphatidic acid (LPA). LPA acts as a mitogen to activate NHE1[98] which increases cell proliferation, as shown in Figure 9E, while 2BP pretreatment resulted in ablation of LPA stimulated cell proliferation compared to no pretreatment control demonstrating 2BP blocks the effect of LPA on cell proliferation. Taken together, this data shows inhibiting palmitoylation, using 2BP, in PSN cells significantly reduces stress fiber formation leading to reduced cell migration and proliferation.

Figure 9 PSN Cell Migration and Proliferation are Hindered by 2BP

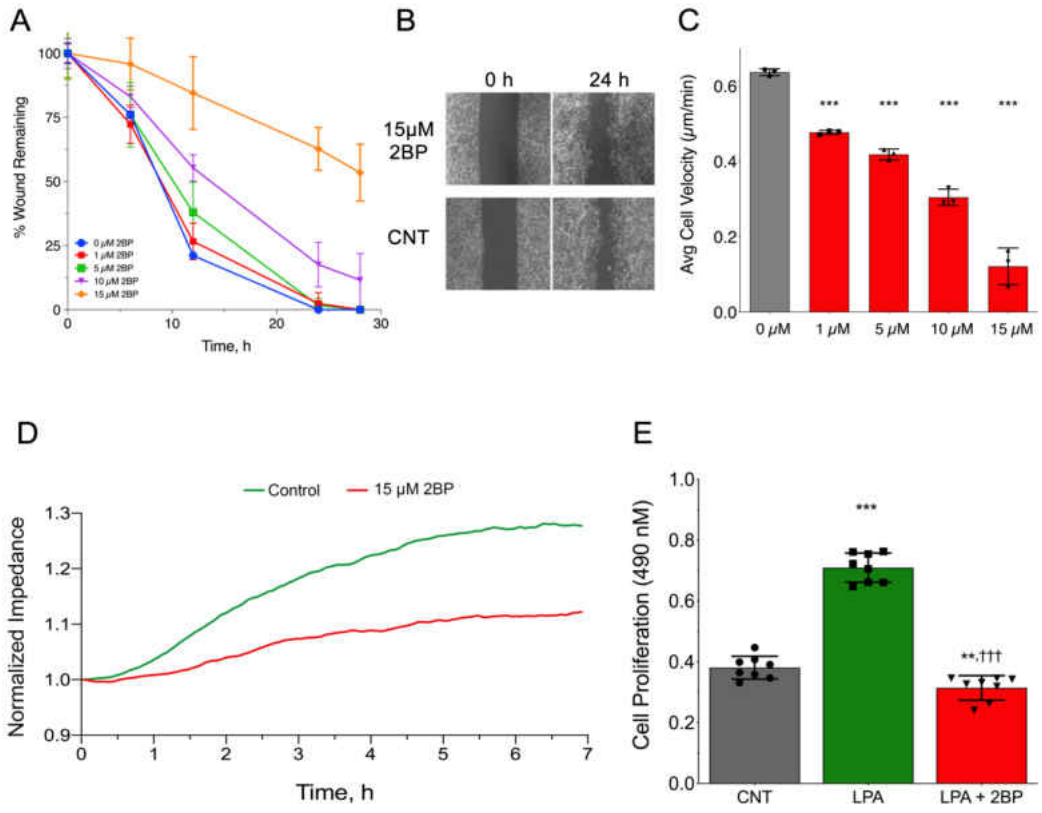


Figure 9. PSN Cell Migration and Proliferation are Hindered by 2BP. Cell migration was evaluated by measuring ability of cells to close the wound over 28 h. A, quantification of wound closure after increasing concentrations of 2BP. B, representative image of the initial wound at 0 h with 2BP treatment resulting in reduced would closure after 24h. C, quantification of average cell velocity using calculated using wound healing data. D, representative trace of cell migration measured by electric cell impedance sensing showing 15 µM 2BP treatment decreases the ability of cells to migrate over 7 h compared to control cells. E, Treatment with 15 µM 2BP for 18 hours results in significantly decreased PSN cell proliferation after 15 min LPA stimulation (10 µM) compared to LPA alone treated and control cells. Quantifications done as mean ± SD of at least three independent experiments relative to control (CNT), * < p 0.05, ** < p 0.01, *** < p 0.001 and ††† < p 0.001 versus LPA (one-way ANOVA with Tukey's post hoc test, Mean ± S.D).

Discussion

In this study, we show for the first time that NHE1, a SLC9 transporter, is acylated in both cells and rat tissues providing a novel mechanism of NHE1 regulation, which is critical in multiple cellular properties including cellular pH, stress fiber formation, migration, and proliferation. Using the PAT inhibitor, 2BP, we showed NHE1 acylation decreases in a time-dependent manner consistent with the irreversible nature of the inhibitor (Fig. 6A), and using metabolic labeling we identified the incorporation of [^{3}H]palmitate into NHE1 confirming palmitoylation of NHE1 also demonstrated using the ABE method (Fig. 4). NHE1 was fairly sensitive to 2BP treatments with decreases in NHE1 palmitoylation seen within 30 min of exposure to 1 μM 2BP which continued through 18 h in a stepwise manner (Fig. 6A). Loss of NHE1 palmitoylation plateaued after 18h of treatment with 1 μM 2BP as increasing the concentration did not result in increased loss of NHE1 palmitoylation Fig. 6B).

Prior to assessing the effect of 2BP and loss of palmitoylation on NHE1 function we established that 2BP had no effect on the viability NHE1 expressing PSN cells or the non-NHE1 expressing parent line (PS120) in the 18h time frame and 2BP concentrations used in our experiments (Fig. 5). This assured that any NHE1 functional effects seen in the presence of 2BP would not be attributable to cell viability but to the loss of palmitoylation. Since loss of palmitoylation can affect protein turnover[147] we utilized 2BP concentrations (1-15 μM) and an 18 h incubation period where no loss of total cellular NHE1 was detected (see Fig. 6) assuring that changes NHE1 functional effects would not be attributable to loss of NHE1 itself.

With this in mind, we found that 2BP treatments led to decreased NHE1 palmitoylation and altered cellular properties associated with NHE1 including cellular pH, stress fiber formation, migration, and proliferation. We found a concentration dependent decrease in intracellular pH in response to inhibition of palmitoylation by 2BP. However, there was not a close correlation between loss of NHE1 palmitoylation and decreased pH_i where 1-15 µM 2BP treatments led to equivalent loss of NHE1 palmitoylation while 1 µM 2BP had no effect on pH_i and 10-15 µM 2BP significantly decreased pH_i (Fig. 7A). This result suggests that palmitoylation of NHE1 may not directly influence NHE1 transport capacity, however, this remains to be explored because NHE1 transport is dependent on cell surface levels of the exchanger in addition to intrinsic exchanger kinetics. The cell surface transport capacity of NHE1 may be sufficient to maintain cellular pH in the presence of lower concentrations of 2BP but not with higher concentrations suggesting that binding partner interactions and their palmitoylation status may be relevant in maintaining cellular pH via NHE1 activity.

To begin assessing the effect of NHE1 palmitoylation on NHE1-associated cellular functions, we measured the effect of 2BP on pH_i after stimulation with LPA. LPA is a well-known agonist of NHE1 increasing activity and thus increasing pH_i[98]. When cells are first treated with 2BP then challenged with LPA we found a reduced ability of LPA to stimulate NHE1 activity (Fig. 7B). This indicates LPA plays a role in regulation of NHE1 activity in a palmitoylation driven manner that is 2BP concentration dependent. This effect is consistent with the 2BP-dependent loss of LPA-stimulated cell proliferation another well-

known NHE1-dependent function that is absent in the presence of 2BP suggesting NHE1 palmitoylation is necessary for LPA stimulation cell proliferation and increased pH_i.

NHE1 also facilitates protein-protein interactions which regulate stress fiber formation through interactions with the cytoskeleton, specifically the ERM complex[70,148]. PSN cells that that express human NHE1 show reduced stress fiber formation when treated with 2BP (Fig. 8). In addition, LPA stimulates stress fiber formation and incubation of PSN cells with 2BP abolished LPA-induced stress fiber formation consistent with 2BP effects seen with cell proliferation and pH_i. The formation of stress fibers in combination with increased cellular pH, regulated through NHE1 activity and localization, is critical for directed cell migration[143,149]. We show in Figure 9 that 2BP treatment also decreases PSN cell migration demonstrated by reduced wound healing even at the lowest concentration of 2BP (1 μM) and further thwarts wound closure as 2BP concentration increases. Additionally, cell velocity is hindered by 2BP in a manner similar to migration with velocity decreasing in a stepwise manner relative to 2BP concentration. Subsequently, this provides preliminary evidence that palmitoylation regulates cell migration and stress fiber formation through a NHE1 mediated mechanism. This suggests that palmitoylation plays a large role in regulation of the NHE1 and of the multiple vital cellular processes associated with the exchanger.

