CHARACTERIZATION OF THE INTERACTION BETWEEN
NA⁺/H⁺ EXCHANGER ISOFORM 7 (NHE7) AND CALMODULIN (CAM)

by

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Abstract

Na⁺/H⁺ exchangers (NHEs) are a family of transmembrane antiporters that catalyze the electroneutral exchange of Na⁺ and H⁺. They are primarily involved in regulation of pH and ion homeostasis and are themselves regulated by several signaling pathways, including Ca²⁺ signaling. NHE isoform 7 (NHE7) is activated by increases in intracellular Ca²⁺, mediated by binding of calmodulin (CaM) to the second intracellular loop (IL2) of NHE7. This interaction between NHE7 IL2 and CaM is unconventional, as NHE7 IL2 is an extremely short CaM-binding site (~10 amino acids) unlike previously characterized CaM-binding sites. We showed that the NHE7 IL2-CaM interaction is likely pH-independent by means of pulldown assays with GST fusion peptides of NHE7 IL2 and immobilized CaM beads performed under pH 7.3 and pH 5.8. We also showed that the NHE7 IL2-CaM interaction is mediated by positively charged and hydrophobic amino acids by means of pulldown assays with GST fusion peptides of mutant NHE7 IL2 (NHE7 IL2 KKPL, NHE7 IL2 KKRAAA and NHE7 IL2 FFAA) and immobilized CaM beads.

The interaction between NHE7 IL2 and CaM has potential for relevance to the nervous system, which is highlighted by the numerous NHE isoforms that have been implicated in neuronal function: NHE1, NHE6 and NHE9. However, much future research needs to be done to elucidate the exact nature of the contribution to physiology and the nervous system of the NHE7-CaM interaction.
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<tr>
<td>ΔH</td>
<td>Change in Enthalpy</td>
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<tr>
<td>ΔS</td>
<td>Change in Entropy</td>
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<tr>
<td>ADHD</td>
<td>Attention Deficit Hyperactivity Disorder</td>
</tr>
<tr>
<td>ASIC</td>
<td>Acid-Sensing Ion Channel</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CA II</td>
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<td>CaM</td>
<td>Calmodulin</td>
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<td>CBDA</td>
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<td>CBDB</td>
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<td>C-CaM</td>
<td>C-lobe of Calmodulin</td>
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<td>Calcineurin Homologous Protein</td>
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<td>Dithiothreitol</td>
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<td>EDTA</td>
<td>Ethylene Diamine Tetraacetic Acid</td>
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<td>EGTA</td>
<td>Ethylene Glycol Tetraacetic Acid</td>
</tr>
<tr>
<td>EIPA</td>
<td>5-(N-ethyl-N-isopropyl) Amiloride</td>
</tr>
<tr>
<td>EL</td>
<td>Extracellular Loop</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
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<td>IL</td>
<td>Intracellular Loop</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1 Thiogalactopyranoside</td>
</tr>
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<td>ITC</td>
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</tr>
<tr>
<td>Kd</td>
<td>Dissociation Constant</td>
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<tr>
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<td>K\textsubscript{Na}</td>
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<td>NCX</td>
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<td>NHERF</td>
<td>Na\textsuperscript{+}/H\textsuperscript{+} exchanger regulatory factor</td>
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<tr>
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<tr>
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<td>Phosphate Buffered Saline</td>
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<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Fluoride</td>
</tr>
<tr>
<td>RSK</td>
<td>p90 Ribosomal S6 Kinase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SWE</td>
<td>Slow Wave Epilepsy</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-Buffered Saline with 0.075% Tween 20</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane Segment</td>
</tr>
<tr>
<td>V-ATPase</td>
<td>Vacuolar type H\textsuperscript{+}-ATPase</td>
</tr>
<tr>
<td>XLMR</td>
<td>X-linked Mental Retardation</td>
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1. Introduction

1.1 pH regulation: physiological relevance and potential cross-talk with Ca\textsuperscript{2+} signaling

