Review

Structural and Functional Changes in the Na⁺/H⁺ Exchanger Isoform 1, Induced by Erk1/2 Phosphorylation

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Abstract: The human Na⁺/H⁺ exchanger isoform 1 (NHE1) is a plasma membrane transport protein that plays an important role in pH regulation in mammalian cells. Because of the generation of protons by intermediary metabolism as well as the negative membrane potential, protons accumulate within the cytosol. Extracellular signal-regulated kinase (ERK)-mediated regulation of NHE1 is important in several human pathologies including in the myocardium in heart disease, as well as in breast cancer as a trigger for growth and metastasis. NHE1 has a N-terminal, a 500 amino acid membrane domain, and a C-terminal 315 amino acid cytosolic domain. The C-terminal domain regulates the membrane domain and its effects on transport are modified by protein binding and phosphorylation. Here, we discuss the physiological regulation of NHE1 by ERK, with an emphasis on the critical effects on structure and function. ERK binds directly to the cytosolic domain at specific binding domains. ERK also phosphorylates NHE1 directly at multiple sites, which enhance NHE1 activity with subsequent downstream physiological effects. The NHE1 cytosolic regulatory tail possesses both ordered and disordered regions, and the disordered regions are stabilized by ERK-mediated phosphorylation at a phosphorylation motif. Overall, ERK pathway mediated phosphorylation modulates the NHE1 tail, and affects the activity, structure, and function of this membrane protein.

Keywords: ERK (extracellular signal-regulated kinase); intrinsically disordered protein; Na⁺/H⁺ exchanger; pH regulation; phosphorylation; membrane transport; scaffolding

1. Introduction

The mammalian Na⁺/H⁺ exchanger (NHE) is a ubiquitously expressed membrane protein that maintains intracellular pH (pHᵢ), removing one intracellular H⁺ ion in exchange for a single extracellular Na⁺ ion [1]. NHE1 (isoform one) in the heart is implicated in both myocardial damage from ischemia/reperfusion injury and in promoting hypertrophy (reviewed in the works of [2,3]). This membrane protein consists of two domains. In humans, the 500 amino acid membrane domain transports ions and a 315 amino acid cytosolic domain regulates the membrane domain and is a target for protein interactions and phosphorylation [3,4] (Figure 1). Ten isoforms of NHEs exist; isoform 1 (NHE1) is the only plasma membrane isoform present in the heart and several other tissues [3–9].
1.1. NHE Structure and Subtypes Distribution

There are ten mammalian NHE isoforms (NHE1–10) that are products of different genes, with unique tissue distribution and physiological roles [4,9,10]. The first NHE cloned was NHE1 [11], and it is ubiquitously expressed in mammalian cells. NHE1 was identified by our laboratory as the predominant isoform in the myocardium [5,6], where it is concentrated along the intercalated discs and transverse tubule system, and is the only plasma membrane form of the protein present [12]. Many cell types lack NHE2–5 and 10 [13–16], while NHE6–9 are localized to intracellular organelle membranes such as mitochondria, endosomes, and the Golgi network [17,18]. NHE2 and NHE4 are mainly expressed in the gastrointestinal tracts, where NHE2 functions in Na\(^+\) reabsorption and may act with NHE4, promoting osmoregulation of renal inner medullary cells. NHE3 targets apical membranes such as mitochondria, endosomes, and the Golgi network [17,18]. NHE2 and NHE4 are

1.2. NHE1 Physiological and Pathological Roles

NHE1 has a variety of roles in many cell types (see the works of [4,19–21] for reviews). Knockout of NHE1 from cells demonstrates its role in cell growth [22]. In a consanguineous human family, a deficiency in NHE1 caused ataxia and deafness [23], which was similar to NHE1-deficient mice [24,25]. NHE1 is also important in cell cycle progression [26,27] and in cell differentiation [28,29]. The tail of NHE1 anchors to the cytoskeleton via interactions with ERM (ezrin, radixin, moesin) proteins.
(which crosslink actin with the plasma membrane), affecting cytoskeletal structure, focal adhesion, and cell migration [30–34]. The role of NHE1 apoptosis varies with cell type. In mouse β-cells, trophic factor withdrawal triggers pH_{I} dysregulation and apoptosis [35]. Activation of NHE1 leads to apoptosis in isolated cardiomyocytes [36]. NHE1 is involved in altering the pH_{I} of malignant cells. NHE1-dependent alkalization plays a pivotal role in the development of a transformed phenotype [37–40]. NHE1 activation has been implicated as a key player in breast cancer cell invasion [41–46]. During ischemia, anaerobic glycolysis results in the production of protons, decreasing pH_{I} and activating NHE1. Activated NHE1 exchanges internal H^{+} for extracellular Na^{+}, leading to a rapid accumulation of Na^{+} in cells [47–50]. The high Na^{+} concentration drives an increase in Ca^{2+} via reversal of the Na^{+}/Ca^{2+} exchanger. The resulting buildup of Ca^{2+} triggers various pathways leading to cell death. A huge body of evidence indicates that inhibition of NHE1 during ischemia and reperfusion protects the myocardium from this Ca^{2+} overload [47–50] (and see the works of [50,51] for reviews). NHE1 inhibition by the drugs cariporide, amiloride, and other benzoylguanidines is cardioprotective [52–54]. Activation of NHE1 regulatory pathways is important in NHE1-mediated damage to the myocardium [55]. Similarly, several studies have also shown that NHE1 inhibition prevents cardiac hypertrophy in vivo in rats [56,57] and mice [58–65].

1.3. The Na^{+}/H^{+} Exchanger Structural Aspects

Transmembrane Na^{+}/H^{+} exchange is ubiquitous across all phyla and kingdoms, so NHEs play an important role in many species. NHEs are grouped into the monovalent cation proton antiporter (CPA) superfamilies of CPA1, CPA2, and NaT-DC (Na-transporting carboxylic acid decarboxylase) [21]. The CPA1 family catalyzes Na^{+}, Li^{+}, K^{+}, or Rb^{+} in the electroneutral exchange for a proton. CPA1 includes mammalian NHE1-9. The CPA2 family can catalyze electrogenic or electroneutral activity. This includes Na^{+}, K^{+}/H^{+} exchangers and the electrogenic E. coli NhaA antiporter. Additionally, it includes fungal antiporters and the mammalian electroneutral NHA1 and NHA2 proteins. NaT-DC transporters are a smaller group that export 1–2 Na^{+} in exchange for an extracellular H^{+} as part of a complex that catalyzes decarboxylation of oxaloacetate, malonyl/CoA, or glutaconyl/CoA [21].

The structures of four plasma membrane bacterial transporters Na^{+}/H^{+} antiporters, E. coli NhaA, [66], NapA of Thermus thermophilus [67], MjNhaP1 of Methanocaldococcus jannaschii [68], and PaNhaP of Pyrococcus abyssi [69], have been elucidated by crystallography. The first known structure solved, E. coli NhaA, suggested that Na^{+}/H^{+} antiporters have a novel fold. It consists of two transmembrane segments with a helix-extended region–helix conformation, which was TM4 and TM11 in the E. coli protein [70]. The E. coli protein also had a dimerization or scaffolding subdomain and a six-helix bundle cylindrical transport subdomain [66,71]. The NhaA fold was also found in ThhNapA [67], MjNhaP1 [72], and PaNhaP [69]. EcNhaA is a dimer [73], as is MjNhaP1 [72]. Dutta et al. [70] recently published an alignment of Na^{+}/H^{+} antiporters. The identity of various antiporters varied, being as low as 18% when comparing eukaryotic antiporters with E. coli NhaA. A yeast (S. pombe) Na^{+}/H^{+} antiporter SpNHE1 aligned reasonably with the 13 transmembrane segments of PaNhaP and was predicted to have 13 transmembrane segments. Similarly, the plant Na^{+}/H^{+} antiporter of Arabidopsis, SOS1, was aligned with a number of Na^{+}/H^{+} antiporters and a 13 transmembrane segment topology was also predicted [74].

The topology of the hNHE1 isoform of the Na^{+}/H^{+} exchanger is not yet deduced and is controversial. One model was made using cysteine-scanning accessibility and suggested a 12 transmembrane segment model with amino acids 15–36 N-terminal and cytosolic. [75]. Later, a 3D model was made using homology modeling with EcNhaA [76]. Both models were similar except for different topology assignments of, and near, amino acids comprising TM9, 341–362. Later work suggested that amino acids 363–410 are EL5, with amino acids 341–362 preceding it as TM9 [77,78]. Recently, a newer molecular modeling of NHE1 also mapped amino acids 363–410 to the extracellular surface and also docked NHE inhibitors to sites on the protein [79]. The region between TM9 and TM10 (extracellular loop 5, approximately amino acids 362–411) was shown to be extracellular based on cysteine scanning...
and accessibility experiments. Part of this segment was suggested to be associated with the lipid bilayer based on its hydrophobicity and a lack of accessibility of a few residues in cysteine accessibility studies. However, the position of flanking amino acids confirmed it does not traverse the membrane [75,77]. It has since not been well studied.

The mammalian NHE isoforms share a greater identity within the membrane domain as opposed to the cytosolic regulatory domain. In the membrane domain, identity is conserved between transmembrane segments. This varies depending on the isoforms compared, but is often around 55, and rises to above 90% when comparing the same isoform in different species. In the hydrophilic C-terminal domain, the identity is approximately 24–31% [80]. It should be noted, however, that the structure of the cytosolic tail domain shows conservation (discussed below) [34].

