

## Physiological role and regulation of the Na<sup>+</sup>/H<sup>+</sup> exchanger

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**Abstract:** In mammalian eukaryotic cells, the Na<sup>+</sup>/H<sup>+</sup> exchanger is a family of membrane proteins that regulates ions fluxes across membranes. Plasma membrane isoforms of this protein extrude 1 intracellular proton in exchange for 1 extracellular sodium. The family of Na<sup>+</sup>/H<sup>+</sup> exchangers (NHEs) consists of 9 known isoforms, NHE1–NHE9. The NHE1 isoform was the first discovered, is the best characterized, and exists on the plasma membrane of all mammalian cells. It contains an N-terminal 500 amino acid membrane domain that transports ions, plus a 315 amino acid C-terminal, the intracellular regulatory domain. The Na<sup>+</sup>/H<sup>+</sup> exchanger is regulated by both post-translational modifications including protein kinase-mediated phosphorylation, plus by a number of regulatory-binding proteins including phosphatidylinositol-4,5-bisphosphate, calcineurin homologous protein, ezrin, radixin and moesin, calmodulin, carbonic anhydrase II, and tescalcin. The Na<sup>+</sup>/H<sup>+</sup> exchanger is involved in a variety of complex physiological and pathological events that include regulation of intracellular pH, cell movement, heart disease, and cancer. This review summarizes recent advances in the understanding of the physiological role and regulation of this protein.

*Key words:* Na<sup>+</sup>/H<sup>+</sup> exchanger, pH regulation, membrane proteins, phosphorylation, ischemia, heart hypertrophy.

**Résumé :** Dans les cellules eucaryotes des mammifères, l'échangeur Na<sup>+</sup>/H<sup>+</sup> est une famille de protéines membranaires qui régule les flux ioniques à travers les membranes. Les isoformes de cette protéine sur la membrane plasmique expulsent un proton intracellulaire en échange de sodium extracellulaire. La famille d'échangeurs Na<sup>+</sup>/H<sup>+</sup> (NHE) est composée de neuf isoformes connues, NHE1–NHE9. La première à avoir été découverte, NHE1, est la mieux caractérisée et est présente sur la membrane plasmique de toutes les cellules de mammifères. Elle contient un domaine membranaire de 500 acides aminés en position N-terminale qui transporte les ions et un domaine régulateur intracellulaire de 315 acides aminés en position C-terminale. L'échangeur Na<sup>+</sup>/H<sup>+</sup> est régulé par des modifications post-translationnelles, y compris la phosphorylation véhiculée par les protéines kinases, et par plusieurs protéines de liaison régulatrices telles que le phosphatidylinositol-4,5-bisphosphate, la protéine homologue de la calcineurine, l'ezrine, la radixine et la moesine, la calmoduline, l'anhydrase carbonique II et la tescalcine. L'échangeur Na<sup>+</sup>/H<sup>+</sup> participe à divers événements pathologiques et physiologiques complexes, comme la régulation du pH intracellulaire, le mouvement des cellules, la maladie cardiaque et le cancer. La présente synthèse résume les récentes avancées dans la compréhension du rôle physiologique et de la régulation de cette protéine.

*Mots clés :* échangeur Na<sup>+</sup>/H<sup>+</sup>, régulation du pH, protéines membranaires, phosphorylation, ischémie, hypertrophie cardiaque.

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### Introduction

#### Intracellular pH in mammalian cells

Intracellular pH (pH<sub>i</sub>) is finely controlled in mammalian cells as many basic systems depend on it. Proteins require a specific pH for optimal functioning, normal and pathological gene expression is regulated by pH<sub>i</sub>, and ion concentrations are affected since many channels and pumps are involved in

H<sup>+</sup> transport and are affected by pH<sub>i</sub> (Ives and Rector 1984; Lagarde and Pouyssegur 1986; Putney and Barber 2004). To regulate pH<sub>i</sub> mammalian cells express a number of proteins at their plasma membrane that transport H<sup>+</sup> and HCO<sub>3</sub><sup>-</sup> into and out of the cell (Puceat 1999). Important participants in the precise system of pH<sub>i</sub> regulation include the Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE) family of proteins and various bicarbonate transporters and exchangers.

Proton efflux is a major factor in pH<sub>i</sub> maintenance as normal cellular metabolism generates an intracellular acid load. NHE proteins are activated by acidic cellular conditions to catalyze the electroneutral exchange of 1 extracellular sodium ion for 1 intracellular proton and as such, constitute a key component in the protection against cellular acidosis. This exchange process is dependent on extracellular Na<sup>+</sup>

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and ATP, although the protein does not directly consume metabolic energy (Aharonovitz et al. 2000; Orłowski and Grinstein 2004). The family of NHE proteins includes 9 isoforms, each with unique tissue and cellular distribution, inhibitor sensitivities, regulatory elements, and ensuing physiological roles. NHE1, the first isoform identified, is distinct in that it is ubiquitously expressed in all mammalian cells and plays a housekeeping role.

## The Na<sup>+</sup>/H<sup>+</sup> exchanger family

### NHE1

As early as 1982, Pouyssegur et al. had identified a "Na<sup>+</sup>/H<sup>+</sup> exchange system" that played an important role in regulation of pH<sub>i</sub> and had a major influence on growth factor action (Pouyssegur et al. 1982). Further studies identified biochemical characteristics of this system, for example, the addition of external Na<sup>+</sup> to a cell caused the release of internal H<sup>+</sup> with a 1:1 stoichiometry. The exchange of sodium and protons was also amiloride sensitive, and this exchange system existed on the plasma membrane of cells (Paris and Pouyssegur 1983). In 1984, Pouyssegur and his group developed an H<sup>+</sup>-suicide technique that allowed them to select for a cell line deficient in Na<sup>+</sup>/H<sup>+</sup> exchange activity. This proved to be a useful model for identifying the actual roles of this exchanger (Pouyssegur et al. 1984).

In 1989, Sardet et al. cloned the first Na<sup>+</sup>/H<sup>+</sup> exchanger isoform, NHE1, and based on the cDNA sequence, the amino acid sequence was predicted (Sardet et al. 1989). Since the initial cloning of NHE1, many studies have looked at protein expression and localization, mechanistic and regulatory features, and physiological roles for the protein. NHE1 is ubiquitously expressed in all mammalian cells and is considered the "housekeeping" isoform. After its initial discovery, NHE1 was also identified as the predominant sarcolemmal isoform expressed in the myocardium (Fliegel et al. 1991), although as stated above, its expression is not limited to the myocardium. In addition to the protein being expressed ubiquitously in mammalian cells, NHE1 cellular localization also varies depending on the cell type. For fibroblasts in which NHE1 is involved in migration and anchoring, the protein is predominantly localized in lamellipodia (Denker and Barber 2002; Denker et al. 2000). NHE1 is distributed to the basolateral membrane of epithelial cells (Biemesderfer et al. 1992; Orłowski and Grinstein 2004) and within myocardial tissue it is concentrated in the intercalated disks and along the transverse tubular system (Petrecca et al. 1999).

NHE1 is the isoform most sensitive to amiloride and its derivatives (Masereel et al. 2003). Amiloride was the first drug identified as an NHE inhibitor although it does have an inhibitory effect on various other channels and exchangers. Amiloride is thought to bind to the external Na<sup>+</sup> binding site since inhibitory potency is reduced in high Na<sup>+</sup> concentrations (Masereel et al. 2003). There have since been a number of drugs developed that are more selective for NHE1 including the benzoylguanidine inhibitors. This group of inhibitors includes HOE694, cariporide, and eniporide; the latter 2 have been studied in the clinical setting (Theroux et al. 2000; Zeymer et al. 2001).