Intracellular and extracellular pH serve as important physiological signals. For example, in *Caenorhabditis elegans* intestine rhythmic, oscillatory changes in the pH of the extracellular space regulate the similarly rhythmic patterns of muscle contraction that carry the food through the intestine (Pfeiffer et al. 2008). Also, changes in extracellular pH regulate the excitability of neurons (Chesler 2003) due to various pH-sensitive ion channels. For example, there are voltage-gated Ca\textsuperscript{2+} channels that are sensitive to extracellular pH in neurons (Tombaugh and Somjen 1996, Tombaugh and Somjen 1997). These channels are activated by alkaline pH and inactivated by acidic pH, allowing for regulation of Ca\textsuperscript{2+} current by extracellular pH. Such Ca\textsuperscript{2+} current is excitatory, as it depolarizes the cell. Therefore, neuronal activity is stimulate by alkaline pH (when the Ca\textsuperscript{2+} channels are activated) and inhibited by acidic pH (when the Ca\textsuperscript{2+} channels are inactivated). Indeed, this is the general pattern for regulation of neuronal excitability by pH: acidic pH depresses and alkaline pH stimulates neurons. As an example that goes against this rule, there are acid-sensing ion channels (ASICs) that let cations into the cell when the extracellular space is acidified in some neurons, especially nociceptive neurons that encode the sensation of pain (Wu et al. 2004). Acidification of the extracellular space can occur due to robust synaptic activity, as synaptic vesicles are acidic and fusion of many such vesicles leads to a transient acidification of the synaptic cleft. This in turn opens ASICs, which let in cations (mostly Na\textsuperscript{+}, but also K\textsuperscript{+} and Ca\textsuperscript{2+}), depolarize the
cell and lead to action potentials (Wu et al. 2004). Interestingly, different ASIC channels are formed from four genes and six major transcripts by formation of dimers or trimers. These different ASIC channels are gated at different pHs, except for the homodimer of ASIC4, which has been shown to be pH-independent (Chen et al. 2007). Also, some ASIC isoforms can be inhibited by extracellular Ca\(^{2+}\) and hence are regulated by both pH and Ca\(^{2+}\) (Wu et al. 2004).

A more recent study has suggested that the vacuolar type H\(^{+}\)-ATPase (V-ATPase) provides a point of intersection between Ca\(^{2+}\) and pH. The V-ATPase has at least two functions: a proton-pumping dependent regulation of organellarpH and a proton-pumping independent regulation of vesicle fusion and/or secretion. In yeast it has been shown that at least the latter function is dependent on the Ca\(^{2+}\)-binding protein calmodulin (CaM), a key component of Ca\(^{2+}\) signaling (see 1.5 for details) (Zhang et al. 2008). Furthermore, in Drosophila it has been shown that the V-ATPase V\(_0\) subunit a1, which encodes for the biggest subunit of the transmembrane pore of the V-ATPase and is neuron-enriched if not neuron-specific, directly binds to CaM and recruits CaM to synapses (Zhang et al. 2008). Whether this binding to CaM is important for just one or both functions of V-ATPase is still unclear (Zhang et al. 2008). But it is clear that pH regulation and Ca\(^{2+}\) signaling can both be mediated by the V-ATPase.

### 1.2 Na\(^{+}\)/H\(^{+}\) Exchangers (NHEs)

The Na\(^{+}\)/H\(^{+}\) exchangers (NHEs) are a family of twelve-transmembrane-segment antiporters that facilitate the electroneutral transport of Na\(^{+}\) in one direction and H\(^{+}\) in the other (Kinsella and Aronson 1980). NHEs are widely
accepted as the most prominent pH regulators and they play crucial roles in maintaining pH homeostasis in most organisms. NHEs are highly conserved among different species and in mammals nine different isoforms, termed NHE1 to NHE9, have been identified so far (Orlowski and Grinstein 2004, Brett et al. 2005a). In mammals, NHEs share the same secondary structure, consisting of the N-terminal twelve transmembrane segments (TMs) followed by a hydrophilic C-terminal tail (see Figure 1). The functional unit of NHEs is a dimer (Hisamitsu et al. 2006) and indeed they are found as dimers in intact cells (Fliegel et al. 1993, Fafournoux et al. 1994). All NHEs are acutely regulated by intracellular H+, which binds and greatly increases transporter activity (Aronson et al. 1982). Indeed, at neutral pH NHEs are almost inactive and only become activated upon acidification. Hence, in terms of pH regulation under physiological conditions, they are acid extruders rather than acid loaders.

The regulation of pH mediated by NHEs is physiologically important and aberrant activation of NHEs can initiate Ca²⁺-mediated signaling under pathological conditions. For example, in the heart the NHEs are a key mechanism mediating damage from ischemia and reperfusion. Ischemia occurs when there is an interruption in blood flow and reperfusion occurs when blood flow is restored but results in inflammation and oxidative damage through the induction of oxidative stress. During ischemia, NHEs remove intracellular H⁺ that accumulates due to metabolism in exchange for extracellular Na⁺. The resultant Na⁺ overload leads to reverse-mode activity of the Na⁺/Ca²⁺ exchanger (NCX) and Ca²⁺ overload (Luo et al. 2005, Hwang et al. 2008). This causes cell toxicity and eventually cell death.
Figure 1. General structure of the Na⁺/H⁺ Exchangers (NHEs).
With NHE inhibitors, however, this sequence does not occur and neither does the Ca\(^{2+}\) overload, resulting in improvement in heart function post-ischemia. Hence, the NHEs are clearly physiologically important in the heart and mediate the damage from ischemia, as evidenced by the effects of NHE inhibitors.