The nature of palmitoylation is similar to that of phosphorylation as both modifications regulate proteins in a rapid and dynamic manner and are themselves regulated by enzymes which are influenced various external and internal factors. Multiple studies have shown palmitoylation and phosphorylation occurring on the same proteins

and working in a reciprocal manner to regulate various protein functions[125,150]. Phosphorylation regulates multiple NHE1 functions including activity, stress fiber formation, cell migration, and cell proliferation all of which are also regulated by palmitoylation. This suggests NHE1 regulation by palmitoylation may work in concert with phosphorylation to regulate these critical cellular functions. The initial characterization of the effects of 2BP on NHE1 mediated cellular processes provided in this study establishes an important role for palmitoylation in regulating NHE1 and NHE1 mediated cellular processes. Multiple studies including the identification of NHE1 palmitoylation sites and the PATs that catalyze the modification of these sites are necessary to fully understand the effect of NHE1 palmitoylation on cellular processes and diseases related to NHE1.

Chapter III

PHOSPHORYLATION AND PALMITOYLATION OF THE SODIUM HYDROGEN EXCHANGER ISOFORM 1 (NHE1)

Abstract

The sodium hydrogen exchanger isoform 1 (NHE1) is a ubiquitously expressed transmembrane protein that regulates pH and plays a vital role in multiple cellular processes. Additionally, NHE1 is a scaffolding protein that organizes protein complexes to regulate various signaling pathways within the cell. Therefore, it is important to understand the factors contributing to NHE1 regulation as maintenance of these critical cellular functions is vital to ensure a healthy cell. Phosphorylation and palmitoylation are both dynamic reversible posttranslational modifications (PTMs) that regulate proteins to influence critical cellular processes. Previous work in our lab has shown NHE1 is regulated by palmitoylation and that inhibition of palmitoylation in cells expressing NHE1 decreases NHE1 activity, as well as NHE1 associated cellular functions such as stress fiber formation and cell migration. NHE1 is also regulated by phosphorylation of multiple sites through various kinase pathways. To better understand the relationship between palmitoylation and phosphorylation on NHE1 we used various stimuli known to regulate multiple kinase pathways that control NHE1 phosphorylation and measured the impact on NHE1 palmitoylation. Using serum, lysophosphatidic acid (LPA) and phorbol 12-myristate (PMA) to stimulate NHE1 phosphorylation led to increased palmitoylation of NHE1, while activation of the NHE1 phosphorylation with insulin led to decreased NHE1

palmitoylation. Additionally, we found that inhibition of PI3K, AKT, Rsk, and ROCK kinases all increased NHE1 palmitoylation, while inhibition of ERK1/2 decreased NHE1 palmitoylation. Using this information, we have begun to understand how the presence or absence of palmitoylation and phosphorylation in a coordinated manner contributes to a barcode that dictates a regulatory outcome and cellular response involving the sodium hydrogen exchanger isoform 1 (NHE1).

Introduction

The sodium hydrogen exchanger isoform 1 (NHE1) is a ubiquitously expressed transmembrane protein that regulates pH by exchanging an intracellular proton for an extracellular sodium ion[75]. NHE1 also plays a vital role in multiple cellular processes in addition to regulating pH such as cellular migration and proliferation, control of cell volume, and stress fiber formation[121,122]. Additionally, NHE1 is a scaffolding protein that organizes protein complexes to regulate various signaling pathways within the cell[123,124]. Therefore, it is important to understand the factors contributing to NHE1 regulation and its role in maintaining critical cellular functions vital to cell health. Regulation of NHE1 occurs on the large intracellular C-terminus where binding partners and posttranslational modifications (PTMs) influence the exchanger in both short- and long-term manners. Major binding partners include PIP_2[100] and ERM[71] which are important for association of the tail to the inner membrane leaflet and the formation of stress fibers, respectively[123]. Ca^{2+}/calmodulin binding sites also on the C-terminal tail have been shown to play an important role in proton affinity and thus regulate NHE1 activity[107]. Additionally, multiple phosphorylation sites are also present on the C-terminus which have varying effects on

NHE1 activity and the multiple cellular processes in which NHE1 plays a role. Together these many regulatory factors control NHE1 activity, localization, and expression, all of which are important for regulation of critical cellular processes.

Phosphorylation and palmitoylation are both dynamic reversible posttranslational modifications (PTMs) that regulate proteins through which many critical cellular processes are influenced. Multiple studies have shown phosphorylation and palmitoylation can work together in a barcode to regulate proteins and alter cellular functions[125,150–153]. Phosphorylation is the reversible addition of a phosphoryl group from adenosine triphosphate (ATP) to primarily serine, threonine, or tyrosine residue and is broadly considered the most common posttranslational modification[2,8]. Phosphorylation introduces a charged phosphate group which induces an altered conformational change that can affect catalytic activity of proteins, the tendency of proteins to aggregate, and the ability to recruit binding partners, all of which result in altered protein function and cell signaling[2,5]. S-Palmitoylation is the addition of a 16-carbon fatty acid to a cysteine residue via a thioester bond. Palmitoylation is a unique lipid modification as it is reversible, with palmitoyl acyltransferases (PATs) catalyzing the addition of palmitate while acyl protein thioesterases (APTs) and palmitoyl-protein thioesterases (PPTs) catalyzing the removal of palmitate via a thioester bond at intracellularly available cysteine residues. The most well studied function of palmitoylation is trafficking of proteins between the organelles and increasing membrane association of cytosolic proteins. Recent studies have demonstrated that palmitoylation has multiple other

effects on proteins, particularly transmembrane proteins where effects include regulation of protein activity, membrane microlocalization and protein-protein interactions[125,126].

Previous work in our lab has shown NHE1 is regulated by palmitoylation and that inhibition of palmitoylation in cells expressing NHE1 decreases intracellular pH, which is associated with NHE1, as well as NHE1 associated cellular functions such as stress fiber formation and cell migration (Chapter II). Many binding partners and kinases interact directly or indirectly to regulate NHE1 activity. Identifying and understanding how these complex pathways are coordinated resulting in NHE1 regulation is on-going. Various growth factors and hormones stimulate NHE1 phosphorylation including thrombin, serum, epidermal growth factor, insulin, angiotensin II, phorbol esters, and lysophosphatidic acid (LPA). Figure 10 shows diagrams a few of the pathways that lead to NHE1 phosphorylation. We hypothesize these same growth factors, that stimulate NHE1 phosphorylation, will also alter NHE1 palmitoylation. To address this hypothesis, we stimulated NHE1 using serum, insulin, LPA, and phorbol 12-myristate (PMA). Serum, LPA, and PMA all increased NHE1 phosphorylation as well as palmitoylation. Conversely, stimulation of NHE1 phosphorylation with insulin led to decreased NHE1 palmitoylation. Additionally, we inhibited several kinases that directly and indirectly lead to NHE1 phosphorylation, to aid in understanding how different phosphorylation pathways participate in regulation of NHE1 by palmitoylation. This information has allowed us to begin understanding how the presence or absence of palmitoylation and phosphorylation in a coordinated manner contributes to a barcode that dictates a regulatory outcome and cellular response involving the NHE1.