2. Regulation of NHE1 Isoform of the Na\(^+\)/H\(^+\) Exchanger, General

2.1. Rationale for Study of NHE1 Regulation

Regulation of the NHE1 isoform is extremely important. Not only is it important from the point of view of understanding fundamentally how the protein works, but also in human pathology. For example, it has been shown that artificially activating NHE1 activity by modulating regulation of the protein accentuates the damage the protein causes in pathology in the heart [59,81–84] and in breast cancer [46,85,86]—two common diseases. Alternatively, it has been suggested that targeting regulation of NHE1 warrants investigation to treat disease [87]. At the same time, while direct inhibition of NHE1 has been suggested to treat human disease, there have been detrimental off target side effects of NHE1 inhibitors in at least one clinical trial, though suppression of NHE1 activity still remains a potentially effective therapeutic approach to the treatment of human disease [88]. Clearly there is a need for a better understanding of NHE1 structure and regulation and application of this knowledge towards the treatment of human disease.

2.2. NHE1 Regulation

Various extracellular agonists mediate their effects through several cell surface receptors and signaling networks that modify the NHE1 C-terminal cytosolic regulatory domain. Modifications include binding of regulatory proteins that control transport activity by altering the affinity of the transport domain for intracellular H\(^+\) [10]. The particular agonists and coupling of receptors vary with the tissues involved. The pH dependence of NHE1 is usually shifted to a more alkaline range. Agonists include \(\alpha_1\)-adrenergic stimulation (phenylephrine) and hormones such as endothelin [89,90] (ET-1), thrombin, epidermal growth factor, angiotensin II, and lysophosphatidic acid [4,10,91–97]. This stimulation results in various binding proteins and protein kinases interacting directly and indirectly with NHE1 (Figure 2). An early study used deletion analysis and revealed that the distal 180 amino acids of the 315 amino acid C-terminal tail of NHE1 contain phosphorylation sites responsible for part of the growth factor induced activation of NHE1. Protein–protein interactions mediate other activation [98]. However, many of the protein binding sites and phosphorylation sites overlap [34]. Activation of NHE1 results in a shift of the pH sensitivity curve such that, at a given more alkaline pH, the protein is more active.
Regulatory binding proteins were reviewed earlier [34]. Briefly, calmodulin is one of the most important of these and binds to two sites in the cytoplasmic tail of NHE1. One is a high affinity and one a low affinity site in amino acids 636–700 [99,100]. Calcium dependent binding to the high affinity site (636–656) blocks auto-inhibition, and thereby activates NHE1 [101]. A serine residue (Ser648) within the high affinity calmodulin binding site is phosphorylated by protein kinase B/Akt, reducing calmodulin binding [102]. Specific mutations to the auto-inhibition site can produce a hyper activated protein with significant physiological consequences [59,86].

Another regulatory binding cofactor is phosphatidylinositol 4, 5-bisphosphate. It binds to NHE1 and may function to stabilize NHE1 in a physiologically optimal conformation, promoting protein stability and cell surface targeting [107].

Other proteins binding to the NHE1 tail include carbonic anhydrase II, which increases NHE1 activity and is dependent on the phosphorylation state of NHE1 [108]. The ERM family (ezrin, radixin, and moesin) forms links between actin filaments of the cytoskeleton and integral proteins of the plasma membrane [109], and NHE1 has ERM binding motifs in amino acids 552–560 of its cytoplasmic tail. Hsp70 and Hsp90 also bind to the C-terminus of NHE1 and may participate in protein folding [110–113].

Another regulatory binding cofactor is phosphatidylinositol 4, 5-bisphosphate. It binds to NHE1 in two juxtamembrane cationic binding regions, amino acid segments 513–520 and 556–564 (of the rat protein, equivalent to 509–516 and 552–560, respectively, of the human protein) [114]. Mutation of the sites did not prevent surface targeting but decreased efficiency of transport [114]. The interaction, induced by ATP depletion, inhibits NHE1 [114–116]. Aside from polyphosphoinositides, cationic regions of NHE1 also interact with acidic phospholipids including phosphatidylserine. Region 542–598 is thought to be a lipid interacting domain with the hydrophobic sequence 573LIAFY577 predicted to bind lipids [107,117]. Deletion or mutation of these residues results in decreased NHE1 activity.

2.3. NHE1 Regulation, Phosphorylation

2.3.1. Phosphorylation, General

As noted above, phosphorylation of NHE1 is critical to the stimulation of activity and a number of different protein kinases have been shown to phosphorylate the cytosolic regulatory

Figure 2. Regulation of Na\(^+\)/H\(^+\) exchanger isoform 1 (NHE1). Through agonists, receptor mediated activation of the Na\(^+\)/H\(^+\) exchanger, isoform 1, via protein regulatory interactions or through phosphorylation of the cytosolic C-terminus.

[Diagram showing the regulation of Na\(^+\)/H\(^+\) exchanger isoform 1 (NHE1)].

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Phosphorylation is suggested to account for about half of the growth factor induced regulation of NHE1 and is mostly thought to occur in the 180 amino acid distal region of the carboxyl terminal tail [4,118]. Earlier, we and others [3,4,34,44,119] reviewed phosphorylation-mediated regulation of NHE1 in several tissues. Briefly, some of the more well-characterized protein kinases that have been implicated in regulation through phosphorylation of the cytosolic domain are β-Raf (in the C-terminal 180 amino acids) [120], p38 Mitogen Activated Protein Kinase (MAPK) (Thr718, Ser723, Ser725, Ser729, rabbit sequence changed to equivalent human) [35], and protein kinase B/Akt (Ser648, [102,121]) (a more extensive list includes MS-based phosphoproteomics and is found in the work of [34]).

2.3.2. ERK Mediated Regulation, General

The ERK cascade is a central pathway transmitting signals from many extracellular agents such as hormones to regulate a variety of processes including proliferation and differentiation. Signaling via this cascade pathway travels through sequential phosphorylation involving activation of protein kinases in different levels of the cascade. Briefly described, the main components of the chain of phosphorylation are the Raf kinases, MEK1, ERK1/2 (the ERKS), and ribosomal protein S6 kinase (RSKs) (Figure 3). There are also other components including spliced forms and different proteins that participate depending on conditions, for details see the works of [122,123].

![Figure 3. Simplified schematic of the ERK signaling cascade. Activation processes are indicated. Raf family members bind Ras/GTP (not shown). Raf isoforms activate ERK kinase (MEK). Activated ERK has many substrates. Downstream of ERK1/2 is the RSK family with various isoforms, which participate in ERK signaling and diversification of the signal. Adapted from the works of [122,123].](image-url)

The ERK pathway is important in many aspects of human health and pathology with a number of similarities to the role of NHE1. ERK1/2 are important in cell growth and differentiation [123], as is NHE1 [4]. Mutations in the ERK pathway are important in triggering cancer [123] and activated...
NHE1 is important in cell growth and has been implicated as a trigger in several kinds of cancers including ovarian, breast, and prostate cancer [44,120,124–126]. Similarly, the ERK pathway is involved in several aspects of heart disease including cardiac hypertrophy and ischemia reperfusion damage to the heart [127,128], and NHE1 is involved in the aggravation of both of these forms of myocardial disease [3,107]. The disease states of ischemia and hypertrophy in the myocardium and metastatic breast cancer are known to have activated ERK pathways that activate the Na\(^{+}/H^{+}\) exchanger [3,86,129].

Below, we describe evidence that demonstrates that ERK directly phosphorylates and regulates NHE1 in two disease states.

3. ERK Mediated Regulation of NHE1

3.1. ERK Pathway Regulation of NHE1 in the Myocardium

Early studies showed that ERK-dependent phosphorylation of NHE1 was hormonally regulated and occurred in the cytosolic domain [130]. Later, it was demonstrated that ischemia and reperfusion of the mammalian heart activated ERK1/2 and increased their activity towards the Na\(^{+}/H^{+}\) exchanger. Both ERK1/2 and p90\(^{RSK}\) activity towards NHE1 was increased [55]. Ischemia causes intracellular acidosis, which activates the ERK1/2 pathway. It was thus also demonstrated that artificially inducing acidosis in isolated cardiomyocytes activates the ERK1/2 pathway and increases Na\(^{+}/H^{+}\) exchanger activity. This was through the Ras/Raf/MEK pathway [131]. Another form of activation is through bursts in reactive oxygen species that occur in cardiac ischemia reperfusion. One of these, H(2)O(2), has also been shown to activate ERK1/2 and increase phosphorylation and activation of the Na\(^{+}/H^{+}\) exchanger in cardiomyocytes [132–134].

The sites of phosphorylation of NHE1 in this pathway have obviously attracted interest. Some of the first studies showed that Ser\(^{703}\) is directly phosphorylated by p90 ribosomal S6 kinase (p90\(^{RSK}\)), causing activation of NHE1. Additionally, phosphorylation of this site (RIGSDP) causes association of the protein 14-3-3 and activation of NHE1 [135]. It is notable that inhibiting p90\(^{RSK}\)-mediated phosphorylation of this site reduces myocardial infarction caused by coronary artery occlusion [136]. Clearly, phosphorylation at this site plays an important physiological role in the heart, a point that has been verified pharmacologically [128].