The physiological roles of NHE1 have been thoroughly studied, and have proven to be diverse. The most basic role of NHE1 is in pH<sub>i</sub> and cell volume control, but these factors can have wide ranging implications on numerous cellular processes. NHE1 is involved in normal processes such as cell proliferation, growth, migration, and apoptosis and pathological processes such as cancer cell invasion and heart failure (Harguindey et al. 2005; Karmazyn et al. 2005).

### NHE2–NHE9

Rat NHE2 was first identified as having an amino acid sequence that shared 42% identity with NHE1 (Wang et al. 1993), but since that discovery, in depth studies on the human protein have provided us with a more thorough understanding of this isoform (Malakooti et al. 1999). NHE2 is an apical membrane protein that is expressed in the epithelial tissue of the intestinal tract and kidney, skeletal muscle, and the testis (Malakooti et al. 1999). Within the digestive tract, NHE2 is quite prominent with strong expression in the jejunum, ileum, and colon (Bookstein et al. 1997). Specific expression in renal tissue has been localized to the cortical thick ascending limbs, distal convoluted tubules, connecting tubules, and the macula densa cells (Chambrey et al. 1998; Peti-Peterdi et al. 2000). This isoform has similar inhibition constants for both amiloride and nonamiloride compounds as that observed for NHE1 (Yu et al. 1993). NHE2 is expressed in the parietal cells of the gastric epithelium, and the *Nhe2*<sup>-/-</sup> mouse shows that although NHE2 is not required for acid secretion by these cells, it is essential for their long-term viability (Schultheis et al. 1998). Overall, NHE2 appears to be involved in several secretory processes.

The Na<sup>+</sup>/H<sup>+</sup> exchanger isoforms 3 and 4 were both identified in 1992 and were found to have 39% and 42% amino acid identity to NHE1, respectively (Orłowski et al. 1992). Furthermore, both NHE3 and NHE4 were identified as having significant levels of expression in the kidney and gastrointestinal tract. In the gastrointestinal tract, NHE3 has higher expression in the intestine, whereas NHE4 appears to be localized predominantly in the stomach. In renal tissue, NHE3 has been identified as an apical membrane protein in the proximal tubule and thick ascending limb (Amemiya et al. 1995), whereas the NHE4 isoform has basolateral epithelial distribution and is found specifically in the inner medulla of the kidney (Bookstein et al. 1997; Pizzonia et al. 1998). More recently, both isoforms have also been identified in the submandibular gland in rats, with NHE3 having apical expression in duct cells and NHE4 having basolateral expression in acinar and ducts cells (Oehlke et al. 2006). Both NHE3 and NHE4 are relatively resistant to amiloride and nonamiloride inhibitors (Brant et al. 1995; Yu et al. 1993). NHE4 requires hyperosmolarity for activation (Bookstein et al. 1994; Bookstein et al. 1996). Mouse knockout models of NHE3 and NHE4 show that NHE3 is the major absorptive Na<sup>+</sup>/H<sup>+</sup> exchanger in the kidney and intestine, whereas NHE4 is involved in regulating normal levels of gastric acid secretion (Gawenis et al. 2005; Nakamura et al. 1999).

Studies on NHE5 have revealed that its expression is restricted to the brain in both rats and humans. Sequence analysis demonstrated that NHE5 has 39% amino acid identity to NHE1, but also 53% identity to NHE3. In addition to having similar primary structure to NHE3, NHE5 also has

similar inhibitor sensitivities to NHE3 (Attaphitaya et al. 1999; Baird et al. 1999). Overall, NHE5's restricted expression in the brain suggests a specialized role for the isoform, but as of yet no physiological role has been elucidated.

NHE isoforms 6–9 are unique in that they are localized to the membranes of the Golgi and post-Golgi endocytic compartments, whereas NHE1–5 are localized on the plasma membrane of cells. Primarily, NHE6 is expressed in the early recycling endosomes, NHE7 in the trans-Golgi network, NHE8 in the mid- to trans-Golgi, and NHE9 in the late recycling endosomes, although there is some overlap in distribution. It is likely that these NHE isoforms contribute to pH maintenance of these intracellular organelles (Nakamura et al. 2004).

### Structural features of the Na<sup>+</sup>/H<sup>+</sup> exchanger

The human NHE1 cDNA predicts an 815 amino acid protein. The first 500 residues are predicted to be transmembrane (TM)-spanning segments and the remaining 315 residues are thought to constitute an intracellular regulatory domain (Sardet et al. 1989). Using cysteine-accessibility analysis, Wakabayashi et al. (2000) confirmed a 12TM-spanning N-terminal domain that differed from the initial Kyte–Doolittle model that had been predicted using hydrophathy analysis (Kyte and Doolittle 1982; Wakabayashi et al. 2000). Whereas, the Kyte–Doolittle model predicted 12 transmembrane regions, Wakabayashi's model also predicted 1 extracellular and 2 intracellular membrane-associated loops, and more conclusively identified the amino acids in each TM-spanning region (Fig. 1). The only region of the protein that has had its structure defined has been TM-IV. Based on high-resolution nuclear magnetic resonance (NMR) analysis, this essential TM-spanning segment is structured, but not as a traditional  $\alpha$ -helix. TM-IV is predicted to start with a series of  $\beta$ -turns, followed by an extended region, and ends as a helix (Slepkov et al. 2005).

Further analysis of the membrane domain has also identified it as being the region responsible for NHE1 quaternary structure. NHE1 forms isoform-specific homodimers *in vivo*, although the individual dimer subunits do function independently of each other (Fafournoux et al. 1994; Fliegel et al. 1993).

Circular dichroism (CD) has been a useful tool in preliminary analysis of the C-terminal tail structure. The C-terminal 315 amino acids of human NHE1 were expressed in *Escherichia coli* and purified. They were found to be approximately 35%  $\alpha$ -helix, 16%  $\beta$ -turn, and 49% random coil (Gebreselassie et al. 1998). Based on CD analysis of different protein fragments, the membrane proximal region was predicted as more compact and structured, possibly aiding in interaction with the membrane. The more distal region was flexible and unstructured, possibly allowing for structural changes associated with phosphorylation and protein interactions (Gebreselassie et al. 1998; Li et al. 2003).

The complete structure of NHE1 has not been solved by high-resolution crystallography or NMR analysis, but the structure of the *E. coli* Na<sup>+</sup>/H<sup>+</sup> antiporter NhaA has recently been published. Hunte et al. (2005) describes NhaA as having a unique fold consisting of 2 dense transmembrane domains that show weak sequence homology but prove to be

related structurally. The domains form periplasmic and cytoplasmic funnels that are not continuous in their structures, but do point towards each other. They suggest that a conformational change may open 1 tunnel to the other highlighting a possible mechanism (Hunte et al. 2005). The eukaryotic NHEs share little homology to the prokaryotic Na<sup>+</sup>/H<sup>+</sup> antiporters, but they exchange sodium for protons and are regulated by pH, so the essential features of NhaA structure and function may also apply to mammalian NHE1.

### Regulation of the NHE1 by cofactors and binding proteins

The C-terminal tail of the NHE1 has been shown to comprise the regulatory domain of the Na<sup>+</sup>/H<sup>+</sup> exchanger (Wakabayashi et al. 1992). The regulatory role the cytoplasmic domain plays is greatly influenced by its involvement with a number of binding proteins (Fig. 1) that may act to alter the configuration of the tail and its association with the membrane domain, as well as acting to convey cellular messages to the exchanger.