1.3 NHE structure

Hydrophobicity analysis predicts that NHEs encompass the N-terminal twelve TMs and a hydrophilic C-terminal tail. Wakabayashi et al. (2000) proposed a membrane topology model of NHE1, the most widely studied NHE isoform, by using cysteine scanning mutagenesis and hydrophobicity analysis. More recently Landau et al. (2007) proposed another model by using a fold-recognition approach based on the crystal structure of NhaA from *E. coli* and multiple alignment programs. Although both models feature twelve TMs and agree on the location of nine of these TMs, these models assign the extreme N-terminal TMs differently. The differences in the two models reflect a controversy about the extreme N terminus of NHE1. Landau et al. (2007) suggest that NHE1 contains a signal peptide that is cleaved off during processing of the protein, and TM1 begins at Val\(^{125}\), which corresponds to TM3 of the Wakabayashi et al. (2000) model. Another major difference between the two models revolves around TM7-TM9 originally assigned by Wakabayashi et al. (2000). TM9 originally assigned by Wakabayashi et al. (2000) was suggested to contain TM7, TM8 and the extracellular loop in between in the Landau et al. (2007) model. The intracellular loop following this region has ambiguous patterns of accessibility to extracellular agents and is assumed to be intramembrane spanning in both models. This segment may participate in ion translocation, and therefore may be
accessible from both extracellular and cytosolic side. Even though TM9 of the Wakabayashi et al. (2000) model [and TM7 and TM8 in the Landau et al. (2007) model] has been studied structurally using nuclear magnetic resonance (NMR) (Reddy et al. 2008), it is still unclear which model is correct. This is due to the region having been studied in isolation and the results obtained being compatible with either model (Reddy et al. 2008). Resolution of the controversy awaits the structure of a larger portion of NHE1.

1.3.1 The N terminal transmembrane segments (TMs)

The N-terminal, transmembrane segments of NHEs are responsible for antiporter activity. Based on the available structural information about the N terminus of NHE1, it appears that functionally important amino acid residues are found in unstructured and flexible regions of the molecule (Slepkov et al. 2005, Ding et al. 2006, Ding et al. 2007, Lee et al. 2009). The regions implicated in NHE transporter activity include TM4 (Slepkov et al. 2004, Slepkov et al. 2005, Slepkov et al. 2007a), TM11 (Slepkov et al. 2007b, Lee et al. 2009) and TM9 (Khadilkar et al. 2001, Reddy et al. 2008). Also, two membrane associated regions [intracellular loop 2 (IL2) (Khadilkar et al. 2001, Mukherjee et al. 2006) and extracellular loop 5 (EL5) (Slepkov et al. 2007b)] have been implicated in transporter activity.

Intriguingly, a cluster of highly conserved titratable residues (ie. acidic or basic residues) is found in the core TMs of NHEs (Landau et al. 2007). These same residues have also been shown to be important for the function of the protein in many cases. In particular, a cluster of four negatively-charged amino acids in the middle of otherwise hydrophobic TMs are seen in all known NHEs (Landau et al.
These residues are expected to be involved in the coordination and transport of Na\textsuperscript{+} and/or H\textsuperscript{+} by the transporter. There are also other functionally important, conserved residues in the N terminus that appear to be involved in maintaining the structure of NHEs. For example, some hydrophobic amino acids found inside TMs are expected to contribute to the overall structure of NHEs (Slepkov et al. 2007b).