While Ser\(^{703}\) plays an important role in the myocardium, it is not the only site phosphorylated via the ERK dependent pathway. Two groups studied this. We [137–140] identified direct phosphorylation of the NHE1 cytosolic domain by ERK1/2. This was demonstrated in four general regions, 1, Ser\(^{693}\); 2, Thr\(^{718}\); Ser\(^{723}\); Ser\(^{726}\); Ser\(^{729}\); 3, Ser\(^{766}\); Ser\(^{770}\); Ser\(^{771}\); and 4, Thr\(^{779}\); Ser\(^{785}\), which were identified by mass spectrometry [3,139] (Figure 4). ERK-dependent phosphorylation of Ser\(^{770}\) and Ser\(^{771}\) was demonstrated in rat cardiomyocytes and Chinese hamster ovary (CHO) cells in response to hormonal stimulation and sustained acidosis [137,140]. ERK-dependent sustained intracellular acidosis was shown to activate the NHE1 protein in CHO cells and in cardiomyocytes, even when Ser\(^{703}\) had been mutated to Ala, demonstrating that the ERK-dependent pathway can activate NHE1 without p90\(^{RSK}\) and Ser\(^{703}\) phosphorylation [141]. A second group [142] similarly demonstrated phosphorylation of the NHE1 tail by ERK2. The six sites Ser\(^{693}\), Ser\(^{723}\), Ser\(^{726}\), Ser\(^{771}\) and Thr\(^{779}\), and Ser\(^{785}\) were phosphorylated in a distinct temporal order with varying rate constants. Phosphorylation of residues Ser\(^{693}\) and Thr\(^{779}\) occurred first and simultaneously, and other phosphorylation appeared later (Figures 1 and 4).

An indication that phosphorylation results in structural changes in the cytosolic regulatory domain was first suggested in 2013 [143]. By making phosphomimetic mutations, it was demonstrated that the full-length protein had altered accessibility to trypsin digestion. The same phenomenon occurred in a peptide fragment. Hydrogen/deuterium exchange mass spectrometry showed that the phosphomimetic changes (S770D, S771D) appear to stabilize a sequence between residues Met\(^{764}\) and Thr\(^{779}\). Conversely, the region between amino acids Tyr\(^{577}\)–Leu\(^{588}\) was less stable in the phosphomimetic protein [143].

Another member of the ERK pathway, β-Raf, has also been shown to phosphorylate the C-terminal domain of the Na\(^{+}/H^{+}\) exchanger and activate the protein. Originally, it was demonstrated that in
patients with the V600E mutation of β-Raf, there is aberrant pH regulation in malignant melanoma cells due to stimulation of NHE1 [120,144]. Later, it was shown that in cardiomyocytes, inhibition or depletion of β-Raf inhibited NHE1 activity. Additionally, it was demonstrated that β-Raf bound to NHE1 and phosphorylated amino acid Thr$^{653}$ (Figures 1 and 4) [120].

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Affinity chromatography and co-immunoprecipitation confirmed an interaction between NHE1 and 14-3-3 proteins [145]. The authors of [145] have identified four predicted helices, according to the authors of [34]. Yellow helices above amino acids indicate transient helices induced by ERK-mediated phosphorylation, according to the authors of [34]. Green helices above amino acids are the approximate location of four predicted helices, according to the authors of [34]. Yellow helices above amino acids indicate transient helices induced by ERK-mediated phosphorylation, according to the authors of [145].

**3.2. ERK Pathway Regulation of Breast Cancer Metastasis**

NHE1 is a trigger for metastasis in triple negative breast cancer cells [44,85,124,146–152]. It facilitates metastasis in part, by acidifying the extracellular matrix region promoting activity of extracellular digestive enzymes [37,44,148,153–155]. The extracellular acidification is thought to be necessary for protease activation, which facilitates the digestion and remodeling of the extracellular matrix [149,153,155], critical in metastasis. Elevated NHE1 activity occurs in triple negative breast cancer cells, which facilitates the growth and metastasis of triple negative breast cancer cells [85]. Inhibition or knockout of NHE1 can reduce growth and metastasis of triple negative breast cancer cells [85,86]. Loss or inhibition of NHE1 increases the susceptibility of breast cancer cells to paclitaxel in triple negative breast cancer cells [85].

A key regulatory site of NHE1 in breast cancer is Ser$^{703}$, which is phosphorylated by p90$^{RSK}$ (Figures 1 and 4). Mutation of this p90$^{RSK}$ phosphorylation site on NHE1’s cytosolic C-terminal domain, to the non-phosphorylatable Ala, alters the morphology of invasive mesenchymal-like MDA-MB-231 cells to the less invasive, smaller, rounder, and non-invasive epithelial phenotype. There is also a loss of expression of the protein and mRNA of the mesenchymal marker vimentin [86]. Affinity chromatography and co-immunoprecipitation confirmed an interaction between NHE1 and 14-3-3 [156], which binds to this site. Pharmacological inhibition of p90$^{RSK}$ also inhibited the metastatic potential of invasive MDA-MB-231 cells [86]. Thus, in this disease model, the ERK pathway and certainly Ser$^{703}$ play an important role in the pathology of the disease. It has also been shown that expression of a
truncated, constitutively active ErbB2 tyrosine kinase receptor in MCF-7 breast cancer cells leads to increased ERK1/2 and p90<sup>RSK</sup> activity. This mutant protein is associated with increased metastatic potential and cell motility and caused increased phosphorylation of Ser<sup>703</sup> of NHE1 [157]. It is also worth mentioning that an association of elevated NHE1 expression with p90<sup>RSK</sup> has also been shown in primary metastatic basal triple negative breast cancer tumors [156].

4. NHE1 Scaffolding, ERK1/2, and Disordered Domains

4.1. Scaffolding and ERK1/2

As noted above, the tail of NHE1 has several interacting proteins. The regulatory tail of NHE1 may assemble signaling complexes in special plasma membrane domains, in order to coordinate signaling pathways and complexes [19,158] that promote signaling, or regulatory events and complex formation [159]. Meima [19] postulates that the NHE1 scaffolding ensemble is not static, but varies in response to different stimuli. This has not yet been well documented empirically. The NHE1 “interactome” was examined by affinity chromatography and immunoprecipitation in MDA-MB-231 breast cancer cells. NHE1 was associated with 14-3-3 protein, AKT kinase, alpha-enolase, CHP1, and heat shock proteins HSP70 and HSP90 [156]. A similar approach with renal tissue also demonstrated the presence of interacting heat shock proteins and 14-3-3 [111]. One group [158] used siRNA knockdowns and yeast two-hybrid screening to demonstrate that NHE1 is a MAPK scaffold for several members of the MAPK family, including ERK and Raf [158]. More recently, another group [142] identified the NHE1 tail as a scaffold for ERK1/2. These S/T kinases interact with targets and regulators via two kinds of pathways, D-domains and F-sites. D-domains (also known as docking sites for ERK) have the sequence of 2–5 basic residues (R/K), which is spaced by 1 to 6 residues, followed by a motif hydrophobic amino acid -X-hydrophobic amino acid (Φ-X-Φ, the hydrophobic is usually V, L, or I) (Figure 4). F-sites, also called DEF (docking site for ERK, FXFP)-domains, have the canonical FXFP sequence. They allow for aromatic residues at the P1 (F, W) and P3 positions (F, Y, W). NHE1 has three D-domains and two F-sites (Figure 4) identified in its tail sequence. Their results showed that ERK2 interacted with the D3 domain and two F-sites [142]. Mutation of the D-domains from Φ-X-Φ to AXA showed that only the D3 domain was important for ERK interactions in vitro and in CHO (Chinese hamster ovary) cells. Mutation of the F sites from FTP778–780 to ATP778–780 and FP811–812 to AA811–812 both affected ERK interactions in vitro and mutation of the F1 site affected interactions in CHO cells (F2 was not tested). Thus, the D3 domain and two F-sites are ERK interaction sites.

4.2. ERK1/2 Scaffolding and the Disordered Domains of NHE1

One interesting structural aspect of the Na<sup>+</sup>/H<sup>+</sup> exchanger family is found in the cytosolic regulatory, 315 amino acid tail. This region is poorly conserved among isoforms. This probably lends specificity to the regulation of the isoforms. The proximal part of the NHE1 tail is ordered and four alpha helices are predicted from amino acids 506 to 590. However, this is followed by a predicted disordered region (amino acids 591–625). There are two helices predicted between amino acids 626–685, but the distal part of the tail from amino acids 686–815 is predicted to be intrinsically disordered (Figure 4). This character is common and conserved in the NHE tails of various types [34]. Intrinsic disorder often results from charged amino acids and from their low hydrophobicity, which leads to increased flexibility. This flexibility leads to a variety of interchangeable conformations that are key to binding interactions that assist in molecular recognition. In many cases, there may be linear short binding motifs within the flexible regions and these intrinsically disordered proteins may fold when other proteins bind [34,160,161].