Figure 10 Pathways Involved in the Phosphorylation of NHE1

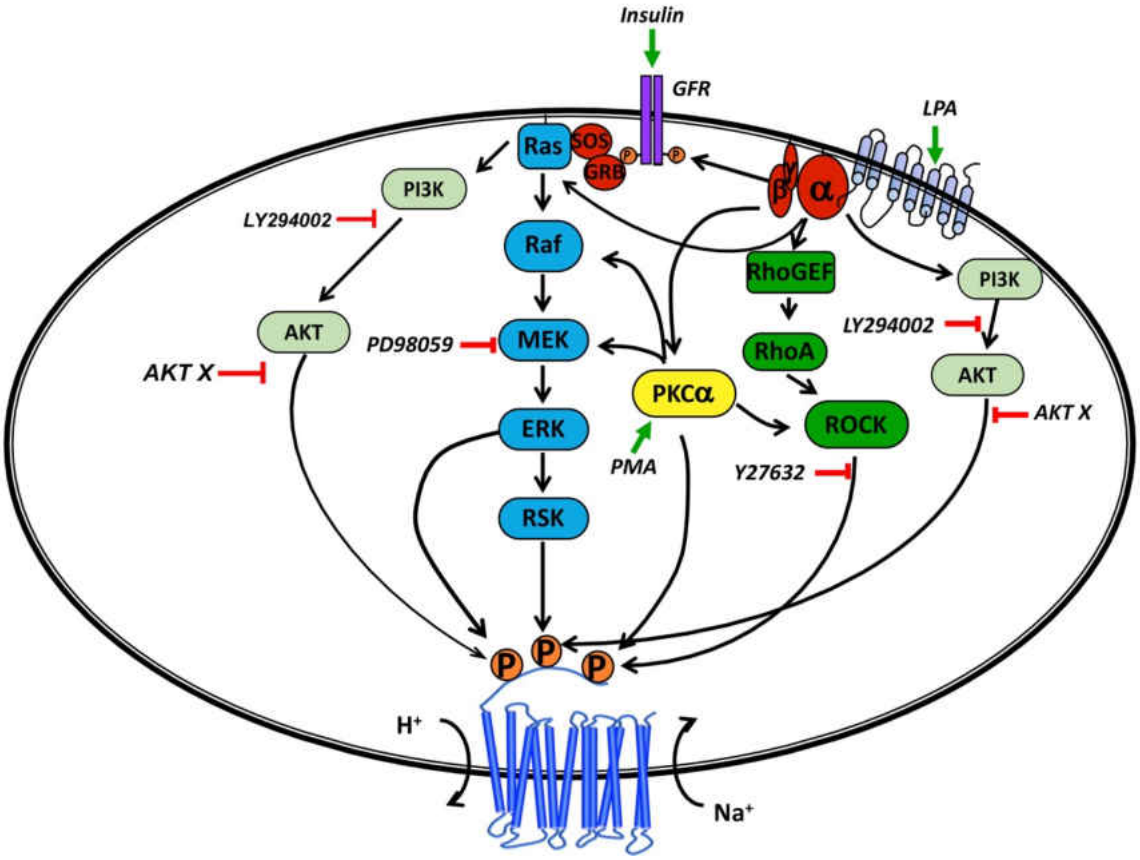


Figure 10. Pathways Involved in the Phosphorylation of NHE1. Diagram of the multiple pathways involved in NHE1 phosphorylation. Stimuli can activate one or more of these pathways initiating kinase cascades resulting in the phosphorylation of NHE1 at various residues.

Materials

Colorburst molecular mass standard, 2- bromopalmitate (2BP), and mouse anti-flag tag antibody were from Sigma Millapore and anti-NHE1$_{(54)}$ mouse monoclonal antibody (sc-136239) was from Santa Cruz Biotechnology. High capacity NeutrAvidin ®-agarose resin, and bicinchoninic acid protein (BCA) assay reagent were from Thermo Scientific. HPDP-biotin was from APExBio, BCECF-AM 2',7'-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein was from Molecular Probes and Nigericin was from Tocris Bioscience. LPA in the form of 14:0 1-myristoyl-2-hydroxy-sn-glycero-3phosphate (sodium salt) was from Avanti Polar Lipids. LY294002, PD98059, BID-1870, Y27632, AKT1X were from Adooq Biosciences. Human insulin was from Alfa Aesar and PMA was from Sigma Millapore.

Methods

Cell Culture

NHE1-null cells (PS120 cells, a gift from D. Barber, University of California, San Francisco) derived from Chinese hamster lung fibroblasts CCL339 were used to generate a cell line stably expressing human NHE1 with an HA DDK epitope tag on the C terminus (PSN cells). These cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 20% fetal bovine serum, 2 mM L-glutamine, and 200 µg/ml G418 at 37^{o}C in a humidified 5% CO_2 incubator after clonal isolation [98].

Cell Treatments and Membrane Preparation for Palmitoylation Detection

PSN cells were subcultured, seeded on 150 mm culture dishes, and grown to confluency followed by treatment with experimental conditions as described. After

treatments, cells were washed twice with 3 mL of ice-cold buffer B (0.25 M sucrose, 10 mM triethanolamine, 10 mM acetic acid, pH 7.8) and then scraped to one side of the plate using 0.5 mL of buffer B and transferred to a 2 mL microcentrifuge tube on ice. Cells were then pelleted by centrifugation at 3,000 x g for 5 min at 4°C. The supernatant fraction was removed and 1 mL of ice-cold buffer C (0.25 M sucrose, 10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, pH 7.8) containing protease inhibitors was added to break the pellet loose. The solution containing the loose pellet was then homogenized in a Dounce homogenizer with 30 up and down strokes on ice. The resulting homogenate was centrifuged at 800 x g for 10 min at 4°C to pellet and remove nuclei and cell debris. The supernatant fraction was transferred to a new tube which was centrifuged at 16,000 x g for 12 min at 4°C to precipitate cell membranes. The supernatant fraction was removed and the pellet containing cell membranes was resuspended in 1 mL sucrose-phosphate (SP) buffer (10 mM sodium phosphate, 0.32M sucrose, pH 7.4) and assayed for protein content.

Palmitoylation Detection using Acyl-Biotinyl Exchange (ABE)

Adapted from Wan et al[134] palmitoylated proteins are detected using three steps. (i) Free cysteine thiols are blocked; membranes isolated from PSN cells were solubilized in lysis buffer (50 mM HEPES pH 7.0, 2% SDS (w/v), 1 mM EDTA) containing protease inhibitors and 25 mM N-ethylmaleimide (NEM) and incubated for 20 minutes at 37°C, followed by acetone precipitation. Precipitated proteins were again solubilized in lysis buffer containing protease inhibitors and NEM and incubated at room temperature with end over end mixing overnight. NEM was removed by three consecutive acetone

precipitations in which the protein pellet was resuspended in 4SB buffer (4% SDS, 50 mM Tris, 5 mM EDTA, pH 7.4) followed by acetone precipitation. (ii) Thioester linked palmitoyl groups are specifically removed using hydroxylamine (HAM); The resuspended protein sample is split equally into negative control (50 mM Tris-treated) and HAM-treated (0.7 mM) halves and incubated at room temperature for 15 min with end-over-end mixing. (iii) The previously palmitoylated and now free sulfhydryl groups are then biotinylated by the addition of sulfhydryl specific HPDP-biotin (0.4 mM, 50 mM Tris-HCl, pH 7.4) for 1 h at room temperature with end over end mixing, HAM and unbound biotin was removed with three sequential acetone precipitations; the biotinylated proteins were then affinity purified using NeutrAvidin® resin. Bound proteins were eluted, subjected to SDS-PAGE, and immunoblotted with mouse anti-Flag M2 primary antibody to detect tagged NHE1.

Results

Serum Stimulation Increases NHE1 Palmitoylation

To study the influence of growth factors on NHE1 palmitoylation we began by looking at low serum (0.5%) and high serum (20%) conditions. Previous studies have shown that serum stimulates NHE1 phosphorylation through ERK signaling with Rsk kinase directly phosphorylating NHE1 at S703 in fibroblast cells[111,154]. PSN cells were plated, allowed to attach and after 18 h the media was replaced with media containing either 20% or 0.5% serum. After an additional 24 h incubation, membranes we prepared and NHE1 palmitoylation was analyzed using the ABE method. Figure 11 shows serum stimulated NHE1 is 25 ± 8.8 % more palmitoylated than when serum starved.

Lysophosphatidic Acid (LPA) Increases NHE1 Palmitoylation

LPA, a component found in serum, acts as a mitogen signaling through G-protein coupled receptors activating growth factor pathways via Ras-ERK. Previous studies have shown LPA stimulates NHE1 phosphorylation by RhoA/ROCK and ERK/Rsk pathways[98]. In Figure 12 we show LPA stimulation increase NHE1 palmitoylation after 10 min by 79.7 ± 16.5 % compared to no treatment. This continues in a time dependent manner with LPA stimulation increasing NHE1 palmitoylation 133.1 ± 18.8 % after 15 min. Moreover, when cells are treated with 2BP, an inhibitor of palmitoylation, for 30 min prior to LPA stimulation the ability of LPA to increase NHE1 palmitoylation is diminished. As both NHE1 phosphorylation and palmitoylation are increased by LPA they may work in a cooperative manner to integrate the LPA stimulated pathway regulating NHE1 activity and function. Figure 13 shows both 5 and 15 μM 2BP plus LPA treatment decreased NHE1 palmitoylation by 62.0 ± 9.5% and 50.6 ± 8.3 % versus LPA alone respectively. Showing 2BP can abrogate the effect of LPA on NHE1 is consistent with previous data (Chapter II - similar effects as those shown in Figure 8 with respect to pH_i).

Insulin Decreases NHE1 Palmitoylation

Insulin, another growth factor, signals through the insulin receptor to activate the PI3K/AKT phosphorylation pathway. Barber et al 2009 showed AKT phosphorylates NHE1 at residue S648 to modulate stress fiber formation through NHE1 phosphorylation[70]. Here we show stimulation of cells for 10 min with 100 nM insulin decreased NHE1 palmitoylation by 38.0 ± 5.7 % (Figure 14). Insulin treatment is known to increase NHE1 phosphorylation and here we show the same stimulation decreases NHE1 palmitoylation.

This indicates that palmitoylation and phosphorylation may work in a reciprocal manner to regulate NHE1.