1.3.2 The C terminal cytosolic tail

The C-terminal, cytosolic segment of NHEs is mainly responsible for regulation of the antiporter activity (Bertrand et al. 1994, Gebreselassie et al. 1998). In terms of structure, this region contains many subdomains that bind to specific proteins that regulate transporter activity. These include calmodulin (CaM) (Bertrand et al. 1994, Wakabayashi et al. 1994), calcineurin homologous proteins 1-3 (CHP1-3) (Lin and Barber 1996, Barroso et al. 1996, Mailander et al. 2001, Pang et al. 2002), carbonic anhydrase II (CA II) (Li et al. 2002, Li et al. 2006), p42/44 mitogen-activated protein kinases (Bianchini et al. 1997), p90 ribosomal S6 kinase (RSK)(Takahashi et al. 1997, Cuello et al. 2007), Nck-interacting kinase (Yan et al. 2001) and phophatidylinositol 4,5-bisphophate (PIP2)(Aharonoviz et al. 2000). RSK (Takahashi et al. 1997, Cuello et al. 2007) and Nck-interacting kinase (Yan et al. 2001) both phosphorylate NHE1 once they bind to the C terminus and lead to activation of transporter activity. On the other hand, p42/44 mitogen-activated protein kinases do not themselves phosphorylate NHE1 but their binding to the C terminus does lead to activation of NHE1 transporter activity (Bianchini et al. 1997). CaM binds to the C terminus in the presence of elevated intracellular Ca\textsuperscript{2+} and activates transporter activity (Bertrand et al. 1994, Wakabayashi et al. 1994), CHP1-
are essential cofactors for transporter activity and are always bound to the C terminus of NHE1 under physiological conditions (Lin and Barber 1996, Barroso et al. 1996, Mailander et al. 2001, Pang et al. 2002), CA II binds to the C terminus of NHE1 when it is phosphorylated by RSK and activates transporter activity (Li et al. 2002, Li et al. 2006) and PIP2 is another cofactor that binds to the C terminus of NHE1 under physiological conditions and activates transporter activity (Aharonovitz et al. 2000).

The C terminus of NHEs has a preponderance of β-structure and random coil (Gebreselassie et al. 1998, Li et al. 2003), suggesting a compact configuration of antiparallel β-sheets joined by β-turns (Li et al. 2003). This structure is suggested to be flexible, altering its conformation due to protein-protein interactions. For example, binding of CHP1-3 to the C terminus favors formation of an α-helix in the domain mediating that binding (Mishima et al. 2007). Intriguingly, the different isoforms (ie. NHE1 to NHE9) differ most dramatically in the C terminus, suggesting they are differentially regulated. This may help to explain their differential physiological functions (Orlowski and Grinstein 2004).

1.4 The mammalian NHE gene family

Evolutionarily, the NHEs can be grouped into two general classes. The first class is the plasmallelmal NHEs, which are functional NHEs across the plasma membrane (Orlowski and Grinstein 2004). Mammalian NHE1-5 are categorized in this class. Generally, this class of NHEs is specific for transport of Na⁺ (Orlowski and Grinstein 2007). The different isoforms within this class (ie. NHE1-5) differ in their
tissue distribution; NHE1 being ubiquitously expressed (Tse et al. 1991), while NHE2-5 are all expressed in specific tissues. NHE2 is expressed in the stomach and intestines (Tse et al. 1991, Collins et al. 1993), NHE3 is expressed in the kidney and intestines (Orlowski et al. 1992, Tse et al. 1992), NHE4 is expressed primarily in the stomach (Orlowski et al. 1992), and NHE5 is expressed almost exclusively in the nervous system (Klanke et al. 1995, Baird et al. 1999).

The second class of NHEs is the organellar NHEs. This class includes NHE6-9, is found intracellularly in specific organelles, and functions primarily in organelar pH and ion homeostasis. It has been suggested that organellar NHEs have higher affinity for K\(^+\) than Na\(^+\), serving as K\(^+\)/H\(^+\) exchangers. This is of potential physiological significance as K\(^+\) is the predominant monovalent cation in the cytosol (Orlowski and Grinstein 2007). The different isoforms within this class (ie. NHE6-9) exhibit differential subcellular localization. Physiological function, pharmacological inhibitor profiles, and regulation of organellar NHEs are significantly different from that of plasmalemmal NHEs. For example, amiloride derivatives that effectively block plasmalemmal NHEs do not inhibit organellar NHEs (Orlowski and Grinstein 2004).

Although basic structure with the N terminal TMs and the C terminal cytosolic tail are conserved between the two classes, there are several amino acid residues that are conserved among organellar NHEs but not plasmalemmal NHEs and vice versa. Functions of such class-specific amino acid residues may include docking for distinct binding proteins and locations for distinct post-translational modifications that permit isoform-specific regulation. Furthermore, in yeast it has been shown
that these class-specific amino acid residues can be key for function of the proteins. It was found that if conserved, organellar-NHE amino acid residues are mutated to the plasmallemmal-NHE version in the context of the organellar NHE Nhx1p, this leads to a complete loss of function (Mukherjee et al. 2006). It would be intriguing to test whether mutation of plasmallemmal-NHE amino acid residues to the organellar-NHE version in the context of a plasmallemmal NHE would also lead to loss of function.