#### Phosphatidylinositol 4,5-bisphosphate

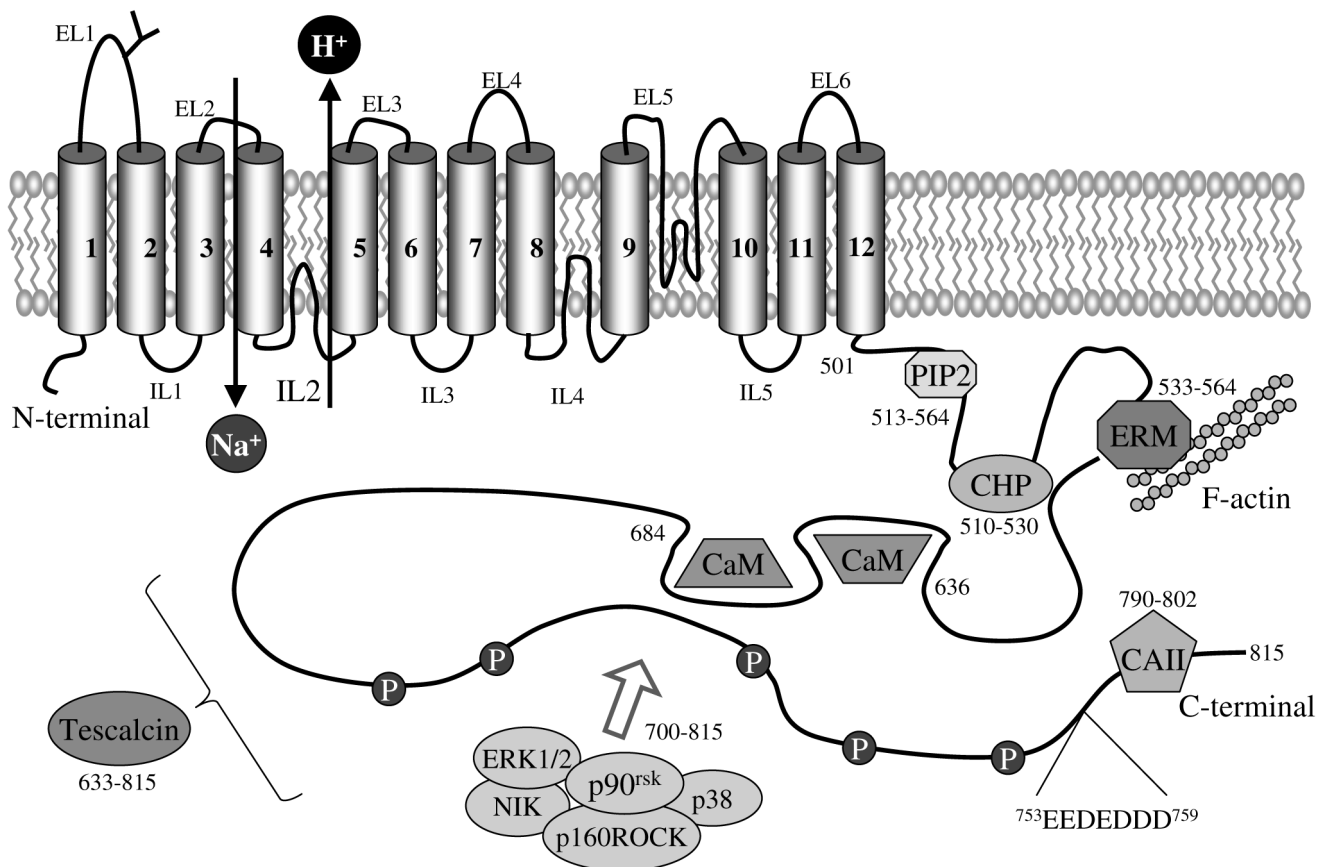
Phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) is a signaling phospholipid in eukaryotic plasma membranes. PIP<sub>2</sub> can also be converted to a number of other signaling molecules including inositol 1,4,5-triphosphate (IP<sub>3</sub>) and diacylglycerol, both of which are important 2<sup>nd</sup> messengers. The level of cellular PIP<sub>2</sub> is directly affected by the amount of available ATP since PIP<sub>2</sub> concentrations are dynamic and dependent on the activity of kinases and phosphatases (Suh and Hille 2005). It has been well documented that NHE1, although not directly consuming ATP, is affected by cellular ATP depletion (Goss et al. 1994). There are several pieces of evidence that suggest that the NHEs ATP dependence is due to an association with PIP<sub>2</sub>, the first being PIP<sub>2</sub> plasmalemmal reduction being concurrent with ATP depletion (Aharonovitz et al. 2000). Additionally, NHE1 has 2 potential PIP<sub>2</sub> binding sites, amino acids 513–520 and 556–564, and mutation of these sites reduces the Na<sup>+</sup>/H<sup>+</sup> exchange capability. Finally, PIP<sub>2</sub> can bind the C-terminal tail of NHE1 *in vitro* (Aharonovitz et al. 2000). On the whole, PIP<sub>2</sub> is an essential factor in pH<sub>i</sub> regulation by NHE1 although it is yet to be determined *in vivo* whether this is via a direct or indirect interaction.

#### Calcineurin homologous protein 1

Calcineurin homologous protein 1 (CHP1) was originally identified as an NHE1-binding protein by screening an expression library with the C-terminal domain of NHE1 (Lin and Barber 1996). It was later determined that CHP1 interacted with a hydrophobic cluster of residues between amino acids 510–530 of the NHE1 cytoplasmic domain, a region proximal to the membrane domain (Pang et al. 2001). The association between NHE1 and CHP1 has been proposed to be an essential interaction, as NHE1 or CHP1 mutants that impede binding result in a dramatic loss of Na<sup>+</sup>/H<sup>+</sup> exchange activity. Characterization of the CHP1–NHE1 association suggests that it is a Ca<sup>2+</sup>-dependent, high-affinity interaction that occurs at the plasma membrane (Pang et al. 2004). CHP1 possess 4 EF-hand motifs, which are very

**Fig. 1.** Topology of the NHE1 isoform of the  $\text{Na}^+/\text{H}^+$  exchanger and its regulatory elements. EL1–EL6, extracellular loops 1–6; IL1–IL5, intracellular loops 1–5; EEDEDDD, amino acids glutamic acid (E) and aspartic acid (D). Regulatory elements and their approximate location of binding are indicated: CaM, calmodulin; CHP, calcineurin homologous protein; ERM, ezrin/radixin/moesin proteins; CAII, carbonic anhydrase isoform II; PIP2, phosphatidylinositol biphosphate. Putative protein kinases suggested to phosphorylate the  $\text{Na}^+/\text{H}^+$  exchanger are indicated: ERK1/2, p90rsk, NIK, p160ROCK, p38. Topology was adapted from (Wakabayashi et al. 2000).

## Extracellular



## Intracellular

common calcium binding motifs, although it appears as though only EF3 and EF4 actually coordinate  $\text{Ca}^{2+}$ . Mutation of either EF3 or EF4 reduces NHE1  $\text{pH}_i$  sensitivity, and mutation of both results in poor interaction with NHE1. Additionally, NHE1 levels dictate the amount of endogenous CHP1 produced, and appear to be the main factor in recruitment of the protein to the plasma membrane (Pang et al. 2004).

CHP2, an isoform that has 61% amino acid identity to CHP1, has also been shown to interact with NHE1 (Pang et al. 2002). This protein is expressed at high levels in tumor cells and the association of CHP2 with NHE1 protects cells from serum deprivation-induced death by increasing  $\text{pH}_i$ . It is proposed that the CHP2–NHE1 association maintains the malignant state of transformed cells (Pang et al. 2002).