4.3. ERK1/2 Phosphorylation and Binding and Conformational Changes in the NHE1 C-terminus

As noted above, an early study [143] demonstrated that phosphomimetic mutations in the cytosolic C-terminus of NHE1 caused conformation changes in the tail. A recent and elegant study [145] examined
more directly the effect of phosphorylation of the C-terminal of NHE1 via ERK2 using nuclear magnetic resonance (NMR) spectroscopy. They examined the ERK2 phosphorylation sites Ser\textsuperscript{693}, Ser\textsuperscript{723}, Ser\textsuperscript{726}, Ser\textsuperscript{771}, Thr\textsuperscript{779}, and Ser\textsuperscript{785}, as well as changes in conformation using NMR chemical shifts. A secondary structure analysis by NMR revealed four transient helices between amino acids 694 and 795 in the disordered regions of the tail domain. The ERK2 phosphorylation sites were located N-terminal to transient helices 1–4 at 694–700, 727–743, 758–766, and 786–795. With phosphorylation of the six ERK2 sites, the local transient helices 1 and 4 showed increased helicity. Arg\textsuperscript{790} was important for helix stabilization upon Ser\textsuperscript{785} phosphorylation, while an upstream acid residue (Asp\textsuperscript{784}) was not. The motif [S/T]-P-[3]-[R/K] was suggested to be a helix promoting motif with the Pro as a possible starting point for the helix.

5. Conclusions

5.1. Summary of ERK Pathway Mediated Effects

It is clear that there are a number of regulatory mechanisms and physiological effects of regulation of NHE1 in several tissues. Perhaps the most significant to mankind is the role the protein plays in heart disease and breast cancer, which affect relatively large percentages of the whole population and of women, respectively. Elevated activity of the NHE1 isoform of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger is a trigger for damage in ischemic heart disease and is an important trigger for cell growth and metastasis in breast cancer. The cytosolic C-terminal tail of NHE1 serves as a regulator of transport of the membrane domain, but more than that, it is a focal point for regulation of the protein that occurs principally by protein binding and phosphorylation (and lipid binding). ERK mediated phosphorylation of the C-terminal occurs at multiple sites and affects the structure and function of the protein. The regulatory tail itself possesses both structured and unstructured intrinsically disordered domains. The intrinsically disordered domains are important in the function of the protein and phosphorylation by ERK affects their structure, inducing helical structure in regions adjacent to the phosphorylation site. We suggest that this conformational change in the tail affects the membrane domain, altering its affinity for protons and increasing activity at more alkaline intracellular pH (Figure 5).

![Figure 5](image_url)

**Figure 5.** Simplified schematic of the hypothetical ERK signaling cascade that activates the Na\textsuperscript{+}/H\textsuperscript{+} exchanger. Cell surface receptor activates ERK signaling cascade that causes multiple kinases, including ERK, to phosphorylate the NHE1 regulatory cytosolic domain. ERK1/2 phosphorylates in multiple locations changing the conformation of the cytosolic domain, activating the membrane domain. Downstream of ERK1/2 is the RSK family that participates in ERK signaling and NHE1 phosphorylation, and upstream is the Raf family that also participates in NHE1 phosphorylation.
5.2. Future Studies

The intrinsically disordered regions of the cytosolic domain of the Na\(^+\)/H\(^+\) exchanger are of great interest. Clearly, they are involved in the regulation of the NHE1 protein and can alter their structure upon phosphorylation; however, the mechanism by which ERK phosphorylation alters activity of the Na\(^+\)/H\(^+\) exchanger still needs to be further elucidated. It is also not clear how and where other proteins bind to the intrinsically disordered region and how they alter its structure, and how this can affect function. It has been suggested that a gate exists in NHE1 that is closed upon autoinhibitory interactions with the region of the C-terminal tail [34]. However, this mechanism is based on the model of NHE1, which originated from NhaA, and others have more recently suggested that NhaA is too different from NHE1 to be used as a model [79]. Nevertheless, it seems likely that the tail must interact with the membrane domain to affect function, and regulation by phosphorylation and proteins seem likely to affect this. The structure of the intact full-length protein is highly desirable, though the intrinsically disordered domains are likely to make this difficult. A structure of a complex of NHE1 with regulatory proteins and a structure of NHE1 with induced structure in the intrinsically disordered region would be most desirable. Experiments toward this end are currently underway.

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**Abbreviations**

- AKT: protein kinase B
- CPA: Cation proton antiporter
- ERK: Extracellular signal-regulated kinase
- ERM: Ezrin, radixin, and moesin
- MAPK: Mitogen activated protein kinase
- NHE1: Na\(^+\)/H\(^+\) exchanger isoform one
- NMR: Nuclear magnetic resonance
- pH\(_i\): Intracellular pH

**References**

1. Fliegel, L. The Na\(^+\)/H\(^+\) exchanger isoform 1. *Int. J. Biochem. Cell Biol.* 2005, 37, 33–37. [CrossRef]
2. Karmazyn, M.; Gan, T.; Humphreys, R.A.; Yoshida, H.; Kusumoto, K. The myocardial Na\(^+\)/H\(^+\) exchange: Structure, regulation, and its role in heart disease. *Circ. Res.* 1999, 85, 777–786. [CrossRef]
3. Fliegel, L. Regulation of the Na\(^+\)/H\(^+\) exchanger in the healthy and diseased myocardium. *Expert Opin. Ther. Targets* 2009, 13, 55–68. [CrossRef] [PubMed]
4. Malo, M.E.; Fliegel, L. Physiological role and regulation of the Na\(^+\)/H\(^+\) exchanger. *Can. J. Physiol. Pharmacol.* 2006, 84, 1081–1095. [CrossRef] [PubMed]
5. Fliegel, L.; Dyck, J.R.B.; Wang, H.; Fong, C.; Haworth, R.S. Cloning and analysis of the human myocardial Na\(^+\)/H\(^+\) exchanger. *Mol. Cell. Biochem.* 1993, 125, 137–143. [CrossRef] [PubMed]
6. Fliegel, L.; Sardet, C.; Pouysségur, J.; Barr, A. Identification of the protein and cdna of the cardiac Na\(^+\)/H\(^+\) exchanger. *FEBS Lett.* 1991, 279, 25–29. [CrossRef]
7. Fliegel, L. Functional and cellular regulation of the myocardial Na\(^+\)/H\(^+\) exchanger. *J. Thromb. Thrombolysis* 1999, 8, 9–14. [CrossRef]
8. Orlowski, J.; Kandasamy, R.A.; Shull, G.E. Molecular cloning of putative members of the Na\(^+\)/H\(^+\) exchanger gene family. *J. Biol. Chem.* 1992, 267, 9331–9339. [PubMed]
9. Lee, S.H.; Kim, T.; Park, E.S.; Yang, S.; Jeong, D.; Choi, Y.; Rho, J. Nhe10, an osteoclast-specific member of the Na\(^+\)/H\(^+\) exchanger family, regulates osteoclast differentiation and survival [corrected]. *Biochem. Biophys. Res. Commun.* 2008, 369, 320–326. [CrossRef] [PubMed]
32. Denker, S.P.; Huang, D.C.; Orlowski, J.; Furthmayr, H.; Barber, D.L. Direct binding of the Na-H exchanger nh1 to erb proteins regulates the cortical cytoskeleton and cell shape independently of H+ translocation. *Mol. Cell* **2000**, *6*, 1425–1436. [CrossRef]

33. Tominaga, T.; Barber, D.L. Na-H exchange acts downstream of rhoa to regulate integrin-induced cell adhesion and spreading. *Mol. Biol. Cell.* **1998**, *9*, 2287–2303. [CrossRef]

34. Hendus-Altenburger, R.; Kragelund, B.B.; Pedersen, S.F. Structural dynamics and regulation of the mammalian slc9a family of Na+/H+ exchangers. *Curr. Top. Membr.* **2014**, *73*, 69–148. [PubMed]

35. Khaled, A.R.; Moor, A.N.; Li, A.; Kim, K.; Ferris, D.K.; Muegge, K.; Fisher, R.J.; Fliegel, L.; Durum, S.K. Trophic factor withdrawal: P38 mitogen-activated protein kinase activates Nhe1, which induces intracellular alkalinization. *Mol. Cell. Biol.* **2001**, *21*, 7545–7557. [CrossRef]

36. Karki, P.; Fliegel, L. Overexpression of the Nhe1 isoform of the Na+/H+ exchanger causes elevated apoptosis in isolated cardiomyocytes after hypoxia/reoxygenation challenge. *Mol. Biochem. Cardiovasc. Haematol. Disord.* **2005**, *3*, 338, 47–57. [CrossRef]

37. Harguindey, S.; Orive, G.; Luis Pedraz, J.; Paradiso, A.; Reshkin, S.J. The role of pH dynamics and the Na+/H+ antipporter in the etiopathogenesis and treatment of cancer. Two faces of the same coin—one single nature. *Biochim. Biophys. Acta* **2005**, *1756*, 1–24. [CrossRef]

38. Reshkin, S.J.; Bellizzi, A.; Caldeira, S.; Albarani, V.; Malanchi, I.; Poignee, M.; Alunni-Fabbroni, M.; Casavola, V.; Tommasino, M. Na+/H+ exchanger-dependent intracellular alkalinization is an early event in malignant transformation and plays an essential role in the development of subsequent transformation-associated phenotypes. *FASEB J.* **2000**, *14*, 2185–2197. [CrossRef]

39. McLean, L.A.; Roscoe, J.; Jørgensen, N.K.; Gorin, F.A.; Cala, P.M. Malignant gliomas display altered pH regulation by Nhe1 compared with nontransformed astrocytes. *Am. J. Physiol.* **2000**, *278*, C676–C688. [CrossRef]

40. Rich, I.N.; Worthington-White, D.; Garden, O.A.; Musk, P. Apoptosis of leukemic cells accompanies reduction in intracellular pH after targeted inhibition of the Na+/H+ exchanger. *Blood* **2000**, *95*, 1427–1434.