Nine typical NHE isoforms were identified in mammals. In the following, they are summarized.

1.4.1 Plasmallemmal NHEs

1.4.1.1 NHE1

The first NHE to be cloned (Sardet et al. 1988), NHE1 has also been the most extensively studied isoform. It is ubiquitously expressed and functions as a "housekeeping" protein to help cells recover after acid load. When the cytoplasm is acidified, NHE1 facilitates the transport of H+ out of the cytoplasm, bringing the cytosolic pH back to baseline (see Figure 2 for a typical pH trace). NHE1 exhibits a Na+ affinity (K_{Na}) of 10.0 ± 1.4 mM and a half-maximal activation value of pH 6.75 ± 0.05 (Orlowski 1993). It is inhibited by amiloride with a k_{0.5} of 1.6 ± 0.1 μM (Orlowski 1993). NHE1 can also have structural roles in cytoskeleton and signaling complexes independent of transporter activity (Meima et al. 2007), a function so far only known for NHE1.
Figure 2. Na\(^+\)/H\(^+\) exchanger isoform 1 (NHE1) mediates recovery of intracellular pH back to baseline following acidification. Figure modified from Hug and Bridges 2001.
Disruption of NHE1 in the mouse is found in the naturally occurring Slow Wave Epilepsy (SWE) mutant mouse (Cox et al. 1997). In accordance, the NHE1 knockout mouse (Bell et al. 1999) exhibited seizures and other neurological symptoms, which were found to be caused by hyperexcitability of neurons (Bell et al. 1999). This suggests NHE1 is most important in the nervous system despite its ubiquitous expression in most tissues. The neuronal hyperexcitability observed in NHE1 deficient mice is mediated by lack of NHE1 somehow upregulating the Na⁺ channel density specifically in hippocampal CA1 and cortical neurons (Xia et al. 2003). Intriguingly, these are the same regions that normally express the highest levels of NHE1 (Ma and Haddad 1997), suggesting NHE1 may normally depress Na⁺ channel density. The increase in Na⁺ channel density seen with lack of NHE1 leads to an increased Na⁺ current and hyperexcitability of neurons, which in turn leads to the seizures and other neurological symptoms of these mice.

1.4.1.2 NHE2

NHE2 is found on the apical membrane of epithelial cells of the digestive tract (ie. stomach and intestines) and is unique among the NHEs to be inhibited by extracellular H⁺ (Yu et al. 1993). It is also inhibited by amiloride with a k_{0.5} of 0.6 μM (Honda et al. 1993), has a K_{Na} of 18 ± 1 mM (Levine et al. 1993) and a half maximal activation value of pH 6.90 (Yu et al. 1993).

Targeted disruption of NHE2 in the mouse leads to reduced viability of gastric parietal cells and loss of net acid secretion (Schultheis et al. 1998a), suggesting NHE2 is most important in the stomach. Interestingly, NHE2 is not required for acid
secretion by the parietal cell, but is essential for its long-term viability. Perhaps this is mediated by the unique sensitivity of NHE2 to inhibition by extracellular H\(^+\), which would allow upregulation of its activity by the increased extracellular alkalinity that accompanies acid secretion and might enable NHE2 to play a specialized role in maintaining the long-term viability of the parietal cell.

1.4.1.3 NHE3

NHE3 is found on the apical membrane of epithelial cells of renal proximal tubules (Ambuhl et al. 1996) and intestines (Booksten et al. 1994). NHE3 exhibits a K\(_{\text{Na}}\) of 4.7 ± 0.6 mM and a half-maximal activation value of pH 6.45 ± 0.08 (Orlowski 1993). It is inhibited by amiloride with a \(k_0.5\) of 101 \(\mu\)M (Orlowski 1993).

NHE3 possesses some unique regulatory mechanisms. For example, NHE3 is inhibited by Protein Kinase A (PKA) and Protein Kinase C (PKC), whereas NHE1 is activated by PKC and PKA. This negative regulation of NHE3 is mediated through the Na\(^+\)/H\(^+\) exchanger regulatory factor (NHERF) family. NHERFs are multi-PDZ domain containing proteins that anchor transmembrane molecules to the actin cytoskeleton and thereby regulate their localization. In the case of regulation by PKA, upon PKA activation NHERF1 binds to NHE3 (Lamprecht et al. 1998) and NHE3 is relocated from the plasma membrane to clathrin-coated pits (Kocinsky et al. 2005), where it is expected to be endocytosed. This removal of NHE3 from the plasma membrane then results in inhibition of transporter activity. In the case of regulation by PKC, upon PKC activation NHERF2 binds to NHE3 and, similarly to NHERF1, stimulates the
endocytosis of NHE3, thereby inhibiting NHE3 transporter activity (Lee-Kwon et al. 2003).