PMA Increases NHE1 Palmitoylation

PMA (Phorbol 12-myristate 13-acetate) is a phorbol ester (PE) that binds and activates PKC. Multiple studies about the effects of PEs, including PMA, have shown PEs are critical for regulating the lipid-interacting domain (LID) of NHE1[118,155]. Some studies have also shown treatment with PEs increases NHE1 phosphorylation however, no direct PKC phosphorylation site has been found on NHE1[69]. In Figure 15 we show treatment of cells with 10 µM PMA increases NHE1 palmitoylation by 23.5 ± 5.0 % after 20 min of exposure. PMA treatment promotes the interaction of the LID region of NHE1 with the membrane[118]. Here we show PMA stimulation also increases NHE1 palmitoylation indicating palmitoylation could play a critical role in the association of NHE1 with the membrane, specifically in the LID region which contains cysteine residue 561 which could be a potential palmitoylation site.

Figure 11 NHE1 is More Palmitoylated in High Serum Conditions

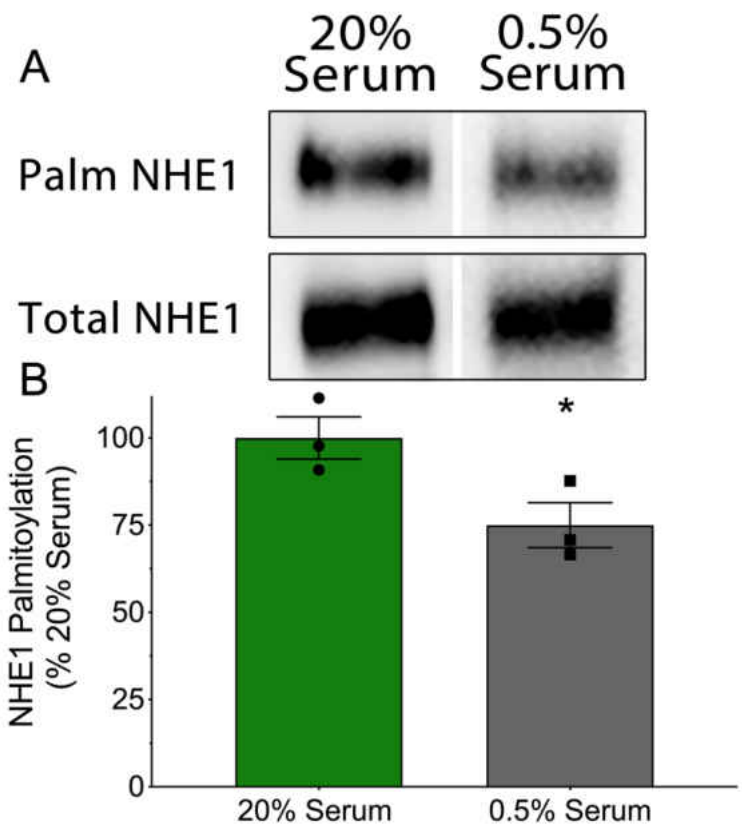


Figure 11. NHE1 is More Palmitoylated in High Serum Conditions. **A.** Western blot representative of at least three independent experiments showing increased palmitoylation after 24 h serum stimulation as total NHE1 levels remain the same. **B.** Quantification of NHE1 palmitoylation (mean ± S.E. of at least three independent experiments relative to 0.5% Serum) * $p < 0.05$ versus 0.5% Serum (Student's t-test).

Figure 12 LPA Increases NHE1 Palmitoylation

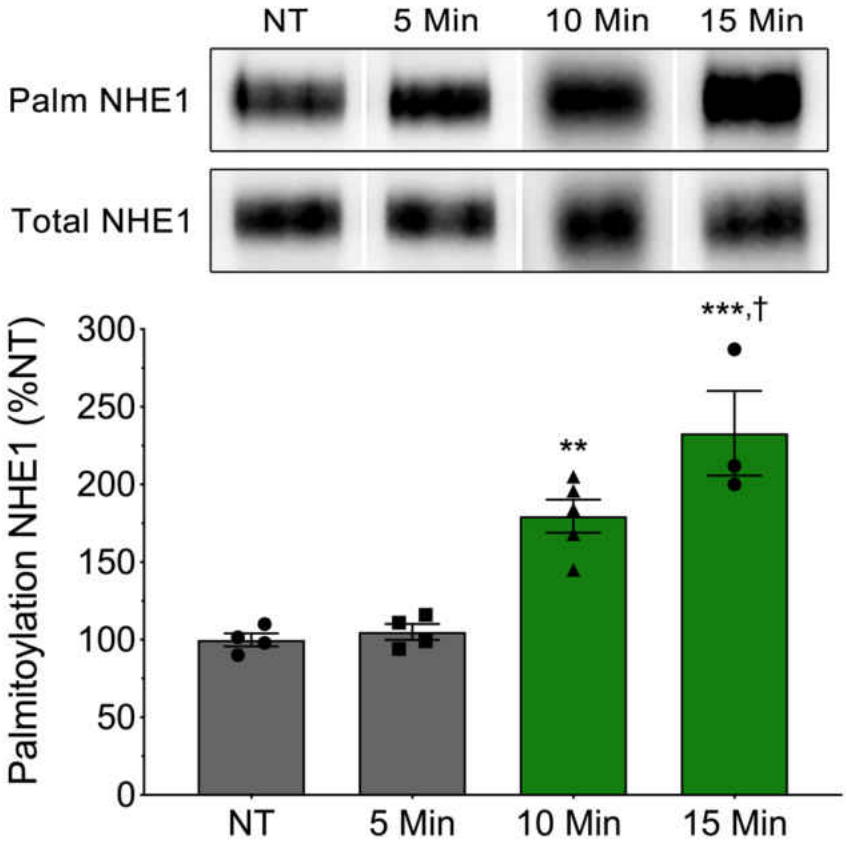


Figure 12. LPA Increases NHE1 Palmitoylation A. Western blot representative of at least three independent experiments for each treatment showing palmitoylated NHE1 increasing in a time dependent manner following LPA treatment as total NHE1 levels remain the same **B.** Quantification of NHE1 palmitoylation normalized to total NHE1 present (mean ± S.E. of at least three independent experiments relative to control (NT) normalized to 100%.) ** $p < 0.01$ and **** $p < 0.0001$ versus NT and † $p < 0.05$ versus 10 min (one-way ANOVA with Tukey's post hoc test).

Figure 13 The Effect of LPA and 2BP Treatment on NHE1 Palmitoylation

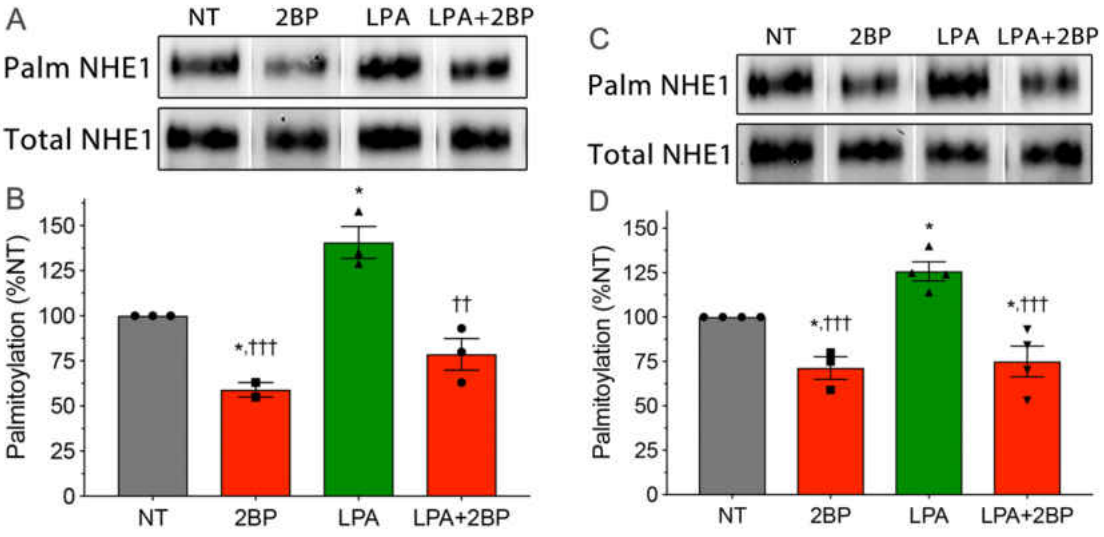


Figure 13. The Effect of LPA and 2BP Treatment on NHE1 Palmitoylation. A/C. Western blot representative of at least three independent experiments for each treatment showing 5 and 15 µM 2BP decrease NHE1 palmitoylation, LPA increase NHE1 palmitoylation and pretreatment with 2BP abrogates the ability of LPA to stimulate NHE1 palmitoylation. **B/D**. Quantification of NHE1 palmitoylation normalized to total NHE1 (mean ± S.E. of at least three independent experiments relative to vehicle (NT) normalized to 100%.) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus NT, † $p < 0.05$, ††† $p < 0.001$ versus LPA (one-way ANOVA with Tukey's post hoc test).