41. Reshkin, S.J.; Bellizzi, A.; Albarani, V.; Guerra, L.; Tommasino, M.; Paradiso, A.; Casavola, V. Phosphoinositide 3-kinase is involved in the tumor-specific activation of human breast cancer cell Na+/H+ exchange, motility, and invasion induced by serum deprivation. *J. Biol. Chem.* **2000**, *275*, 5361–5369. [CrossRef]

42. Cardone, R.A.; Bagorda, A.; Bellizzi, A.; Busco, G.; Guerra, L.; Paradiso, A.; Casavola, V.; Zaccolo, M.; Reshkin, S.J. Protein kinase a gating of a pseudopodial-located rhoa/rock/p38/Nhe1 signal module regulates invasion in breast cancer cell lines. *Mol. Biol. Cell* **2005**, *16*, 3117–3127. [CrossRef]

43. Paradiso, A.; Cardone, R.A.; Bellizzi, A.; Bagorda, A.; Guerra, L.; Tommasino, M.; Casavola, V.; Reshkin, S.J. The Na+/H+ exchanger-1 induces cytoskeletal changes involving reciprocal rhoa and rac1 signaling, resulting in motility and invasion in mda-mb-435 cells. *Breast Cancer Res.* **2004**, *6*, R616–R628. [CrossRef]

44. Amith, S.R.; Fliegel, L. Regulation of the V exchanger (Nhe1) in breast cancer metastasis. *Cancer Res.* **2013**, *73*, 1259–1264. [CrossRef]

45. Amith, S.R.; Fliegel, L. Na+/H+ exchanger-mediated hydrogen ion extrusion as a carcinogenic signal in triple-negative breast cancer etiopathogenesis and prospects for its inhibition in therapeutics. *Semin. Cancer Biol.* **2017**, *33*, 35–41. [CrossRef]

46. Amith, S.R.; Fliegel, L. The Na+/H+ exchanger in metastasis. *Aging (Albany, NY)* **2016**, *8*, 1291. [CrossRef]

47. Allen, D.G.; Xiao, X.H. Role of the cardiac Na+/H+ exchanger during ischemia and reperfusion. *Cardiovasc. Res.* **2003**, *57*, 934–941. [CrossRef]

48. Lazarduski, M.; Frelin, C.; Vigne, P. The sodium/hydrogen exchange system in cardiac cells. Its biochemical and pharmacological properties and its role in regulating internal concentrations of sodium and internal ph. *J. Mol. Cell. Cardiol.* **1985**, *17*, 1029–1042. [CrossRef]

49. Avkiran, M. Protection of the ischaemic myocardium by Na+/H+ exchange inhibitors: Potential mechanisms of action. *Basic Res. Cardiol.* **2001**, *96*, 306–311. [CrossRef]

50. Karmazyn, M.; Sawyer, M.; Fliegel, L. The Na+/H+ exchanger: A target for cardiac therapeutic intervention. *Curr. Drug Targets Cardiovasc. Haematol. Disord.* **2005**, *5*, 323–335. [CrossRef]

51. Avkiran, M.; Marber, M.S. Na+/H+ exchange inhibitors for cardioprotective therapy: Progress, problems and prospects. *J. Am. Coll. Cardiol.* **2002**, *39*, 747–753. [CrossRef]

52. Karmazyn, M. Amiloride enhances post ischemic recovery: Possible role of Na+/H+ exchange. *Am. J. Physiol.* **1988**, *255*, H608–H615.
53. Scholz, W.; Albus, U.; Counillon, L.; Gogelein, H.; Lang, H.J.; Linz, W.; Weichert, A.; Scholkens, B.A. Protective effects of hoe642, a selective sodium-hydrogen exchange subtype 1 inhibitor, on cardiac ischaemia and reperfusion. Cardiovasc. Res. 1995, 29, 260–268. [CrossRef]

54. Gumina, R.J.; Mizumura, T.; Beier, N.; Schelling, P.; Schultz, J.J.; Gross, G.J. A new sodium/hydrogen exchange inhibitor, emd 85131, limits infarct size in dogs when administered before or after coronary artery occlusion. J. Pharmacol. Exp. Ther. 1998, 286, 175–183.

55. Moor, A.; Gan, X.T.; Karmazyn, M.; Fliegel, L. Activation of Na⁺/H⁺ exchanger-directed protein kinases in the ischemic and ischemic-reperfusion rat myocardium. J. Biol. Chem. 2001, 276, 16113–16122. [CrossRef]

56. Kusumoto, K.; Haist, J.V.; Karmazyn, M. Na⁺/H⁺ exchange inhibition reduces hypertrophy and heart failure after myocardial infarction in rats. Am. J. Physiol. 2001, 280, H738–H745. [CrossRef]

57. Yoshida, H.; Karmazyn, M. Na⁺/H⁺ exchange inhibition attenuates hypertrophy and heart failure in 1-wk postinfarction rat myocardium. Am. J. Physiol. 2000, 278, H300–H304. [CrossRef]

58. Kilic, A.; Velic, A.; De Windt, L.J.; Fabritz, L.; Voss, M.; Mitko, M.; Baba, H.A.; van Eickels, M.; Schlammer, E.; et al. Enhanced activity of the myocardial Na⁺/H⁺ exchanger Nhe-1 contributes to cardiac remodeling in atrial natriuretic peptide receptor-deficient mice. Circulation 2005, 112, 2307–2317. [CrossRef] [PubMed]

59. Mraiche, F.; Oka, T.; Gan, X.T.; Karmazyn, M.; Fliegel, L. Activated Nhe1 is required to induce early cardiac hypertrophy in mice. Basic Res. Cardiol. 2011, 106, 603–616. [CrossRef] [PubMed] [CrossRef]

60. Karmazyn, M.; Liu, Q.; Gan, X.T.; Brix, B.J.; Fliegel, L. Aldosterone increases Nhe-1 expression and induces nhe-1-dependent hypertrophy in neonatal rat ventricular myocytes. Hypertension 2003, 42, 1171–1176. [CrossRef]

61. Cingolani, H.E.; Alvarez, B.V.; Ennis, I.L.; Camilion de Hurtado, M.C. Stretch-induced alkalization of feline papillary muscle: An autocrine-paracrine system. Circ. Res. 1998, 83, 775–780. [CrossRef]

62. Camilion de Hurtado, M.C.; Portiansky, E.L.; Perez, N.G.; Rebolledo, O.R.; Cingolani, H.E. Regression of cardiomyocyte hypertrophy in shr following chronic inhibition of the Na⁺/H⁺ exchanger. Cardiovasc. Res. 2002, 53, 862–868. [CrossRef]

63. Baartscheer, A.; Hardziyenka, M.; Schumacher, C.A.; Belterman, C.N.; van Borren, M.M.; Verkerk, A.O.; Coronel, R.; Fiolet, J.W. Chronic inhibition of the Na⁺/H⁺ exchanger-directed protein kinases in SHR我的心室肥大和心肌电生理学重建。Br. J. Pharmacol. 2008, 154, 1266–1275. [CrossRef]

64. Cingolani, H.E.; Ennis, I.L. Sodium-hydrogen exchanger, cardiac overload, and myocardial hypertrophy. Circulation 2007, 115, 1090–1100. [CrossRef]

65. Dulce, R.A.; Hurtado, C.; Ennis, I.L.; Garciaarena, C.D.; Alvarez, M.C.; Caldiz, C.; Pierce, G.N.; Portiansky, E.L.; Chiappe de Cingolani, G.E.; Camilion de Hurtado, M.C. Endothelin-1 induced hypertrophic effect in neonatal rat cardiomyocytes: Involvement of Na⁺/H⁺ and Na⁺/Ca²⁺ exchangers. J. Mol. Cell. Cardiol. 2006, 41, 807–815. [CrossRef]

66. Hunte, C.; Scerpanti, E.; Venturi, M.; Rimon, A.; Padan, E.; Michel, H. Structure of a Na⁺/H⁺ antiporter and insights into mechanism of action and regulation by ph. Nature 2005, 435, 1197–1202. [CrossRef]

67. Lee, C.; Kang, H.J.; von Ballmoos, C.; Newstead, S.; Uzdavinys, P.; Dotson, D.L.; IWata, S.; Beckstein, O.; Cameron, A.D.; Drew, D. A two-domain elevator mechanism for sodium/proton antiporter. Nature 2013, 501, 573–577. [CrossRef]

68. Paulino, C.; Kuhlbrandt, W. Ph- and sodium-induced changes in a sodium/proton antiporter. eLife 2014, 3, e01412. [CrossRef]

69. Wohler, D.; Kuhlbrandt, W.; Yildiz, O. Structure and substrate ion binding in the sodium/proton antiporter panhap. eLife 2014, 3, e03579. [CrossRef]

70. Dutta, D.; Fliegel, L. Structure and function of yeast and fungal Na⁺/H⁺ antiporters. IUBMB Life 2018, 70, 23–31. [CrossRef]