### Ezrin, radixin, and moesin protein family

The cytoskeleton of a cell is essential for maintaining cell shape and establishing the mechanical and chemical properties of the plasma membrane. Ezrin, radixin, and moesin (ERM) proteins form important links between the actin filaments that form the structural basis of the cytoskeleton and

integral proteins that are situated within the plasma membrane (Vaheri et al. 1997). The NHE1 C-terminal tail contains 2 ERM protein binding motifs of acidic residues within amino acids 553–564 that mediate a direct interaction between the proteins (Denker et al. 2000). This interaction is pivotal in determining the role that NHE1 plays in a number of important cellular events such as cell migration, formation of signaling complexes, and resistance to apoptosis (Denker and Barber 2002; Denker et al. 2000; Wu et al. 2004). When NHE1 and ERM physically associate, the pro-survival kinase, Akt, is activated and thus apoptosis is stalled. Additionally, association of NHE1 with ERM directs NHE1 localization in the lamellapodia of migrating cells. If this interaction is disrupted, NHE1 is not properly localized and cells develop an irregular shape and poor motility.

### Calmodulin

Calcium is an important 2<sup>nd</sup> messenger in the cell that communicates its message by associating with proteins and altering their functional state. Calmodulin (CaM) is a  $\text{Ca}^{2+}$ -binding protein that plays a major role in regulating NHE1 function in response to  $\text{Ca}^{2+}$  signaling. CaM binds  $\text{Ca}^{2+}$  and

then binds to NHE1 at 2 sites in the cytoplasmic tail: residues 636–656 constitute a high affinity site and residues 657–700 a low affinity site (Bertrand et al. 1994). The high affinity CaM binding site appears to interact with another region of NHE1 in the absence of CaM and  $\text{Ca}^{2+}$  in an auto-inhibitory manner. Residues Leu<sup>639</sup>, Lys<sup>651</sup>, and Tyr<sup>652</sup> are particularly important in the interaction (Wakabayashi et al. 1997). When CaM and  $\text{Ca}^{2+}$  are present, they bind to the high affinity site thereby blocking the autoinhibitory interaction and activating the NHE (Bertrand et al. 1994). Additionally, there are 7 conserved acidic amino acids, <sup>753</sup>EDED<sup>759</sup>, in the distal region of the C-terminal tail that play a role in CaM binding (Li et al. 2004). CaM does not directly bind to this region of the tail but mutation of the sequence results in decreased NHE1 activity following an acid load and reduced CaM binding. It is possible that these residues are important for maintaining a proper conformation of the cytosolic tail that is required for CaM binding.

### Carbonic anhydrase II

Carbonic anhydrase II (CAII) is an enzyme that catalyzes the production of  $\text{HCO}_3^-$  and  $\text{H}^+$  from the hydration of  $\text{CO}_2$ . CAII associates with NHE1 *in vivo* via interaction at residues 790–802 of the C-terminal tail with Ser<sup>796</sup> and Asp<sup>797</sup> forming part of the binding site (Li et al. 2006). Association of these 2 proteins increases NHE1 activity and is dependent on the phosphorylation state of the NHE1 (Li et al. 2002). It appears as though a region upstream of the CAII binding site, when dephosphorylated, interferes with CAII binding (Li et al. 2006). The association of CAII with NHE1 may result in more efficient removal of protons that are produced by CAII activity.

### Tescalcin

Tescalcin is a  $\text{Ca}^{2+}$ -binding protein that is structurally homologous to CHP. The protein has been shown to interact with the C-terminal tail of NHE1 *in vivo* in a  $\text{Ca}^{2+}$ -dependent manner at a site distinct from CaM (Li et al. 2003; Mailander et al. 2001). This association has an inhibitory effect on NHE1 activity.

## Post-translational regulation and modification

### Glycosylation

Sequence analysis of NHE1 predicts 2 potential glycosylation sites in the N-terminal domain (Sardet et al. 1989). Subsequently, carbohydrate digestion and site-directed mutagenesis confirmed the existence and location of these modifications (Counillon et al. 1994; Haworth et al. 1993). An N-linked glycosylation site was found at Asn<sup>75</sup> in the first extracellular loop in the N-terminal domain in addition to O-linked glycosylation sites. When separated by SDS-PAGE, 2 forms of NHE1 are consistently observed: a cell surface, 110-kDa protein that contains N- and O-linked oligosaccharides, and an 85-kDa protein that is likely intracellular and contains only the N-linked, high-mannose oligosaccharide. Although the carbohydrate moieties exist *in vivo* they are not required for ion transport (Haworth et al. 1993).

### NHE1 phosphorylation and regulatory protein kinases

Phosphorylation was identified as an NHE1 regulatory mechanism when Sardet et al. (1990) confirmed that the protein was phosphorylated in response to treatment with thrombin, epidermal growth factor (EGF), phorbol esters, or serum, in a time course concurrent with increased NHE1 activity. The importance of phosphorylation was further reinforced when tryptic mapping of the exchanger identified that a common pattern of phosphorylation and pH, alkalization occurred after treatment of cells with thrombin and okadaic acid, a Ser/Thr protein phosphatase 1 and 2A (PP1 and PP2A) inhibitor (Sardet et al. 1991). The results from these studies suggested that some unknown kinases, activated by growth factors, phosphorylated the NHE1 on serine and (or) threonine residues to mediate  $\text{Na}^+/\text{H}^+$  exchange activation. Using deletion analysis, Wakabayashi et al. (1994) localized the major sites of *in vivo* phosphorylation to the residues 636–815 of the cytoplasmic tail and confirmed the importance of this regulatory mechanism when they found that with its removal, 50% of the stimulatory effect induced by growth factors was lost. It has been postulated that the mechanism by which phosphorylation alters the activation state of NHE1 is by inducing a conformational change in the cytoplasmic tail and thereby altering the association of the tail with the  $\text{H}^+$  sensor of the NHE transmembrane domain (Wakabayashi et al. 1992).

Since identifying NHE1 as a phosphoprotein, there has been considerable interest in isolating the kinases responsible as well as the pathways connecting the extracellular signals to the final event. Since phorbol esters were identified as a stimulator of NHE1 activity and inducer of NHE1 phosphorylation (Sardet et al. 1990), the possibility of protein kinase C (PKC) being an NHE1 kinase was investigated. *In vitro* assays confirmed that PKC did not phosphorylate the last 178 residues of NHE1, nor did PKA, but  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaM kinase II) did. Analysis of the NHE1 amino acid sequence revealed 3 CaM kinase II consensus sequences, suggesting the possibility that phosphorylation by CaM kinase II may also occur *in vivo* (Fliegel et al. 1992). This was the first study that identified a kinase that directly acted on NHE1.

The mitogen-activated protein kinases (MAPK) extracellular-regulated kinases 1 and 2 (ERK1/2) have also been implicated in growth factor NHE1 activation. When inhibiting the ERK signaling cascade, a 50%–60% reduction in growth factor-induced NHE1 stimulation was observed in Chinese hamster fibroblast cells, which agrees with the effect observed when removing the NHE1 phosphorylated region (Bianchini et al. 1997). Fast protein liquid chromatography (FPLC) isolated 4 fractions from rabbit skeletal muscle that were able to phosphorylate the NHE1 C-terminus, and when antibodies were used to immunoprecipitate MAPK from the extracts, 3 of these 4 fractions lost this ability (Wang et al. 1997a). This identified the 3 fractions as containing MAPKs involved in direct phosphorylation of NHE1 *in vitro*. Additionally, a downstream target of the ERK1/2 kinases, ribosomal protein S6 kinase (p90<sup>RSK</sup>), was found to be activated in vascular smooth muscle cells of rats after Ang II treatment, which resulted in increased NHE1 activity (Phan et al. 1997; Takahashi et al. 1997). It was later established that p90<sup>RSK</sup> was a serum-stimulated kinase

that directly phosphorylated NHE1 at Ser<sup>703</sup> in vivo (Takahashi et al. 1999). This confirmed that the ERK1/2 kinases and their downstream target p90<sup>RSK</sup> as being major players in growth factor-stimulated NHE1 activation. Further in vitro work also identified the residues Ser<sup>693</sup>, Ser<sup>766</sup>, Ser<sup>770</sup>, Thr<sup>779</sup>, and Ser<sup>785</sup> as also being phosphorylated by ERK2, but the importance of these residues must be confirmed in vivo (Liu et al. 2004).