Figure 14 Insulin Decreases NHE1 Palmitoylation

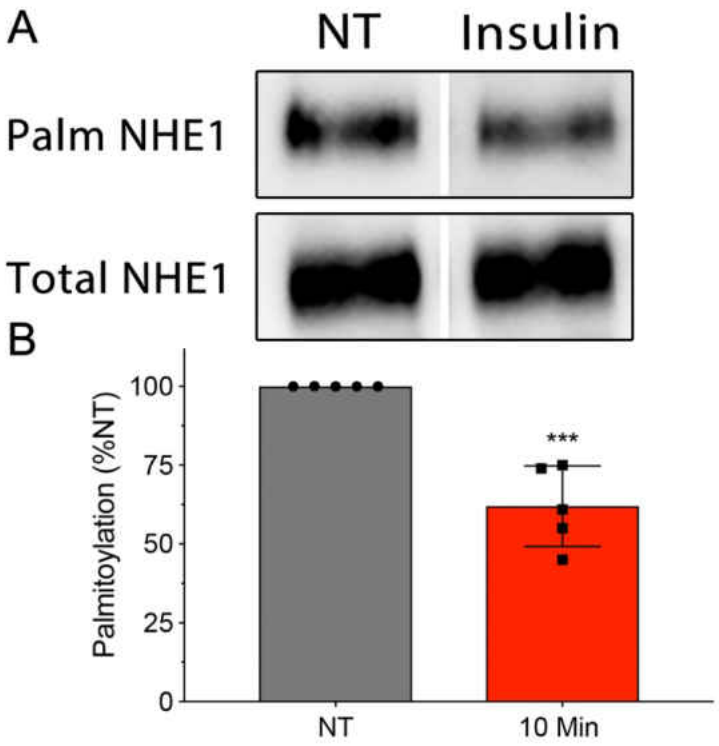


Figure 14. Insulin Decreases NHE1 Palmitoylation A. Western blot representative of at least three independent experiments showing NHE1 palmitoylation decreases after insulin treatment with total NHE1 levels remaining the same. **B.** Quantification of palmitoylated NHE1 normalized to total NHE1 (mean ± S.E. of at least three independent experiments relative to control (NT) normalized to 100%.) *** $p < 0.001$ versus NT (Student's t-test).

Figure 15 PMA Increases NHE1 Palmitoylation

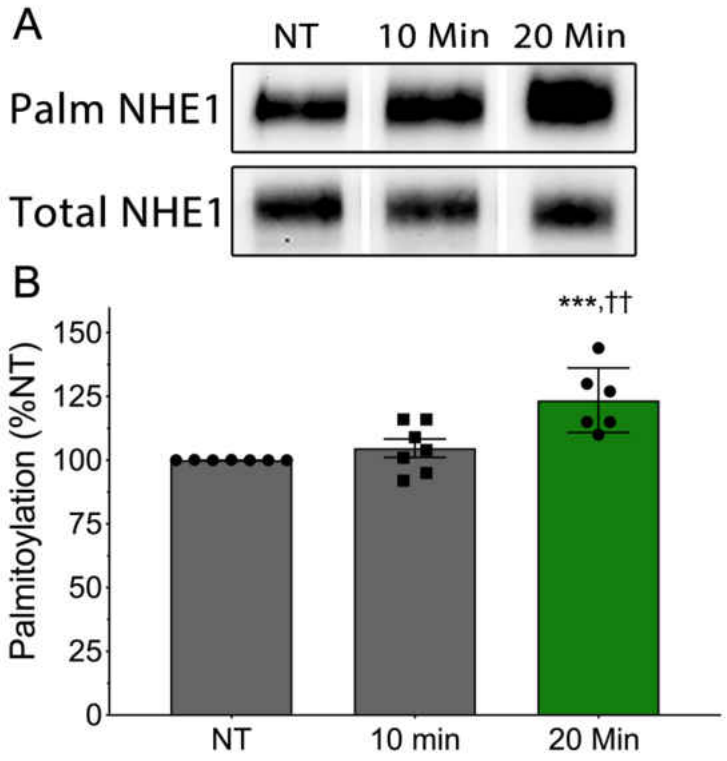


Figure 15. PMA Increases NHE1 Palmitoylation. A. Western blot representative of at least three independent experiments showing NHE1 palmitoylation decreases after PMA treatment with total NHE1 levels remaining the same. **B.** Quantification of palmitoylated NHE1 normalized to total NHE1 (mean ± S.E. of at least three independent experiments relative to control (NT) normalized to 100%.) *** $p < 0.001$ versus NT and ††† $p < 0.001$ versus 10 min (one-way ANOVA with Tukey's post hoc test).

PI3K and AKT Inhibition Increases NHE1 Palmitoylation

Insulin signals through the PI3K/AKT pathway leading to increased NHE1 phosphorylation at S648[70]. To begin assessing the involvement of kinases downstream of insulin binding to its receptor in regulating NHE1 palmitoylation we used LY294002 or AKTX1 to inhibit PI3K or AKT respectively. In Figure 16 we show NHE1 palmitoylation increases 37.0 ± 9.8% after treatment with 10 μM LY294002 for 20 min consistent with insulin pathway stimulation decreasing NHE1 palmitoylation. Additionally, treatment with 10 μM AKTX1 for 1 h increased NHE1 palmitoylation by 69.6 ± 5.9 % (Fig. 17). This aligns with the previous data showing insulin treatment decreases NHE1 palmitoylation, implicating the PI3K/AKT pathway as a major player linking NHE1 palmitoylation and phosphorylation.

MEK Inhibition Decreases NHE1 Palmitoylation

To further explore the role of phosphorylation on NHE1 palmitoylation we used PD98059 to inhibit MEK activity while cells were maintained in 20 % serum. Cells were treated with 10 μM PD98059 for the indicated time and NHE1 palmitoylation was measure using the ABE detection method. We found NHE1 palmitoylation was decreased by 46.6 ± 5.0 % compared to no treatment after 20 min of inhibition and was maintained at 30 min of treatment (Fig. 18). The MEK/ERK/Rsk kinase pathway is involved in direct phosphorylation of NHE1 at S770/771[78]. Here we show inhibition of ERK results in decreased palmitoylation which aligns with our earlier data showing the presence of serum promotes NHE1 palmitoylation. Decreased NHE1 palmitoylation after MEK inhibition is consistent with data in Figure 9 showing serum stimulates NHE1

palmitoylation linking MEK activity and NHE1 phosphorylation at S770/771 to regulation of NHE1 palmitoylation.

Rsk and ROCK Inhibition Increases NHE1 Palmitoylation

Another kinase pathway involved in the activation of NHE1 by phosphorylation is the ERK/Rsk/ROCK pathway. BI-D1870 is a selective Rsk inhibitor that has been used extensively in previous studies to decrease NHE1 phosphorylation at S703 after serum stimulation[156]. In Figure 19 we show cells treated with 10 μM BI-D1870 for 30 min have a 40.6 ± 6.4 % increase in NHE1 palmitoylation. Likewise, NHE1 is directly phosphorylated by ROCK at T653[98] and when cells are treated with 10 μM of ROCK inhibitor Y-27632 for 30 min NHE1 palmitoylation is increased by 52.3 ± 17.3 % (Fig. 20). This increase in NHE1 palmitoylation after both ROCK and Rsk inhibition suggests stimuli increasing phosphorylation at S703 and T653 have an opposite effect on NHE1 palmitoylation than stimulus that increase phosphorylation at the ERK specific site S770/771.

Figure 16 Inhibition of PI3K Increases NHE1 Palmitoylation

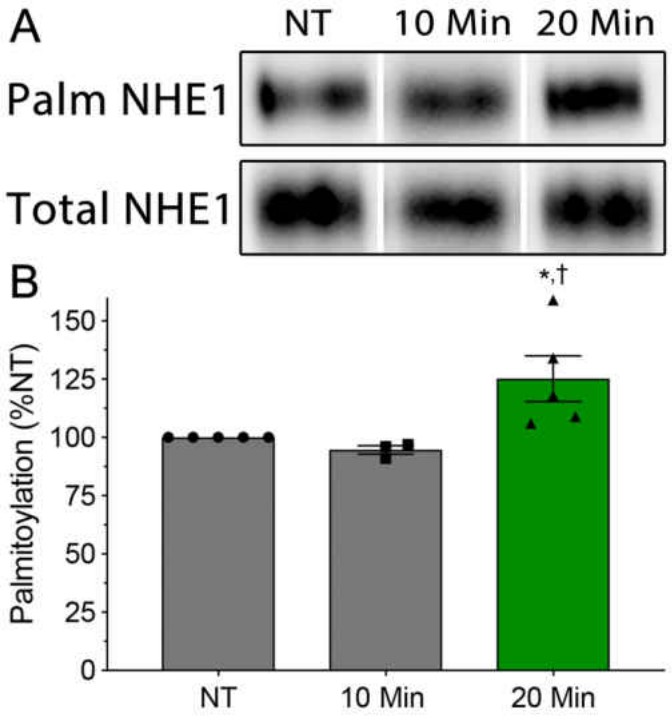


Figure 16. Inhibition of PI3K Increases NHE1 Palmitoylation. A. Western blot representative of at least three independent experiments showing NHE1 palmitoylation decreases after inhibition of PI3K with LY29400 treatment with total NHE1 levels remaining the same. **B.** Quantification of palmitoylated NHE1 normalized to total NHE1 (mean ± S.E. of at least three independent experiments relative to vehicle (NT) normalized to 100%.) * $p < 0.05$ versus NT and † $p < 0.05$ versus 10 min (one-way ANOVA with Tukey's post hoc test).