Targeted disruption of NHE3 leads to renal and intestinal absorptive defects (Schultheis et al. 1998b). Thus, NHE3 is the predominant absorptive NHE in kidney and the intestine. Specifically, homozygous mutant mice show diarrhea, mild acidosis, reduced blood pressure and severe absorptive defects in the intestine. More recently, electroneutral reabsorption of NaCl from the intestines has been shown to be dependent on NHE3 in addition to the HCO3⁻/Cl⁻ exchanger Downregulated in Adenoma (DRA), whose activity is coupled to that of NHE3 (Musch et al. 2009).

1.4.1.4 NHE4

NHE4 is found on the basolateral membrane of gastric parietal cells. It is inhibited by amiloride with a kₘₐₙ of 813 ± 20 μM (Chambrey et al. 1997). Intriguingly, unlike NHE1-3, NHE4 does not show simple, linear kinetics of interaction with extracellular Na⁺. Instead, sigmoidal kinetics are seen, suggesting there is cooperative binding of extracellular Na⁺ to NHE4 (Bookstein et al. 1996). This may be mediated by dimer formation, with one Na⁺-bound monomer increasing the likelihood of the second monomer binding Na⁺ via a conformational shift.

Targeted disruption of NHE4 leads to impaired gastric acid secretion (Gawenis et al. 2005), suggesting NHE4 is most important in the stomach. Unlike NHE2, which is not required for acid secretion by the parietal cell or proper differentiation of the parietal cell, NHE4 is required for both processes.
1.4.1.5 NHE5

NHE5 mRNA is most abundantly expressed in the brain, especially in neuron-rich regions (Klanke et al. 1995, Baird et al. 1999). It exhibits a $K_{Na}$ of $18.6 \pm 1.6\, \text{mM}$ and half-maximal activation value of pH $6.43 \pm 0.08$ (Szabo et al. 2000). It is inhibited by amiloride with a $k_{0.5}$ of $21\, \mu\text{M}$ (Szabo et al. 2000).

In terms of regulation of NHE5 activity, NHE5 is most similar to NHE3. Like NHE3, NHE5 is inhibited by PKA and PKC (Attaphitaya et al. 2001), although the mechanism of this inhibition has not yet been elucidated. In addition, RACK1 associates with NHE5 in focal adhesions via NHE5's C terminus and positively regulates the transporter activity (Onishi et al. 2007). Beyond this, little is known about the physiological function of NHE5, something our lab is trying to rectify.

1.4.2 Organellar NHEs

NHE6-NHE9 are designated organellar NHEs because of their predominant association to organellar membranes. Specifically, NHE6 is found in the early recycling endosome (Numata et al. 1998, Brett et al. 2002), NHE7 is found in the trans Golgi network (Numata and Orlowski 2001), NHE8 is found in the endoplasmic reticulum and mid to trans Golgi (Goyal et al. 2003, Nakamura et al. 2005) and NHE9 is found in the late recycling endosome (Nakamura et al. 2005). NHE6-9 share a high degree of structural similarity (55-75% amino acid identity), whereas they share only limited similarity with plasmalemmal NHEs (approximately 25% amino acid identity) (Orlowski and Grinstein 2007). By both liposome-based in vitro transporter assays (Nakamura et al. 2005) and cell-based organellar trace influx assays (Numata and Orlowski 2001, Kagami et al. 2008)
organellar NHEs showed higher affinity for K⁺ than Na⁺, leading to a hypothesis that the physiological transporter mode is K⁺/H⁺ exchange. This leads to an interesting possibility that organellar NHEs may pump H⁺ out of acidic organellar lumens by using the K⁺ concentration gradient across organellar membranes, which may at least in part contribute to the establishment of pH gradient along secretory and endocytic pathways.