71. Padan, E. Functional and structural dynamics of nhaa, a prototype for Na⁺ and H⁺ antiporters, which are responsible for Na⁺ and H⁺ homeostasis in cells. Biochim. Biophys. Acta 2014, 1837, 1047–1062. [CrossRef]

72. Goswami, P.; Paulino, C.; Hizlan, D.; Vonck, J.; Yildiz, O.; Kuhlbrandt, W. Structure of the archaeal Na⁺/H⁺ antiporter nhap1 and functional role of transmembrane helix 1. EMBO J. 2011, 30, 439–449. [CrossRef]

73. Padan, E.; Danieli, T.; Keren, Y.; Alkoby, D.; Masrati, G.; Haliloglu, T.; Ben-Tal, N.; Rimon, A. Nhaa antiporter functions using 10 helices, and an additional 2 contribute to assembly/stability. Proc. Natl. Acad. Sci. USA 2015, 112, E5575–E5582. [CrossRef]
89. Karmazyn, M.; Mohamed, I.A.; Alemrayat, B.; Al-Sulaiti, F.; Mlih, M.; Mraiche, F. Novel structure of the Na+/H+ exchanger isoform 1. J. Biol. Chem. 2000, 275, 7942–7949. [CrossRef]

76. Landau, M.; Herz, K.; Padan, E.; Ben-Tal, N. Model structure of the Na+/H+ exchanger 1 (Nhe1): Functional and clinical implications. J. Biol. Chem. 2007, 282, 37854–37863. [CrossRef]

75. Wakabayashi, S.; Pang, T.; Su, X.; Shigekawa, M. A novel topology model of the human Na+/H+ exchanger isoform 1. J. Biol. Chem. 2000, 275, 7942–7949. [CrossRef]

74. Ullah, A.; Dutta, D.; Fliegel, L. Expression and characterization of the sos1 arabidopsis salt tolerance protein. Mol. Cell. Biochem. 2016, 415, 133–143. [CrossRef]

81. Jaballah, M.; Mohamed, I.A.; Alemrayat, B.; Al-Sulaiti, F.; Mlih, M.; Mraiche, F. Novel structure of the Na+/H+ exchanger isoform 1. J. Biol. Chem. 2000, 275, 7942–7949. [CrossRef]

79. Dutta, D.; Fliegel, L. Molecular modeling and inhibitor docking analysis of the Na+/H+ exchanger isoform one. Biochem. Cell Biol. 2019, in press. [CrossRef]

80. Orlowski, J.; Shull, G. Characteristics of the plasma membrane Na+/H+ exchanger isoform 1. J. Biol. Chem. 1997, 272, 2385–2393. [CrossRef]

83. Mraiche, F.; Fliegel, L.; Gadeau, A.P.; Fliegel, L.; Lopaschuk, G.; Mlih, M.; Abdulrahman, N.; Fillmore, N.; Mraiche, F. Elevated expression of activated Na+/H+ exchanger protein induces hypertrophy in isolated rat neonatal ventricular cardiomyocytes. Mol. Cell. Biochem. 2015, 290, 18173–18186. [CrossRef] [PubMed]

77. Liu, Y.; Basu, A.; Li, X.; Fliegel, L. Topological analysis of the Na+/H+ exchanger. Int. J. Mol. Sci. 2019, 20, 2378. [CrossRef]

82. Mohamed, I.A.; Gadeau, A.P.; Fliegel, L.; Lopaschuk, G.; Mlih, M.; Abdulrahman, N.; Fillmore, N.; Mraiche, F. Elevated expression of activated Na+/H+ exchanger isoform 1 induced cardiomyocyte hypertrophy involves activation of p90 ribosomal s6 kinase. PLoS ONE 2015, 10, e0122230. [CrossRef] [PubMed]

84. Xue, J.; Mraiche, F.; Zhou, D.; Karmazyn, M.; Oka, T.; Fliegel, L.; Haddad, G.G. Elevated myocardial Na+/H+ exchanger isoform 1 activity elicits gene expression that leads to cardiac hypertrophy. Physiol. Genom. 2010, 42, 374–383. [CrossRef]

78. Jinadasa, T.; Josephson, C.B.; Boucher, A.; Orlowski, J. Determinants of cation permeation and drug sensitivity in predicted transmembrane helix 9 and adjoining exofacial re-entrant loop 5 of Na+/H+ exchanger Nhe1. J. Biol. Chem. 2015, 290, 2385–2393. [CrossRef]

85. Amith, S.R.; Wilkinson, J.M.; Baksh, S.; Fliegel, L. Na+/H+ exchanger (Nhe1 isoform) in response to growth factors. J. Biol. Chem. 2007, 282, 37854–37863. [CrossRef]

72. Dutta, D.; Fliegel, L. Molecular modeling and inhibitor docking analysis of the Na+/H+ exchanger isoform one. Biochem. Cell Biol. 2019, in press. [CrossRef]

86. Amith, S.R.; Wilkinson, J.M.; Fliegel, L. Na+/H+ exchanger (Nhe1 isoform) in response to growth factors. J. Biol. Chem. 2007, 282, 37854–37863. [CrossRef] [PubMed]

73. Landau, M.; Herz, K.; Padan, E.; Ben-Tal, N. Model structure of the Na+/H+ exchanger 1 (Nhe1): Functional and clinical implications. J. Biol. Chem. 2007, 282, 37854–37863. [CrossRef]

71. Liu, Y.; Basu, A.; Li, X.; Fliegel, L. Topological analysis of the Na+/H+ exchanger. Int. J. Mol. Sci. 2019, 20, 2378. [CrossRef] [PubMed]

87. Avkiran, M.; Cook, A.R.; Cuello, F. Targeting Na+/H+ exchanger regulation for cardiac protection: A rsky approach? Curr. Opin. Pharmacol. 2008, 8, 133–140. [CrossRef] [PubMed]

Karmazyn, M. Nhe-1: Still a viable therapeutic target. J. Mol. Cell. Cardiol. 2013, 61, 77–82. [CrossRef]

Karmazyn, M.; Moffat, M.P. Role of Na+/H+ exchange in cardiac physiology and pathophysiology: Mediation of myocardial reperfusion injury by the ph paradox. Cardiovasc. Res. 1993, 27, 915–924. [CrossRef] [PubMed]

Haworth, R.S.; Avkiran, M. Receptor-mediated regulation of the cardiac sarcolemmal Na+/H+ exchanger. Mechanisms and (patho)physiological significance. In The Na+/H+ Exchanger, From Molecular to Its Role in Disease; Karmazyn, M., Avkiran, M., Fliegel, L., Eds.; Kluwer Academic Publishers: Boston, MA, USA; Dordrecht, The Netherlands; London, UK, 2003; pp. 191–209.

Bianchini, L.; L’Allemain, G.; Pouyssegur, J. The p42/44 mitogen-activated protein kinase cascade determinant in mediating activation of the Na+/H+ exchanger (Nhe1 isoform) in response to growth factors. J. Biol. Chem. 1997, 272, 271–279. [CrossRef]

Phan, V.N.; Kusuhara, M.; Lucchesi, P.A.; Berk, B.C. A 90kd Na+/H+ exchanger kinase has increased activity in spontaneously hypertensive rat vascular smooth muscle cells. Circ. Res. 1997, 29, 1265–1272.

Sardet, C.; Counillon, L.; Franchi, A.; Pouyssegur, J. Growth factors induce phosphorylation of the Na+/H+ antiporter, glycoprotein of 110 kd. Science 1990, 247, 723–726. [CrossRef] [PubMed]

Snabaitis, A.K.; Yokoyama, H.; Avkiran, M. Roles of mitogen-activated protein kinases and protein kinase c in a1α-adrenoreceptor-mediated stimulation of the sarcolemmal Na+/H+ exchanger. Circ. Res. 2000, 86, 214–220. [CrossRef]

Tominaga, T.; Ishizaki, T.; Narumiya, S.; Barber, D.L. P160rock mediates rhoa activation of Na/H exchange. EMBO J. 1998, 17, 4712–4722. [CrossRef] [PubMed]
96. Takahashi, E.; Abe, J.-i.; Berk, B.C. Angiotensin II stimulates p90rsk in vascular smooth muscle cells: A potential Na+/H+ exchanger kinase. *Hypertension* 1997, 29, 1265–1272.

97. Fliegel, L.; Karmazyn, M. The cardiac Na-H exchanger: A key downstream mediator for the cellular hypertrophic effects of paracrine, autocrine and hormonal factors. *Biochem. Cell Biol.* 2004, 82, 626–635. [CrossRef] [PubMed]

98. Wakabayashi, S.; Bertrand, B.; Shigekawa, M.; Fafournoux, P.; Pouyssegur, J. Growth factor activation and “H+”-sensing” of the Na+/H+ exchanger isoform 1 (Nhe1). *J. Biol. Chem.* 1994, 269, 5583–5588.