Figure 17 Inhibition of AKT Increases NHE1 Palmitoylation

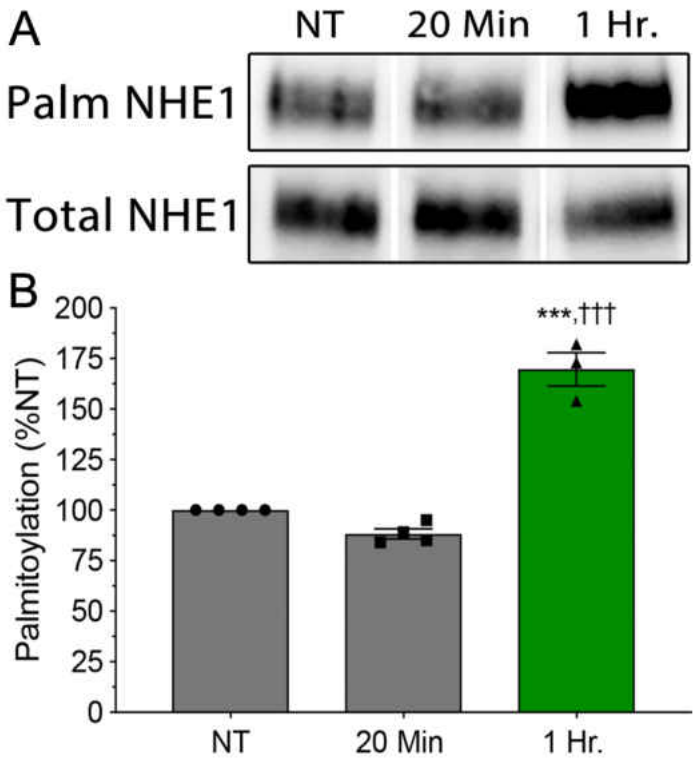


Figure 17. Inhibition of AKT Increases NHE1 Palmitoylation. A. Western blot representative of at least three independent experiments showing NHE1 palmitoylation decreases after inhibition of AKT with AKT1X treatment with total NHE1 levels remaining the same. **B.** Quantification of palmitoylated NHE1 normalized to total NHE1 (mean ± S.E. of at least three independent experiments relative to vehicle (NT) normalized to 100%.) *** $p < 0.001$ versus NT and ††† $p < 0.001$ versus 20 min (one-way ANOVA with Tukey's post hoc test).

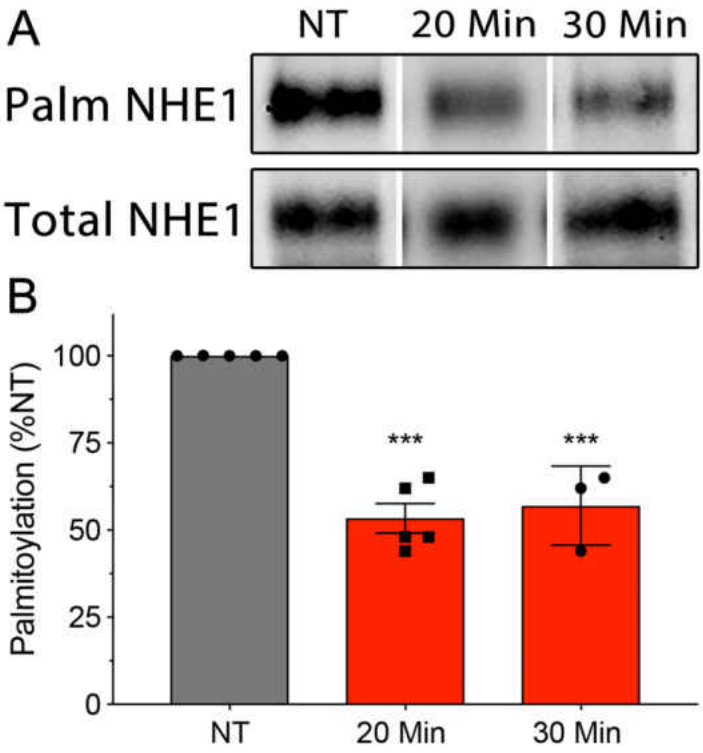


Figure 18. Inhibition of MEK Decreases NHE1 Palmitoylation. A. Western blot representative of at least three independent experiments showing NHE1 palmitoylation decreases after inhibition of MEK with PD98059 treatment with total NHE1 levels remaining the same. **B.** Quantification of palmitoylated NHE1 normalized to total NHE1 (mean ± S.E. of at least three independent experiments relative to control (NT) normalized to 100%.) *** $p < 0.001$ versus NT (one-way ANOVA with Tukey's post hoc test).

Figure 19 Inhibition of Rsk Increases NHE1 Palmitoylation

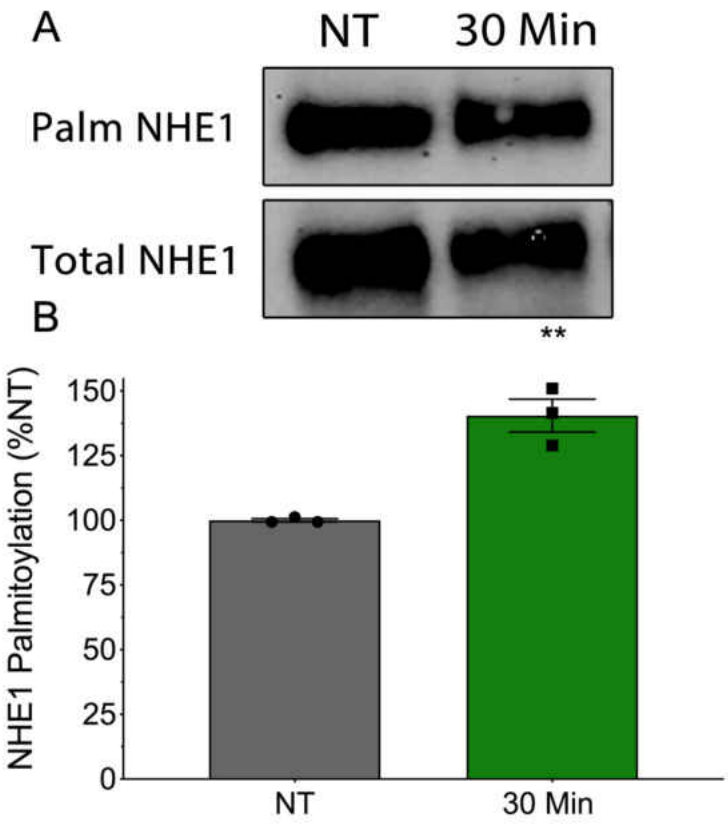


Figure 19. Inhibition of Rsk Increases NHE1 Palmitoylation. A. Western blot representative of at least three independent experiments showing NHE1 palmitoylation decreases after inhibition of Rsk with BI-D1870 treatment with total NHE1 levels remaining the same. **B.** Quantification of palmitoylated NHE1 normalized to total NHE1 (mean ± S.E. of at least three independent experiments relative to control (NT) normalized to 100%.) ** $p < 0.01$ versus NT (one-way ANOVA with Tukey's post hoc test).