This model of NHEs as a proton leak pathway hold up to experimental scrutiny. Activation of NHE6 in mammalian cells by knockdown of RACK1 and redistribution of NHE6 from the plasma membrane to the endosome results in alkalization of the endosome (Ohgaki et al. 2008). Equivalently, overexpression of NHE8 alkalizes the Golgi (Nakamura et al. 2005) and overexpression of NHE9 alkalizes the recycling endosome (Nakamura et al. 2005). Hence, organellar NHEs can regulate organellar pH. This is known to be important for several cellular processes, including vesicle trafficking and protein sorting (Bowers et al. 2000, Brett et al. 2005b, Mukherjee et al. 2006). This is of potential significance in synaptic transmission mediated by secretion and endocytosis of synaptic vesicles. Concomitantly, NHE6, NHE7 and NHE9 are highly enriched in brain tissues and accumulating evidence points to the association of their mutation to neurological disorders (Claes et al. 1997, Shashi et al. 2000, de Silva et al. 2003, Gilfillan et al. 2008, Morrow et al. 2008). See Discussion for more details.
1.5 Regulation of NHEs by Ca\textsuperscript{2+}

NHEs are regulated by various signaling cascades. Of these, one of the most important is Ca\textsuperscript{2+} signaling. Ca\textsuperscript{2+} signaling is ubiquitous in all forms of life, and is key for many cellular processes. Neurotransmitter release in the brain is triggered by Ca\textsuperscript{2+} (Morris et al. 1987), as is muscle contraction (Portzehl 1965, Rose et al. 2006). In addition, changes in Ca\textsuperscript{2+} signaling have been associated with aging in the brain (Foster 2007). Due to its wide-spread physiological effects, it is not surprising to find extensive effects of Ca\textsuperscript{2+}, even on one molecule: NHE1.

As a key secondary messenger, intracellular Ca\textsuperscript{2+} is tightly regulated, such that resting levels are in the nM range. At the same time, significant stores of Ca\textsuperscript{2+} are kept in organelles, such as the sarcoplasmic reticulum in muscle cells, ready to be released upon appropriate stimulation of the cell. Intracellular Ca\textsuperscript{2+} levels can then rise from the nM range into the \mu M or even mM range, triggering many varied signaling events, leading to the many physiological effects of Ca\textsuperscript{2+}.

Intriguingly, many of these Ca\textsuperscript{2+} signaling events are mediated by a key molecule: calmodulin (CaM). CaM is a small, 148-amino acid protein that is conserved in most organisms (Zhang and Yuan 1998). CaM is able to bind at least 40 different target proteins and enzymes including various protein kinases (Martin et al. 2004) and phosphatases, receptors, ion-channel proteins (Saimi and Kung 2002), phosphodiesterases, and nitric oxide synthases (Spratt et al. 2007). CaM consists of two similar domains, termed N-lobe (N-CaM) and C-lobe (C-CaM). N-CaM and C-CaM contain two Ca\textsuperscript{2+}-binding sites, which are made up of so-called EF-hand helix-loop-helix motifs (Zhang and Yuan 1998). Structural analysis revealed that CaM has a
dumbbell shape comprising N-CaM and C-CaM, which are connected by a long, solvent-exposed α-helix, the so-called linker helix (Zhang and Yuan 1998). CaM can exist in either the Ca^{2+}-free state called apoCaM or in various Ca^{2+}-bound states. Most targets of CaM require three or four Ca^{2+} ions to be bound to CaM in order to become activated, so one can simplify the discussion by considering Ca^{2+}-CaM to contain three or four bound Ca^{2+} ions (Jurado et al. 1999). Both Ca^{2+}-CaM and apoCaM (Jurado et al. 1999, Bahler and Roads 2002, Vetter and Leclerc 2003, Fallon et al. 2005) are capable of binding target proteins, and both are involved in responding to intracellular Ca^{2+} levels. ApoCaM generally signals reactions of the cell to low intracellular Ca^{2+} levels and may act as a store of Ca^{2+}-unbound CaM, pre-bound to the target protein, ready to react with Ca^{2+} when it becomes available. By contrast, Ca^{2+}-CaM generally signals reactions of the cell to high intracellular Ca^{2+} levels.

The binding of Ca^{2+} ions causes a conformation change in CaM (Zhang et al. 1995, Zhang and Yuan 1998). Specifically, hydrophobic residues unexposed to the solvent in apoCaM twist and become exposed to the solvent in Ca^{2+}-CaM, creating a large, solvent-exposed hydrophobic surface in each EF-hand domain. These hydrophobic surfaces have been shown to be largely responsible for the binding of CaM to its targets (Zhang et al. 1995, Zhang and Yuan 1998). Some CaM-target proteins have been reported to interact with Ca^{2+}-CaM at two distinct sites in their amino acid sequence, while others interact at just one site (Zhang and Yuan 1998). It seems that the two represent separate classes of CaM binding peptides and bind to different conformations of Ca^{2+}-CaM (Zhang and Yuan 1998, Jurado et al. 1999). At
the center of the hydrophobic surface, CaM contains a deep hydrophobic cavity that anchors bulky aromatic or long alkyl amino acid side chains of the target proteins (Zhang et al. 1995, Zhang and Yuan 1998). In addition to the change in the EF-hand motifs, the binding of Ca\(^{2+}\) to CaM significantly reduces the backbone flexibility of the protein. The binding of protein targets further reduces the backbone flexibility of CaM throughout its entire sequence. Such protein targets of CaM include NHEs.