A number of other kinases have been implicated in NHE1 phosphorylation including p38, which is also a MAPK, p160ROCK, and the Nck-interacting kinase (NIK) (Fig. 1). Evidence for p38 in regulation of NHE1 by phosphorylation is inconsistent. Ang II treatment of rat vascular smooth muscle cells results in rapid activation of p38, resulting in NHE1 inhibition while providing a stimulatory signal to the exchanger via activation of ERK1/2 (Kusuhara et al. 1998). In contrast, the opposite effect was found in studying cytokine withdrawal in mouse  $\beta$ -cells. In this case activation of NHE1 via p38 induced intracellular alkalization and triggered apoptosis, possibly through phosphorylation of NHE1 at Thr<sup>718</sup>, Ser<sup>723</sup>, Ser<sup>726</sup>, and Ser<sup>729</sup> (human sequence) (Khaled et al. 2001). The difference in results is likely due to activation of different pathways in different cell types, suggesting a further level of complexity in kinase and NHE1 activation whereby the same proteins can provide different effects depending on the cell type.

The kinase p160ROCK is a downstream target of the GTPase RhoA, which has been shown to stimulate NHE1 activity (Hooley et al. 1996). RhoA activation of NHE1 is mediated by p160ROCK, likely via a direct phosphorylation of NHE1. When amino acids 636–815 of NHE1 are removed, the effect of p160ROCK is blocked (Tominaga et al. 1998). NIK also activates NHE1 via phosphorylation at a site distal to residue 638, but to mediate its effect it must also bind NHE1 at the residues 538–638 of the cytoplasmic tail (Yan et al. 2001).

For phosphorylation to be considered a plausible mode of regulation for NHE1 activity, a system must also be in place for removing the phosphate group from the protein. A role for the phosphatase protein phosphatase 1 (PPI) has been confirmed (Misik et al. 2005). PPI was found to bind to NHE1 in vivo and reduce its basal level of Na<sup>+</sup>/H<sup>+</sup> exchange activity. Additionally, inhibition of PPI by expression of inhibitor 2 (I2) elevated NHE1 proton efflux rates. These results suggest that PPI is involved in NHE1 regulation through its role in dephosphorylation of the protein. Another phosphatase that has been shown to dephosphorylate the NHE1 is protein phosphatase 2A (PP2A). PP2A was recently shown to dephosphorylate NHE1 in vitro (Misik et al. 2005; Snabaitis et al. 2006). In response to adenosine A<sub>1</sub> receptor agonists, PP2A colocalized with NHE1 in adult rat ventricular myocytes. PP2A activity was also necessary for the inhibitory effect of adenosine A<sub>1</sub> on  $\alpha$ 1-adrenoceptor-mediated increase in NHE1 phosphorylation (Snabaitis et al. 2006). It was suggested that PP2Ac-mediated NHE1 dephosphorylation is a signaling regulatory pathway downstream of the inhibitory adenosine A<sub>1</sub> receptor (Snabaitis et al. 2006). Prevention of dephosphorylation has also been shown to maintain NHE1 in an active state. The ligand 14-3-3 binds NHE1 at Ser<sup>703</sup> after the residue has been phosphorylated

by p90<sup>RSK</sup> as a result of serum stimulation and limits dephosphorylation (Lehoux et al. 2001).

## Growth factors and hormonal control of NHE activity

It was determined many years ago that growth factors and hormones stimulate NHE activity, but it required much work to elucidate the mode by which this occurred. Various binding proteins and protein kinases have been identified that interact directly and indirectly with the exchanger (see above), but identifying how they integrate into the pathways of regulation has been complex. Some of the growth factors and hormones that have been shown to activate NHE1 include thrombin, serum, epidermal growth factor, insulin, angiotensin II, and lysophosphatidic acid (Bianchini et al. 1997; Phan et al. 1997; Sardet et al. 1990; Snabaitis et al. 2000; Tominaga et al. 1998). Thrombin activates the NHE1 via phosphorylation-dependent and -independent mechanisms (Sardet et al. 1990; Wakabayashi et al. 1994). Thrombin is a serine protease that interacts with the protease-activated receptor-1 (PAR-1) with high affinity (Sabri et al. 2000). Interaction of thrombin with its receptor initiates a cascade that activates ERK1/2 via Raf-1 activation of MAP-ERK kinase 1 (MEK1) resulting in NHE1 phosphorylation and activation (Bianchini et al. 1991; Sardet et al. 1991). Additionally, in rat ventricular myocytes, thrombin activates NHE1 by means of a PKC-mediated mechanism, although PKC does not directly phosphorylate the exchanger (Fliegel et al. 1992; Yasutake et al. 1996).

Activation of NHE1 by serum involves a similar pathway as thrombin activation, but an interesting feature of this mechanism has been identified. The ERK1/2-activated p90<sup>RSK</sup> has been shown to phosphorylate NHE1 at Ser<sup>703</sup> (Takahashi et al. 1999) as stated above, but in doing so, it also forms a 14-3-3 ligand binding site (Lehoux et al. 2001). When 14-3-3 binds to the phospho-Ser<sup>703</sup>, it limits dephosphorylation of this residue and as a result, participates in serum-mediated NHE1 activation. Like serum, EGF activation of NHE1 appears to be similar to that of thrombin and results in NHE1 phosphorylation (Sardet et al. 1991). However, Maly et al. challenge the involvement of the MAPK pathway in EGF-mediated NHE1 activation (Maly et al. 2002). Their study only implicated PKC $\alpha$  in EGF-mediated NHE1 activation in mouse NIH3T3 cells. They suggest that that lack of MAPK involvement may be unique to their cell system. The hormone insulin activates NHE1 via a ERK1/2 MAPK pathway in addition to a PKC pathway (Bianchini et al. 1997; Sauvage et al. 2000). In human erythrocytes, insulin activates phosphatidylinositol 3-kinase, which in turn activates PKC $\zeta$ , and ultimately activates NHE1 (Sauvage et al. 2000).

Angiotensin II is a potent vasoconstrictor that plays an important role in determining blood pressure as well as stimulating ion fluxes, protein phosphorylation, contractility, gene expression, and cell growth. It acts via its interaction with AT<sub>1</sub> receptor, a G-protein-coupled receptor (Schmitz and Berk 1997). In rat vascular smooth muscle cells (VSMC), Ang II stimulates NHE1-mediated pH<sub>i</sub> alkalization via p90<sup>RSK</sup>-direct phosphorylation of NHE1 (Phan et al. 1997; Takahashi et al. 1997). The p90<sup>RSK</sup> kinase is activated

via a MEK1→ERK1/2 pathway and is Ca<sup>2+</sup>-dependent, although not PKC-dependent. Ang II-mediated NHE1 activation is also dependent on p38 activation in some tissues. p38 is activated by Ang II in rat VSMC but it is suggested to negatively regulate ERK1/2 and NHE1 (in contrast to stimulatory effects in  $\beta$ -cells (Khaled et al. 2001)). Ang II signaling may therefore activate opposing pathways, and therefore must stimulate NHE1 through a balance of these pathways depending on the tissue type (Kusuhara et al. 1998). In this regard, in isolated adult rat ventricular myocytes, Ang II has complicated opposing effects on NHE1 activity. Ang II acts positively to stimulate NHE1 through AT<sub>1</sub> receptor subtypes but also exerts a negative opposing action through AT<sub>2</sub> receptor subtypes (Gunasegaram et al. 1999). In addition, Ang II exerts a positive inotropic effect on the myocardium, which is abrogated by NHE inhibition (Cingolani et al. 2006). Figure 2 illustrates some putative pathways of angiotensin stimulation of NHE1 activity.