Figure 20 ROCK Inhibition Increases NHE1 Palmitoylation

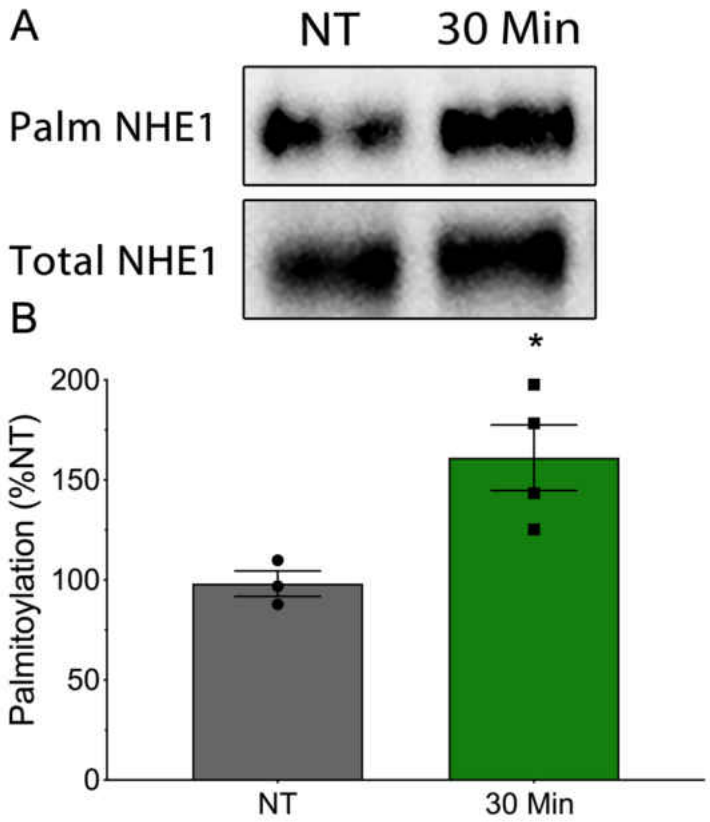


Figure 20. ROCK inhibition increases NHE1 palmitoylation. A. Western blot representative of at least three independent experiments showing NHE1 palmitoylation decreases after inhibition of ROCK with Y-27632 treatment with total NHE1 levels remaining the same. **B.** Quantification of palmitoylated NHE1 normalized to total NHE1 (mean ± S.E. of at least three independent experiments relative to control (NT) normalized to 100%.) ** $p < 0.01$ versus NT (one-way ANOVA with Tukey's post hoc test).

Discussion

NHE1 has a large number of phosphorylation sites that work together to regulate NHE1 activity, localization, and protein-protein interactions. All of these factors are important for maintaining cellular pH_i as well as keeping cells in a homeostatic state. Our previous work has shown that in addition to phosphorylation NHE1 is also palmitoylated, a reversible lipid modification, that we now have linked to regulation of multiple NHE1 associated functions (Figs. 7-9). Cell growth is regulated, in part, by pH_i and stimulated by various growth factors and hormones. Extensive research has been done looking at the role of growth factors, hormones, and cell signaling molecules on NHE1 regulation with a focus on the specific phosphorylation pathways and kinases involved in NHE1 phosphorylation[72,77,78,98,117,120,155,157]. Multiple studies have shown a link between phosphorylation and palmitoylation working to regulate the same protein in either a reciprocal or cooperative manner[125,150,158–161]. Here we show serum, LPA, and PMA, all factors that increase NHE1 phosphorylation at distinct sites, increase NHE1 palmitoylation and insulin, also known to specifically increase NHE1 phosphorylation, decreases NHE1 palmitoylation. We then inhibited several kinases directly involved in NHE1 phosphorylation and examined the effect on NHE1 palmitoylation, which is described below.

Serum contains a number of growth factor that can activate the cell and stimulate NHE1 activity through phosphorylation of S703 and enhance 14-3-3 binding to NHE1. Figure 11 shows NHE1 is more palmitoylated in the presence of high serum. Accordingly,

palmitoylation of NHE1 might contribute to NHE1 being in a more active state in response to serum.

LPA, a component of serum, acts as a mitogen to signal through GPCRs to activate MAPK cascades. LPA activation of ERK results in direct phosphorylation of NHE1 at residues S770/771. LPA treatment increases NHE1 palmitoylation within 10 min and this increase continues in a time-dependent manner (Fig. 12). While LPA signals through multiple pathways leading to the possibility that many factors may be contributing to the increase in NHE1 palmitoylation here we show that LPA stimulates NHE1 palmitoylation and phosphorylation suggesting these modifications may work in a cooperative manner to regulate NHE1. Insulin signaling occurs through the PI3K/AKT phosphorylation pathway to phosphorylate NHE1 at S648. In Figure 14 we show cells treated with 100 nM insulin have decreased NHE1 palmitoylation after 10 min. This implies the AKT phosphorylation pathway results in a reciprocal relationship between NHE1 phosphorylation and palmitoylation.

Phorbol esters such as PMA activate PKC signaling pathways. While no PKC phosphorylation site has been established on NHE, studies have found a defined role for PMA activation of NHE1. A defined binding motif for phorbol esters on NHE1 shows PMA acts in a PKC independent manner to activate NHE1 by increased association of the lipid interacting domain (LID) on NHE1 with the membrane[118]. In Figure 15 we show PMA modestly increases NHE1 palmitoylation suggesting a potential role for palmitoylation facilitating LID membrane interaction through a PKC mediated mechanism. A potential

NHE1 palmitoylation site is located within the LID area at residue C561 supporting this theory.

Taken together we show various stimuli that signal through different kinase pathways and contribute to NHE1 palmitoylation in both cooperative and reciprocal manners. To continue exploring this connection between NHE1 phosphorylation and palmitoylation we used specific kinase inhibitors to inhibit specific signaling pathways and measured the effect on NHE1 palmitoylation.

First looking at the insulin stimulated PI3K/AKT phosphorylation pathway we show inhibition of PI3K or AKT results in increased NHE1 palmitoylation (Figs. 16 and 17 respectively). This is consistent with and supportive of results in Figure 14 showing insulin treatment decreases NHE1 palmitoylation. AKT is responsible for phosphorylation of NHE1 at S648 where stimulation of this pathway using insulin increases NHE1 phosphorylation at S648[70] and decreases NHE1 palmitoylation implying palmitoylation and phosphorylation may work to regulate NHE1 in a reciprocal manner. The ERM protein complex binds NHE1 between residues 550-566 which facilitates the formation of stress fibers. Phosphorylation of S648 on NHE1 through AKT reduces the presence of actin stress fibers by encouraging depolymerization. As shown previously in Figure 8, inhibition of palmitoylation using 2BP results in decreased formation of stress fibers and here we show treatment of cells with insulin which promotes S648 phosphorylation leading to depolymerization of stress fibers, decreases NHE1 palmitoylation (Figure 14). The ERM binding domain is located between residues 550-566, a potential palmitoylation site at residue C561 could offer an explanation for this relationship. Palmitoylation of NHE1

decreases stress fiber formation which could be due to the palmitate inhibiting ERM binding either sterically or by embedding the ERM binding motif into the membrane thus preventing the formation of stress fibers.

LPA activates MEK/ERK phosphorylation pathway resulting in NHE1 phosphorylation at S770/771. Inhibition of MEK using PD98059 decreases NHE1 palmitoylation (Fig. 18) which aligns with LPA increasing NHE1 palmitoylation (Fig. 12). Phosphorylation of NHE1 S770/771 sites increases NHE1 activity in response to sustained acidosis. Figure 16 along with previous data showing inhibition of palmitoylation result in a more acidic pH_i (Fig. 7) provides preliminary evidence that palmitoylation may play a role in cooperating with phosphorylation at these specific sites to increase NHE1 activity.

Serum contains many growth factors and has been shown to activate the ERK/Rsk/ROCK pathway to phosphorylate NHE1. Rsk phosphorylates NHE1 at S703 creating a binding pocket for 14-3-3 protein binding, which when bound decreases the rate of S703 dephosphorylation. Binding of 14-3-3 to NHE1 is necessary for serum activation of NHE1. In conjunction with serum increasing NHE1 palmitoylation (Fig. 11) ERK inhibition decreases NHE1 palmitoylation (Fig. 18). Interestingly when Rsk, the next step in the ERK pathway and kinase that directly phosphorylates S703, is inhibited NHE1 palmitoylation increases (Fig. 19). Additionally, serum promotes phosphorylation of NHE1 at T653 through ROCK, which when inhibited results in increased NHE1 palmitoylation (Fig. 20). While this seems contradictory it is likely due to the complexity of growth factor pathways influencing NHE1. Overall this data provides preliminary evidence that

palmitoylation and phosphorylation work to create specific barcoding patterns that dictate a regulatory outcome and cellular response involving NHE1.

CHAPTER IV

DISCUSSION

Here we show for the first time NHE1, a SLC6A transporter, is acylated in both cells and rat tissue providing a novel mechanism of NHE1 regulation. Using the PAT inhibitor, 2BP, we showed NHE1 acylation decreases in a time dependent manner. While 2BP globally inhibits palmitoylating enzymes (PATs) we show treatment of PSN cells with 2BP in the time and concentration range which decreases NHE1 palmitoylation also alters multiple cellular properties associated with NHE1 including cellular pH, stress fiber formation, migration, and proliferation. We determined the concentration range in which 2BP treatment decreases NHE1 palmitoylation without causing a loss in total NHE1 protein levels begins at a concentration of 1 μM 2BP and continues until 15 μM 2BP. Additionally, we begin to see decreased NHE1 palmitoylation within 30 min at even the lowest concentration (1 μM) of 2BP which continues through 18 h in a stepwise manner. This provides a range of 2BP concentration and times which are effective in reducing NHE1 palmitoylation without significantly effecting total NHE1 levels or cell viability which we subsequently used to examine the effect of decreased palmitoylation on cell physiology attributed to NHE1 function and can continue to be used in future experiments.