99. Bertrand, B.; Wakabayashi, S.; Ikeda, T.; Pouyssegur, J.; Shigekawa, M. The Na+/H+ exchanger isoform 1 (Nhe1) is a novel member of the calmodulin-binding proteins. *J. Biol. Chem.* 1994, 269, 13703–13709. [PubMed]

100. Li, X.; Alvarez, B.; Casey, J.R.; Reithmeier, R.A.; Fliegel, L. Carbonic anhydrase II binds to and enhances activity of the Na+/H+ exchanger isoform 1 (Nhe1). *Mol. Pharmacol.* 2009, 76, 43771–43777. [CrossRef] [PubMed]

101. Pang, T.; Wakabayashi, S.; Shigekawa, M. Expression of calcineurin b homologous protein 2 protects serum deprivation-induced cell death by serum-independent activation of Na+/H+ exchanger. *J. Biol. Chem.* 2002, 277, 43771–43777. [CrossRef] [PubMed]

102. Lin, X.; Barber, D.L. A calcineurin homologous protein inhibits gtpase-stimulated Na/H exchange. *J. Biol. Chem.* 1996, 271, 12631–12636. [CrossRef] [PubMed]

103. Li, X.; Liu, Y.; Kay, C.M.; Muller-Esterl, W.; Fliegel, L. The Na+/H+ exchanger cytoplasmic tail: Structure, function, and interactions with tescalcin. *Biochemistry* 2003, 42, 7448–7456. [CrossRef] [PubMed]

104. Mailander, J.; Muller-Esterl, W.; Dedio, J. Human homolog of mouse tescalcin associates with Na+/H+ exchanger type-1. *FEBS Lett.* 2001, 507, 331–335. [CrossRef]

105. Li, X.; Liu, Y.; Kay, C.M.; Muller-Esterl, W.; Fliegel, L. The Na+/H+ exchanger isoform 1 (Nhe1). *J. Biol. Chem.* 2002, 277, 36085–36091. [CrossRef]

106. Lin, X.; Alvarez, B.; Casey, J.R.; Reithmeier, R.A.; Fliegel, L. Carbonic anhydrase II binds to and enhances activity of the Na+/H+ exchanger. *J. Biol. Chem.* 2002, 277, 36085–36091. [CrossRef]

107. Li, X.; Alvarez, B.; Casey, J.R.; Reithmeier, R.A.; Fliegel, L. Carbonic anhydrase II binds to and enhances activity of the Na+/H+ exchanger. *J. Biol. Chem.* 2002, 277, 36085–36091. [CrossRef]

108. Vaheri, A.; Carpen, O.; Heiska, L.; Helander, T.S.; Jaaskelainen, J.; Majander-Nordenswan, P.; Sainio, M.; Timonen, T.; Turunen, O. The ezrin protein family: Membrane-cytoskeleton interactions and disease associations. *Curr. Opin. Cell. Biol.* 1997, 9, 659–666.

109. Silva, N.L.C.L.; Haworth, R.S.; Singh, D.; Fliegel, L. The carboxyl-terminal region of the Na+/H+ exchanger interacts with mammalian heat shock protein. *Biochemistry* 1995, 34, 10412–10420. [CrossRef]

110. Odunewu-Aderibigbe, A.; Fliegel, L. Protein mediated regulation of the Nhe1 isoform of the Na+/H+ exchanger in renal cells. A regulatory role of hsp90 and akt kinase. *Cell Signal.* 2017, 36, 145–153. [CrossRef] [PubMed]

111. Odunewu-Aderibigbe, A.; Fliegel, L. Heat shock proteins and the Na+/H+ exchanger. *Channels (Austin)* 2017, 11, 380–382. [CrossRef]

112. Xue, J.; Zhou, D.; Yao, H.; Gavrialov, O.; McConnell, M.J.; Gelb, B.D.; Haddad, G.G. Novel functional interaction between Na+/H+ exchanger 1 and tyrosine phosphatase shp-2. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2007, 292, R2406–R2416. [CrossRef]

113. Aharonovitz, O.; Zaun, H.C.; Balla, T.; York, J.D.; Orlowski, J.; Grinstein, S. Intracellular ph regulation by Na+/H+ exchange requires phosphatidylinositol 4,5-bisphosphate. *J. Cell Biol.* 2000, 150, 213–224. [CrossRef]

114. Aharonovitz, O.; Demaurex, N.; Woodside, M.; Grinstein, S. Atp dependence is not an intrinsic property of Na+/H+ exchanger Nhe1: Requirement for an ancillary factor. *Am. J. Physiol.* 1999, 276, C1303–C1311. [CrossRef]

115. Shimada-Shimizu, N.; Hisamitsu, T.; Nakamura, T.Y.; Hirayama, N.; Wakabayashi, S. Na+/H+ exchanger 1 is regulated via its lipid-interacting domain, which functions as a molecular switch: A pharmacological approach using indolocarbazole compounds. *Mol. Pharmacol.* 2014, 85, 18–28. [CrossRef]
117. Wakabayashi, S.; Nakamura, T.Y.; Kobayashi, S.; Hisamitsu, T. Novel phorbol ester-binding motif mediates hormonal activation of Na⁺/H⁺ exchanger. J. Biol. Chem. 2010, 285, 26652–26661. [CrossRef]

118. Fliegel, L. Molecular biology of the myocardial Na⁺/H⁺ exchanger. J. Mol. Cell. Cardiol. 2008, 44, 228–237. [CrossRef] [PubMed]

119. Oduewu-Aderibigbe, A.; Fliegel, L. The Na⁺/H⁺ exchanger and ph regulation in the heart. IUBMB Life 2014, 66, 679–685. [CrossRef] [PubMed]

120. Karki, P.; Li, X.; Schrama, D.; Fliegel, L. B-Raf is associated with and activates the Nhe1 isoform of the Na⁺/H⁺ exchanger. J. Biol. Chem. 2011, 286, 13096–13105. [CrossRef] [PubMed]

121. Meima, M.E.; Webb, B.A.; Witkowska, H.E.; Barber, D.L. The sodium-hydrogen exchanger Nhe1 is an akt substrate necessary for actin filament reorganization by growth factors. J. Biol. Chem. 2009, 284, 26666–26675. [CrossRef] [PubMed]

122. Yoon, S.; Seger, R. The extracellular signal-regulated kinase: Multiple substrates regulate diverse cellular functions. Growth Factors 2006, 24, 21–44. [CrossRef] [PubMed]

123. Kolch, W. Coordinating erk/mapk signalling through scaffolds and inhibitors. Nat. Rev. Mol. Cell Biol. 2005, 6, 827–837. [CrossRef]

124. Amith, S.R.; Fong, S.; Baksh, S.; Fliegel, L. Na⁺/H⁺ exchange in the tumour microenvironment: Does nhe1 drive breast cancer carcinogenesis? Int. J. Dev. Biol. 2015, 59, 367–377. [CrossRef] [PubMed]

125. Wakabayashi, S.; Nakamura, T.Y.; Kobayashi, S.; Hisamitsu, T. Novel phorbol ester-binding motif mediates hormonal activation of Na⁺/H⁺ exchanger. J. Biol. Chem. 2010, 285, 26652–26661. [CrossRef]

126. Sanhueza, C.; Araos, J.; Naranjo, L.; Toledo, F.; Beltran, A.R.; Ramirez, M.A.; Gutierrez, J.; Pardo, F.; Leiva, A.; Sobrevia, L. Sodium/proton exchanger isoform 1 regulates intracellular pH and cell proliferation in human ovarian cancer. Biochim. Biophys. Acta 2017, 1863, 81–91. [CrossRef] [PubMed]

127. Steffan, J.J.; Snider, J.L.; Skalli, O.; Welbourne, T.; Cardelli, J.A. Na⁺/H⁺ exchangers and rhoa regulate acidic extracellular ph-induced lysosome trafficking in prostate cancer cells. Traffic 2009, 10, 737–753. [CrossRef] [PubMed]

128. Shi, X.; O’Neill, M.M.; MacDonnell, S.; Brookes, P.S.; Yan, C.; Berk, B.C. The rsk inhibitor bix02565 limits cardiac ischemia/reperfusion injury. J. Cardiovasc. Pharmacol. Ther. 2016, 21, 177–186. [CrossRef]

129. Lin, Y.; Chang, G.; Wang, J.; Jin, W.; Wang, L.; Li, H.; Ma, L.; Li, Q.; Pang, T. Nhe1 mediates mda-mb-231 cells invasion through the regulation of mt1-mmp. Exp. Cell Res. 2011, 317, 2031–2040. [CrossRef] [PubMed]

130. Wang, H.; Silva, N.L.C.L.; Lucchesi, P.A.; Haworth, R.; Wang, K.; Michalak, M.; Pelech, S.; Fliegel, L. Phosphorylation and regulation of the Na⁺/H⁺ exchanger through mitogen-activated protein kinase. Biochemistry 1997, 36, 9151–9158. [CrossRef] [PubMed]

131. Haworth, R.S.; Dashnyam, S.; Avkiran, M. Ras triggers acidosis-induced activation of the extracellular-signal-regulated kinase pathway in cardiac myocytes. Biochem. J. 2006, 399, 493–501. [CrossRef]

132. Sabri, A.; Byron, K.L.; Samarei, A.M.; Bell, J.; Lucchesi, P.A. Hydrogen peroxide activates mitogen-activated protein kinases and Na⁺/H⁺ exchange in neonatal rat cardiac myocytes. Circ. Res. 1998, 82, 1053–1062. [CrossRef]

133. Wei, S.; Rothstein, E.C.; Fliegel, L.; Dell’Italia, L.J.; Lucchesi, P.A. Differential map kinase activation and Na⁺/H⁺ exchanger phosphorylation by H(2)O(2) in rat cardiac myocytes. Am. J. Physiol. 2001, 281, C1542–C1550. [CrossRef]