Lysophosphatidic acid (LPA) is an anionic, bioactive phospholipid that mediates its signaling by associating with the G-protein-coupled receptors, LPA receptor subtypes 1–4 (LPA<sub>1–4</sub>) (Kooijman et al. 2005). Association with the LPA receptors results in a variety of cellular responses including survival and proliferation, migration, and tumor-cell invasion (Gardell et al. 2006). LPA is able to activate NHE1 by stimulation of G<sub>q/13</sub> and activation of both the RhoA and Cdc42 pathways (Hooley et al. 1996). Activation of the RhoA pathway results in p160ROCK activation, leading to NHE1 stimulation possibly by direct phosphorylation (Tominaga et al. 1998). Activation of the Cdc42 pathway mediates ERK1/2 activation leading to NHE1 stimulation (Hooley et al. 1996; Wallert et al. 2005).

### Other stimulatory mechanisms

Sustained intracellular acidosis treatment of neonatal rat ventricular myocytes results in a significant increase in NHE1 activity (Haworth et al. 2003). Sustained intracellular acidosis was achieved by subjecting the cells to an NH<sub>4</sub>Cl-induced acid load and then washing out the NH<sub>4</sub>Cl with Na<sup>+</sup>-free buffer. If the cells were incubated in the Na<sup>+</sup>-free buffer for a prolonged period of time, the low pH<sub>i</sub> was maintained. To rescue the cells from the acid load, Na<sup>+</sup> was reintroduced. To confirm that this mode of stimulation was not unique to the neonatal rat ventricular myocytes, the authors of this study also looked at the effect on adult ventricular myocytes and the monkey COS-7 non-myocyte cell line. They found that there were no differences from what they observed in the neonatal rat ventricular myocytes (Haworth et al. 2003).

To investigate the role that kinases played in the sustained intracellular acidosis method of NHE1 stimulation, the authors tested the ability of neonatal rat ventricular myocytes cell extracts to phosphorylate an NHE1-GST fusion protein (Haworth et al. 2003). The fusion protein contained the last 178 amino acids of the NHE1 C-terminal tail. They found that the cell extracts contained significant NHE1 kinase activity resulting in increased NHE1 phosphorylation. Further analysis demonstrated that this method of stimulation activated ERK1/2 and p90<sup>RSK</sup> kinases with a timeline corresponding to NHE1 activation. Additionally, inhibition of the ERK1/2 upstream effector MEK1 with PD98059 or U0126,

2 MEK1/2 inhibitors, prevented the stimulatory effect of sustained intracellular acidosis. Based on the results of this study, sustained intracellular acidosis initiates a signaling cascade that activates ERK1/2 and p90<sup>RSK</sup> kinases and promotes NHE1 phosphorylation, ultimately activating Na<sup>+</sup>/H<sup>+</sup> exchange.

Cellular osmotic challenge also activates the NHE (Grinstein et al. 1992). Osmotic stimulation activates a number of MAP kinases, although this occurs in parallel to NHE1 stimulation rather than being the mechanism of NHE1 osmotic activation, which occurs through a phosphorylation-independent mechanism (Gillis et al. 2001). The site that appears to mediate the osmotic stimulation of NHE1 exists between the end of the membrane-associated domain and residue 566 of the tail (Bianchini et al. 1995). This region of the protein is involved in associations with the cellular cytoskeletal network, suggesting the possibility that shrinkage associated with cellular osmotic stress may affect the NHE1 association with the cytoskeleton and therefore alter some yet elucidated regulatory mechanism (Denker et al. 2000).

Other mechanisms that stimulate NHE1 include H<sub>2</sub>O<sub>2</sub> in rat cardiomyocytes (Wei et al. 2001) and, more recently, zinc in colonocytes (Azriel-Tamir et al. 2004). H<sub>2</sub>O<sub>2</sub> causes activation of ERK1/2 kinases, in addition to causing an increase in NHE1 phosphorylation and activation, which may contribute to contractile dysfunction during ischemia-reperfusion injury (Snabaitis et al. 2002). The mechanism by which Zn activates NHE1 occurs with the initial binding of Zn to an extracellular zinc-sensing receptor, which triggers the release of Ca<sup>2+</sup> that subsequently activates ERK1/2 via the IP<sub>3</sub> pathway.