We then used these concentrations and treatment times to establish 2BP decreases pH_i which is associated with decreased NHE1 activity. To begin teasing out specific stimuli that have a greater effect on NHE1 palmitoylation we measured the effect

of 2BP on pH_i after stimulation with LPA. LPA is a well-known agonist of NHE1 increasing activity and thus increases pH_i[98]. When cells are first treated with 2BP and then challenged with LPA we found a reduced ability of LPA to stimulate NHE1 activity. This indicates LPA plays a greater role in regulation of NHE1 activity in a palmitoylation driven manner than serum.

NHE1 also facilitates protein-protein interactions which regulates stress fiber formation through interactions with the cytoskeleton, specifically the ERM complex[70,148]. PSN cells treated with 2BP show reduced stress fiber formation and again when PSN cells were treated with 2BP, the ability of LPA to increase stress fiber formation was abolished as seen with pH_i effects. Additionally, 2BP treatment decreases PSN cell migration shown by reduced wound healing, decreased cell velocity and decreased cell impendence. This suggests that palmitoylation plays a large role in regulation of the NHE1 and of the multiple vital cellular processes associated with the exchanger.

The initial characterization of the effects of 2BP on NHE1 mediated cellular processes provided in this study establishes an important role for palmitoylation in regulating NHE1 and NHE1 mediated cellular processes. The nature of palmitoylation is similar to that of phosphorylation as both modifications regulate proteins in a rapid and dynamic manner and are themselves regulated by enzymes which are influenced various external and internal factors. Multiple studies have shown palmitoylation and phosphorylation occurring on the same proteins and working in a reciprocal manner to regulate various protein functions[125,150].

NHE1 has a large number of phosphorylation sites all of which work together to regulate NHE1 activity, localization, and protein-protein interactions. All of these factors are important for maintaining cellular pH_i as well as keeping cells in a homeostatic state. Various growth factors and hormones stimulate NHE1 phosphorylation. Here we show serum, LPA, and PMA, all factors that increase NHE1 phosphorylation, increase NHE1 palmitoylation and insulin, also known to increase NHE1 phosphorylation, decreases NHE1 palmitoylation. We then inhibited several kinases directly involved in NHE1 phosphorylation and examined the effect on NHE1 palmitoylation which effects cell physiology.

High serum levels and the growth factors within activate the cell and stimulate NHE1 activity through phosphorylation of S703 and 14-3-3 binding of NHE1. Accordingly, palmitoylation of NHE1 might contribute to NHE1 being in a more active state in response to serum. LPA acts as a mitogen, signaling through GPCRs to activate MAPK cascades. While LPA signals through multiple pathways leading to the possibility that many factors may be contributing to the increase in NHE1 palmitoylation here we show that LPA coordinates NHE1 palmitoylation and phosphorylation to work in a cooperative manner on NHE1. Phorbol esters such as PMA activate PKC signaling pathways, while no PKC phosphorylation site has been established on NHE1, studies have found a defined role for PMA activation of NHE1. A defined binding motif for phorbol esters on NHE1 shows PMA acts in a PKC independent manner to activate NHE1 by increased association of the lipid interacting domain (LID) on NHE1 with the membrane[118]. PMA increases NHE1 palmitoylation suggesting a potential role for palmitoylation facilitating LID membrane

interaction through a PMA mediated mechanism. A potential NHE1 palmitoylation site is located within the LID area at residue C561 supporting this theory. Insulin signaling occurs through the PI3K/AKT phosphorylation pathway to phosphorylate NHE1 at S648. Insulin decreases NHE1 palmitoylation indicating the AKT phosphorylation pathway stimulation results in a reciprocal relationship between NHE1 phosphorylation and palmitoylation.

Taken together we show various stimuli that signal through different kinase pathways contribute to NHE1 palmitoylation in both a cooperative and reciprocal manner. This preliminary evidence points to palmitoylation as a possible connection point linking different kinase pathways that phosphorylate NHE1. To continue exploring this connection between NHE1 phosphorylation and palmitoylation we used specific kinase inhibitors and measured the effect on NHE1 palmitoylation.

Looking at the insulin stimulated PI3K/AKT phosphorylation pathway we show inhibition of PI3K and AKT results in increased NHE1 palmitoylation. AKT is responsible for phosphorylation of NHE1 at S648, stimulation of this pathway using insulin increase NHE1 phosphorylation at S648[70] and decreases NHE1 palmitoylation implying palmitoylation and phosphorylation may work to regulate NHE1 in a reciprocal manner. The ERM protein complex binds NHE1 between residues 550-566 which facilitates the formation of stress fibers. Phosphorylation of S648 on NHE1 through AKT reduces actin stress fibers by encouraging depolymerization. Inhibition of palmitoylation using 2BP results in decreased formation of stress fibers and here we show treatment of cells with insulin which promotes S648 phosphorylation leading to depolymerization of stress fibers decreases NHE1 palmitoylation. The ERM binding domain is located between residues 550-566, and

a potential palmitoylation site at residue C561 could offer an explanation for this relationship. Palmitoylation of NHE1 decreases stress fiber formation which could be due to the palmitate inhibiting ERM biding either sterically or by embedding the ERM binding motif into the membrane thus preventing the formation of stress fibers.

Inhibition of MEK using PD98059 decreases NHE1 palmitoylation which aligns with LPA increasing NHE1 palmitoylation. Phosphorylation of NHE1 S770/771 sites increase NHE1 activity in response to sustained acidosis. This along with the fact that inhibition of palmitoylation with 2BP results in a more acidic pH_i provides preliminary evidence that palmitoylation may play a role in cooperating with phosphorylation at these specific sites to increase NHE1 activity. In conjunction with serum increasing NHE1 palmitoylation MEK inhibition decreases NHE1 palmitoylation. Interestingly when Rsk, a step in the MEK pathway and kinase that directly phosphorylates S703, is inhibited NHE1 palmitoylation increases. Additionally, serum promotes phosphorylation of NHE1 at T653 through ROCK, which when inhibited results in increased palmitoylation. While this seems contradictory, it is likely due to the complexity of growth factor pathways influencing NHE1. Overall this data provides preliminary evidence that palmitoylation and phosphorylation work to create specific barcoding patterns that dictate a regulatory outcome and cellular response involving NHE1.

Future Directions

Multiple studies are necessary to fully understand the effect of NHE1 palmitoylation on cellular processes and diseases related to NHE1. A major step forward will be to determine the specific sites where NHE1 is palmitoylated. Once palmitoylation

sites are known multiple studies can be done to understand the role of NHE1 specific palmitoylation in NHE1 transport kinetics, localization, protein interactions, and cell physiology associated with NHE1. Identification of the palmitoylating and depalmitoylating enzymes involved in NHE1 palmitoylation would also provide targets to regulate and study NHE1 function in a manner similar to kinases and phosphatases.

Additional work is necessary to understand how phosphorylation and palmitoylation work in a coordinated manner to regulate NHE1. These studies will focus on using mutations of the palmitoylation sites and phosphorylation sites to measure the effect on the opposite modification. However, this would be time consuming considering the sheer number of phosphorylation sites and potential palmitoylation sites and the inherent issues with mutagenesis having potential off-site effects. Mass spectrometry will be a useful tool to take a snapshot the effect of various stimuli on NHE1 palmitoylation and phosphorylation at the same time without the complications that can accompany mutagenesis.

NHE1 acts as a protein scaffold by interacting with various binding partners including PIP_2, ERM, and CHP1 and 2. NHE1 palmitoylation may alter these interactions, especially considering two potential palmitoylation sites are located in the PIP_2 and ERM binding domains. Gaining a better understanding of the role palmitoylation plays in regulating these binding partners would provide valuable information on how palmitoylation is involved in protein scaffolding and on a larger scale cellular homeostasis and dysfunction.

By pursuing any one of these paths the knowledge gained will have far-reaching impacts on our understanding of cellular homeostasis and on the multiple diseases where NHE1 and palmitoylation are known to play significant roles such as cancer, Alzheimer disease, diabetes, epilepsy, and general neurodegeneration.

Summary Conclusions

Overall, we found NHE1 is palmitoylated and that palmitoylation plays a role in regulation of multiple vital cellular processes associated with the NHE1. Additionally, we gathered preliminary data showing NHE1 palmitoylation and phosphorylation physiological states as well as how NHE1 dysregulation contributes to multiple diseases.

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