134. Rothstein, E.C.; Byron, K.L.; Reed, R.E.; Fliegel, L.; Lucchesi, P.A. H₂O₂-induced Ca²⁺ overload in nrvm involves erk1/2 map kinases: Role for an nhe-1-dependent pathway. Am. J. Physiol. Heart Circ. Physiol. 2002, 283, H598–H605. [CrossRef]

135. Lehoux, S.; Abe, J.; Florian, J.A.; Berk, B.C. 14-3-3 binding to Na⁺/H⁺ exchanger isoform-1 is associated with serum-dependent activation of Na⁺/H⁺ exchange. J. Biol. Chem. 2001, 276, 15794–15800. [CrossRef]

136. Maekawa, N.; Abe, J.; Shishido, T.; Itoh, S.; Ding, B.; Sharma, V.K.; Sheu, S.S.; Blaxall, B.C.; Berk, B.C. Inhibiting p90 ribosomal s6 kinase prevents (Na⁺)⁺H⁺ exchanger-mediated cardiac ischemia-reperfusion injury. Circulation 2006, 113, 2516–2523. [CrossRef]

137. Coccaro, E.; Karki, P.; Cojocaru, C.; Fliegel, L. Phenylephrine and sustained acidosis activate the neonatal rat cardiomyocyte Na⁺/H⁺ exchanger through phosphorylation of amino acids ser770 and ser771. Am. J. Physiol. Heart Circ. Physiol. 2009, 297, H846–H858. [CrossRef]
138. Stupak, J.; Liu, H.; Wang, Z.; Brix, B.J.; Fliegel, L.; Li, L. Nanoliter sample handling combined with microspot maldi-ms for detection of gel-separated phosphoproteins. *J. Proteome Res.* 2005, 4, 515–522. [CrossRef]

139. Liu, H.; Stupak, J.; Zheng, J.; Keller, B.O.; Brix, B.J.; Fliegel, L.; Li, L. Open tubular immobilized metal–ion affinity chromatography combined with maldi ms and ms/ms for identification of protein phosphorylation sites. *Anal. Chem.* 2004, 76, 4223–4232. [CrossRef]

140. Malo, M.E.; Li, L.; Fliegel, L. Mitogen-activated protein kinase-dependent activation of the Na+/H+ exchanger is mediated through phosphorylation of amino acids ser770 and ser771. *J. Biol. Chem.* 2007, 282, 6292–6299. [CrossRef]

141. Karki, P.; Coccaro, E.; Fliegel, L. Sustained intracellular acidosis activates the myocardial Na+/H+ exchanger independent of amino acid ser703 and p90sk. *Biochim. Biophys. Acta* 2010, 1798, 1565–1576. [CrossRef]

142. Hendus-Altenburger, R.; Pedraz-Cuesta, E.; Olesen, C.W.; Papaleo, E.; Schnell, J.A.; Hopper, J.T.; Robinson, C.V.; Pedersen, S.F.; Kragelund, B.B. The human Na+/H+ exchanger 1 is a membrane scaffold protein for extracellular signal-regulated kinase 2. *BMC Biol.* 2016, 14, 31. [CrossRef]

143. Li, X.; Khan, M.F.; Schiønner, D.C.; Fliegel, L. Structural changes in the c-terminal regulatory region of the Na+/H+ exchanger mediate phosphorylation induced regulation. *J. Mol. Cell. Cardiol.* 2013, 61, 153–163. [CrossRef]

144. Fliegel, L. Beta-raf activation of the myocardial Na+/H+ exchanger. *Channels (Austin)* 2017, 11, 181–182. [CrossRef]

145. Hendus-Altenburger, R.; Lambrughri, M.; Torkelsen, T.; Pedersen, S.F.; Papaleo, E.; Lindorff-Larsen, K.; Kragelund, B.B. A phosphorylation-motif for tuneable helix stabilisation in intrinsically disordered proteins - lessons from the sodium proton exchanger 1 (nhe1). *Cell Signal.* 2017, 37, 40–51. [CrossRef]

146. Chang, G.; Wang, J.; Zhang, H.; Zhang, Y.; Wang, C.; Xu, H.; Zhang, H.; Lin, Y.; Ma, L.; Li, Q.; et al. Cd44 targets Na/H exchanger 1 to mediate mda-mb-231 cells' metastasis via the regulation of erk1/2. *Br. J. Cancer* 2014, 110, 916–927. [CrossRef] [PubMed]

147. Cardone, R.A.; Bellizzi, A.; Busco, G.; Weinman, E.J.; Dell’aquila, M.E.; Casavola, V.; Azzariti, A.; Mangia, A.; Paradiso, A.; Reshkin, S.J. The nherf1 pdz2 domain regulates pka-roha-p38-mediated Nhe1 activation and invasion in breast tumor cells. *Mol. Biol. Cell.* 2007, 18, 1768–1780. [CrossRef] [PubMed]

148. Reshkin, S.J.; Greco, M.R.; Cardone, R.A. Role of pH, and proton transporters in oncogene-driven neoplastic transformation. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* 2014, 369, 20130100. [CrossRef] [PubMed]

149. Spugnini, E.P.; Sonveaux, P.; Stock, C.; Perez-Sayans, M.; De Milito, A.; Avnet, S.; Garcia, A.G.; Harguindey, S.; Fais, S. Proton channels and exchangers in cancer. *Biochim. Biophys. Acta* 2015, 1848, 2715–2726. [CrossRef]

150. Reshkin, S.J.; Cardone, R.A.; Harguindey, S. Na+/H+ exchanger, pH regulation and cancer. *Recent Pat. Anticancer Drug Discov.* 2013, 8, 89–95. [CrossRef]

151. Brisson, L.; Drifort, V.; Benoist, L.; Poet, M.; Cournillon, L.; Antelmi, E.; Rubino, R.; Besson, P.; Labbal, F.; Chevalier, S.; et al. Nav1.5 Na+ channels allosterically regulate the Nhe-1 exchanger and promote the activity of breast cancer cell invadopodia. *J. Cell Sci.* 2013, 126, 4835–4842. [CrossRef]

152. Daniel, C.; Bell, C.; Burton, C.; Harguindey, S.; Reshkin, S.J.; Rauch, C. The role of proton dynamics in the development and maintenance of multidrug resistance in cancer. *Biochim. Biophys. Acta* 2013, 1832, 606–617. [CrossRef]

153. Greco, M.R.; Antelmi, E.; Busco, G.; Guerra, L.; Rubino, R.; Casavola, V.; Reshkin, S.J.; Cardone, R.A. Protease activity at invadopodial foci digestive areas is dependent on nhe1-driven acidic pH. *Oncol. Rep.* 2014, 31, 940–946. [CrossRef] [PubMed]

154. Busco, G.; Cardone, R.A.; Greco, M.R.; Bellizzi, A.; Colella, M.; Antelmi, E.; Mancini, M.T.; Dell’Aquila, M.E.; Casavola, V.; Paradiso, A.; et al. Nhe1 promotes invadopodial ecm proteolysis through acidification of the peri-invadopodial space. *FASEB J.* 2010, 24, 3903–3915. [CrossRef]

155. Stock, C.; Cardone, R.A.; Busco, G.; Krahling, H.; Schwab, A.; Reshkin, S.J. Protons extruded by Nhe1: Digestive or glue? *Eur. J. Cell Biol.* 2008, 87, 591–599. [CrossRef] [PubMed]

156. Amith, S.R.; Vincent, K.M.; Wilkinson, J.M.; Postovit, L.M.; Fliegel, L. Defining the Na+/H+ exchanger nhe1 interactome in triple-negative breast cancer cells. *Cell Signal.* 2017, 29, 69–77. [CrossRef] [PubMed]

157. Lauritzen, G.; Stock, C.M.; Lemaire, J.; Lund, S.F.; Jensen, M.E.; Damsgaard, B.; Petersen, K.S.; Wiwel, M.; Ronnov-Jessen, L.; Schwab, A.; et al. The Na+/H+ exchanger nhe1, but not the Na+, HCO3(-) cotransporter nbcn1, regulates motility of mcf7 breast cancer cells expressing constitutively active erbB2. *Cancer Lett.* 2012, 317, 172–183. [CrossRef] [PubMed]
158. Bandyopadhyay, S.; Chiang, C.Y.; Srivastava, J.; Gersten, M.; White, S.; Bell, R.; Kurschner, C.; Martin, C.H.; Smoot, M.; Sahasrabudhe, S.; et al. A human MAP kinase interactome. Nat. Methods 2010, 7, 801–805. [CrossRef] [PubMed]

159. Baumgartner, M.; Patel, H.; Barber, D.L. Na+/H+ exchanger Nhe1 as plasma membrane scaffold in the assembly of signaling complexes. Am. J. Physiol. 2004, 287, C844–C850. [CrossRef]

160. Tompa, P.; Fuxreiter, M. Fuzzy complexes: Polymorphism and structural disorder in protein-protein interactions. Trends Biochem. Sci. 2008, 33, 2–8. [CrossRef] [PubMed]

161. Fuxreiter, M.; Tompa, P.; Simon, I. Local structural disorder imparts plasticity on linear motifs. Bioinformatics (Oxford) 2007, 23, 950–956. [CrossRef]

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