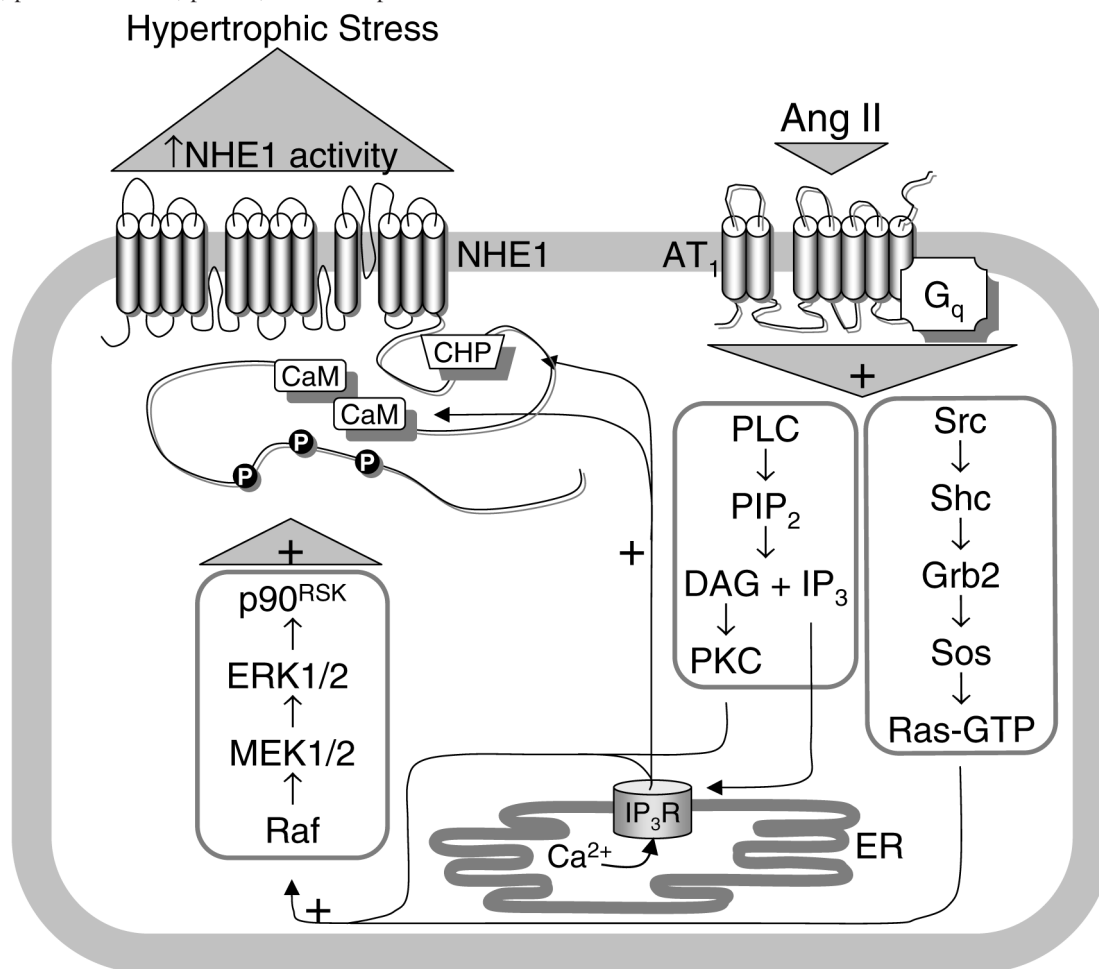
## The NHE in health and disease

### Physiological roles

A number of studies have used knock out or inhibition of NHE1 to determine its physiological role. Early studies examined the role of NHE1 in Chinese hamster lung fibroblasts cells (CCL39) by examining cell growth of cells deficient in NHE1 (Pouyssegur et al. 1984). In the absence of Na<sup>+</sup>/H<sup>+</sup> exchange activity, cells lacked the ability to grow at neutral or acidic pH. To examine the role of NHE1 in intact mice, 2 NHE1 null mice have been studied. Cox et al. (1997) studied a spontaneous mutation that gave rise to an NHE1-null phenotype, whereas Bell et al. (1999) examined a traditional knockout. In both cases, *Nhe1*<sup>-/-</sup> mice experienced a decreased rate of postnatal growth and exhibited ataxia and epileptic-like seizures (Bell et al. 1999; Cox et al. 1997). The role NHE1 plays in cell cycle progression may be the basis for its involvement in cell growth and proliferation. An NHE1-dependent transient increase in pH<sub>i</sub> promotes the G<sub>2</sub>/M transition allowing for S phase to occur in a timely manner (Putney and Barber 2003). In the absence of Na<sup>+</sup>/H<sup>+</sup> exchange, the onset of S phase is delayed and mitosis is stalled. Additionally, NHE1 has been shown to regulate the expression of a number of genes involved in cell cycle progression via its control of pH<sub>i</sub> (Putney and Barber 2004).

NHE1 is also involved in cell differentiation. Treatment of cells with an NHE1 inhibitor prevents retinoic

**Fig. 2.** Hypothetical model for receptor-mediated activation of the NHE1 isoform of the  $\text{Na}^+/\text{H}^+$  exchanger (NHE). Angiotensin II stimulates NHE1 activation via  $\text{p90}^{\text{RSK}}$ -mediated phosphorylation of the exchanger. A  $\text{Ca}^{2+}$ -dependent process though PKC does not directly phosphorylate NHE1 (Phan et al. 1997; Takahashi et al. 1997). Ang II binds to the GPCR  $\text{AT}_1$  and initiates intracellular signaling via the  $\text{G}_q$  protein (Schmitz and Berk 1997). This signaling cascade involves  $\text{Src} \rightarrow \text{Shc} \rightarrow \text{Grb2} \rightarrow \text{Sos} \rightarrow \text{Ras-GTP}$ , and leads to activation of PKC and ERK1/2 MAP kinases.  $\text{AT}_1$  receptor activates signaling that is both  $\text{G}_q$  dependent and  $\text{G}_q$  independent (Rajagopal et al. 2005; Zhai et al. 2005). The  $\text{G}_q$ -independent pathway appears to involve increased cardiac hypertrophy in transgenic mice through a mechanism that activated the  $\text{Src}/\text{Ras}/\text{ERK}$  pathway. The  $\text{G}_q$ -dependent pathway activates PLC that leads to the generation of the 2<sup>nd</sup> messengers DAG and  $\text{IP}_3$ .  $\text{IP}_3$  binds the  $\text{IP}_3$  receptor on the ER causing the release of ER  $\text{Ca}^{2+}$  stores into the cytoplasm. The  $\text{Ca}^{2+}$  signaling activates PKC and other  $\text{Ca}^{2+}$ -binding proteins (CaM and CHP). Ang II, Angiotensin II;  $\text{AT}_1$ , angiotensin receptor subtype 1; CaM, calmodulin; CHP, calcineurin homologous protein; DAG, diacylglycerol; ERK, externally regulated kinase;  $\text{G}_q$ , small G-protein subtype q;  $\text{IP}_3$ , inositol triphosphate;  $\text{IP}_3\text{R}$ ,  $\text{IP}_3$  receptor; MEK, MAP-ERK kinase; NHE1,  $\text{Na}^+/\text{H}^+$  exchanger isoform 1;  $\text{PIP}_2$ , phosphatidylinositol-4,5-bisphosphate; PLC, phospholipase C; PKC, protein kinase C;  $\text{p90}^{\text{RSK}}$ , ribosomal protein kS6 kinase.



acid-induced differentiation (Wang et al. 1997b). The involvement of NHE1 in cell growth, proliferation, and differentiation implicates the protein as being an important player in normal developmental processes.

Anchoring of NHE1 to the cytoskeleton via interactions with the ERM family of proteins also links NHE1 to a role in maintenance of cytoskeletal structure, focal adhesion, and cell migration. NHE1-deficient cells have impaired cell adhesion and cells that express NHE1 but that are unable to interact with the ERM proteins have reduced actin stress fiber organization, irregular cell shape, and impaired migration (Denker and Barber 2002; Denker et al. 2000; Tominaga and Barber 1998).

NHE1 is also thought to be involved in apoptosis, although its role seems to differ depending on the cells

tested. In mouse  $\beta$ -cells during trophic factor withdrawal, which triggers pH<sub>i</sub> dysregulation and apoptosis, NHE1 is activated, leading to cellular alkalization and progression of apoptosis (Khaled et al. 2001). In contrast, NHE1 has also been shown to activate the prosurvival kinase, Akt, in response to apoptotic stress. Further, in breast cancer cells and leukemic cells, inhibition of NHE1 induces apoptosis, although since these are cancer cells, the signaling pathway may not represent a typical mechanism in healthy cells (Reshkin et al. 2003; Rich et al. 2000; Wu et al. 2004).

#### Heart disease

NHE1 is the predominant isoform present on the cardiomyocyte sarcolemma, which suggests a significant role for the protein in myocardial acid control (Fliegel et al. 1991).



In fact, NHE1 was found to be responsible for approximately 50% of proton efflux in isolated perfused ferret hearts and as such it is considered to play a critical role in maintaining pH<sub>i</sub> homeostasis and contractility (Grace et al. 1993). Although NHE1 has a major role in normal cardiac function, it has also been implicated as contributing to several pathological states.

During ischemia and reperfusion in the myocardium, Na<sup>+</sup>/H<sup>+</sup> exchange activity contributes to overall cell damage making it a valuable target for pharmacological intervention. During ischemia, anaerobic glycolysis occurs resulting in the production of protons, which serve to activate NHE1. Activated NHE1 exchanges the H<sup>+</sup><sub>i</sub> for Na<sup>+</sup><sub>e</sub> leading to a rapid accumulation of sodium in the cell (Allen and Xiao 2003; Avkiran 2001; Lazdunski et al. 1985). The high sodium concentration drives the increase in Ca<sup>2+</sup> via reversal of the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger. The ultimate result of this process is that the buildup of Ca<sup>2+</sup> in the cells triggers various pathways leading to cell death. A huge body of evidence suggests that inhibition of NHE1 during ischemia and reperfusion protects the myocardium from calcium overload (Avkiran and Marber 2002). In various animal models, NHE1 inhibition by drugs such as cariporide, amiloride, and EMD 85131 have proven to be cardioprotective (Gumina et al. 1998; Karmazyn 1988; Scholz et al. 1995). Activation of NHE1 regulatory pathways has also been suggested to be important in NHE1-mediated damage to the myocardium. We initially showed that ischemia and reperfusion activate many NHE1 regulatory kinases (Moor et al. 2001). Recently, it has also been demonstrated that inhibition of p90<sup>RSK</sup> activity in the myocardium reduces cardiomyocyte apoptosis in intact hearts subjected to ischemia reperfusion. This may be mediated through reduced activation of NHE1 (Maekawa et al. 2006).

However, clinical trials have not been as promising. The GUARD During Ischemia Against Necrosis (GUARDIAN) study, which covered a wide range of clinical situations with a total of 11 590 patients, found that cariporide demonstrated no overall significant benefit (Theroux et al. 2000). Any benefit observed was limited to the subset of patients who underwent coronary artery bypass graft (CABG) surgery (Avkiran and Marber 2002). These patients received cariporide prior to the onset of ischemia and reperfusion occurred in a timely manner. A much smaller trial (*n* = 100 patients) found that cariporide had the potential to attenuate reperfusion injury when it was administered prior to reperfusion, thereby improving recovery after myocardial infarction (Rupprecht et al. 2000).

The Evaluation of the Safety and Cardioprotective effects of eniporide in the Acute Myocardial Infarction (ESCAMI) study also looked at the effect of NHE1 inhibition on myocardial infarction. Like the GUARDIAN study they found that eniporide did not demonstrate any significant improvements on clinical outcome (Zeymer et al. 2001). The lack of benefit may have been due to the administration of the drug only during the reperfusion therapy (Avkiran and Marber 2002). More recently, the Na<sup>+</sup>/H<sup>+</sup> EXchange Inhibition to Prevent Coronary Events in acute cardiac CONDITIONS (EXPEDITION) study, also looked at the effects of cariporide. It found that although it reduced myocardial infarction owing to coronary artery bypass graft surgery-

induced ischemia, it increased mortality rates overall owing to an increase in cerebrovascular side effects (Karmazyn et al. 2005; Mentzer 2003). Based on the outcome of the animal model studies and clinical trials, it is clear that NHE1 is an important pharmacological target but that more research is necessary for success at the clinical level.

NHE1 activity has also been shown to be involved in the development of cardiac hypertrophy. Hypertrophy is the enlargement of an organ because of the growth of individual cells rather than an increase in the total number of cells. Following myocardial injury, the heart compensates for its loss of functional cells by initiating a hypertrophic response, but this can be a maladaptive response and is one of the components that underlies eventual heart failure (Cingolani et al. 2003).

A number of factors that initiate hypertrophic response have also been shown to be involved in activating Na<sup>+</sup>/H<sup>+</sup> exchange activity or are dependent upon it. For example, altered mechanical load activates NHE1 and is thought to be an early step in hypertrophic initiation. When mechanical stress occurs in the presence of an NHE1 inhibitor, the hypertrophic signaling cascade is inhibited (Yamazaki et al. 1998). Also, stimulation with endothelin-I or via the α<sub>1A</sub>-adrenoreceptor activates NHE1 activity by initiating a signaling cascade that results in ERK1/2 and p90<sup>RSK</sup> activation, in addition to activating hypertrophy (Avkiran and Haworth 2003; Knowlton et al. 1993; Snabaitis et al. 2000; Sugden 2003). Supporting a role for NHE1 in cardiac hypertrophy are the findings that in guanylyl cyclase-A receptor knock-out mice, NHE1 activity was enhanced as was cardiac hypertrophy (Kilic et al. 2005). Finally, in several models, it was shown directly that NHE1 inhibition prevents cardiac hypertrophy. This was demonstrated in vivo in rats subjected to myocardial infarction (Kusumoto et al. 2001; Yoshida and Karmazyn 2000), in mice with guanylyl cyclase-A receptor knock out (Kilic et al. 2005), and in vitro in isolated cardiomyocytes (Karmazyn et al. 2003). Though numerous animal models have shown NHE1 inhibition to be effective in preventing hypertrophy, it is still necessary to establish whether this form of therapy can be beneficial clinically (Karmazyn et al. 2005).

## Cancer

NHE1 is activated in response to a number of mitogenic factors and its activity mediates such processes as cell growth, proliferation, and migration, aberrations of which are characteristic of malignant cells. A key feature of transformed cells is that they have an alkalized pH<sub>i</sub> relative to nontransformed cells, and it has been suggested that this disturbance in pH homeostasis corresponds to an increasing cancerous state (Harguindey et al. 2005). In fact, it has been demonstrated that NHE1 is involved in the altered pH<sub>i</sub> of malignant cells, and that NHE1-dependent alkalization plays a pivotal role in the development of a transformed phenotype, whereas inhibition of the exchanger prevents it (McLean et al. 2000; Reshkin et al. 2000b; Rich et al. 2000).

In addition to establishing an alkaline cellular state, NHE1 has also been implicated as a key player in breast cancer cell invasion. In healthy cells, serum stimulates NHE1 activity, but in breast cancer cells, a conflicting situa-

tion occurs in which serum deprivation activates NHE1 to induce cell motility and invasion (Reshkin et al. 2000a). Cytoskeletal changes that result in the formation of pseudopodia in invasive tumor cells are NHE1-dependent and are prevented via NHE1 inhibition (Lagana et al. 2000). The signaling cascade that initiates the NHE1-mediated cytoskeletal rearrangement involves RhoA phosphorylation and inhibition by PKA, which stops p160ROCK activation. This prevents p38-mediated inhibition of NHE1, ultimately resulting in NHE1 activation and subsequent invasiveness (Cardone et al. 2005; Paradiso et al. 2004).

In accordance with NHE1 being identified as having a role in tumorigenicity, it has garnered some attention as a possible treatment target. Inhibition of NHE1 prevented pH<sub>i</sub> alkalization and resulted in an increase in apoptosis in leukemic cells with more than 90% of the cancer cells killed (Rich et al. 2000). Paclitaxel, a chemotherapy agent used for leukemia and lymphoma, has been shown to activate a pathway involving PKA and p38 inhibition of NHE1, also leading to cancer cell apoptosis (Reshkin et al. 2003). Interestingly, this study also showed that if NHE1 was specifically inhibited simultaneously with paclitaxel treatment, the amount of the drug required for apoptosis to be achieved was significantly reduced. These 2 examples highlight the role NHE1 plays in cancer cell tumorigenicity in addition to its potential in the future of cancer treatment.

### Future studies

A great deal has been learned about NHE; however, a great deal is still to be discovered. Many areas can be explored. Whereas a tremendous potential was shown in animal studies to improve treatment of heart disease with NHE inhibitors, only limited success was shown in clinical studies, partially owing to the discovery of side effects. Would more potent or more specific inhibitors be an improvement for clinical use? This is not yet known. Can NHE inhibition be useful in other clinical scenarios such as breast cancer? This has not yet been well studied. Also, whereas regulation of NHE1 is partially understood, the exact amino acids involved in phosphorylation and regulation of the protein are not entirely elucidated and may vary from 1 tissue to another. Their role in elevation of NHE activity in the disease state still remains to be defined. In addition, whereas great strides have been made in understanding the structure of the prokaryotic NHE, many details of how the eukaryotic NHE functions are not yet clear. So, whereas a great deal of knowledge has been accumulated, much has yet to be learned.

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