1.5.1 NHE1 and Ca\(^{2+}\)-CaM

Ca\(^{2+}\)-CaM binds to the C terminus of NHE1 and activates its transporter activity (Bertrand et al. 1994, Wakabayashi et al. 1994, Wakabayashi et al. 1997). The Ca\(^{2+}\)-CaM interaction is mediated by two CaM binding sites in the C terminus of NHE1, termed the C terminal Calmodulin Binding Domain A and C terminal Calmodulin Binding Domain B domains (CBDA and CBDB, respectively) (Bertrand et al. 1994). CBDA has a dissociation constant (\(k_d\)) of approximately 20 nM while CBDB has a \(k_d\) of approximately 350 nM (Bertrand et al. 1994). Like the majority of CaM-binding domains, CBDA and CBDB comprise a stretch of \(~20\) amino acid residues, contain positively charged residues and hydrophobic residues, and have the potential to form an amphiphilic \(\alpha\)-helix (Bertrand et al. 1994, Zhang and Yuan 1998). The hydrophobic residues of this \(\alpha\)-helix will interact with the two hydrophobic surfaces exposed in Ca\(^{2+}\)-CaM and the \(\alpha\)-helix's positively charged residues can make specific salt bridges with acidic residues in CaM (Zhang and Yuan 1998, Martin et al. 2004). The central linker region in Ca\(^{2+}\)-CaM then unwinds and N-CaM and C-CaM wrap around the \(\alpha\)-helix (Zhang and Yuan 1998, Martin et al. 2004). As a result, the shape of the Ca\(^{2+}\)-CaM-NHE1 complex is globular, as opposed to the dumbbell-shaped
structure of Ca^{2+}-CaM alone. The target α-helix, which may be unstructured in solution, forms an α-helix in the complex and its side chains make intensive interactions with the two hydrophobic patches in Ca^{2+}-CaM. In addition, residues other than those directly binding to CaM are suggested to be important for target interaction with CaM. The binding of Ca^{2+}-CaM to NHE1 leads to activation of the transporter activity (Bertrand et al. 1994, Wakabayashi et al. 1994). Indeed, swapping of CBDA to the equivalent region of the normally Ca^{2+} unresponsive NHE3 confers Ca^{2+} responsiveness to NHE3 (Wakabayashi et al. 1995). Since there are no crystal structures of Ca^{2+}-CaM bound to NHE1 (or indeed any crystal structures of NHE1) available it is unclear how exactly Ca^{2+}-CaM mediates the change in NHE1 transporter activity. One model that has been postulated is that CBDA and CBDB of NHE1 are part of an autoinhibitory domain that normally restricts NHE1’s transporter activity by binding to the N terminus of NHE1. Upon Ca^{2+}-CaM binding, the autoinhibitory domain can no longer bind to the N terminus, thereby activating NHE1 (see Figure 3) (Bertrand et al. 1994, Wakabayashi et al. 1994).

As a final note, there is evidence that regulation can also work the other way. NHE1 activity can lead to oscillations in intracellular Ca^{2+} levels (Yi et al. 2009). This is in the context of integrin signaling and recruitment of NHE1 to specific lipid raft domains of the plasma membrane.

1.5.2 NHE7 and Ca^{2+}-CaM

In contrast to the plasmallemal NHEs, little is known about the regulation mechanisms of NHE7 and other organellar NHEs. To begin to elucidate how NHE7 is regulated, we generated a human breast cancer MDA-MB-231 cells stably expressing
Figure 3. **NHE1 is regulated by a Ca\textsuperscript{2+} signal.** In the absence of Ca\textsuperscript{2+}, the Calmodulin Binding Domain A (CBDA) in the C terminus of NHE1 is part of an autoinhibitory domain that inhibits H\textsuperscript{+} binding to NHE1's ion transporter site, thereby inhibiting transporter activity. Upon signaling that results in elevation of intracellular Ca\textsuperscript{2+}, CaM binds to Ca\textsuperscript{2+} and can now bind CBDA, which can no longer bind to the N-terminal part of NHE1 and inhibit transporter activity.
C terminally tagged NHE7. We next immunoprecipitated the NHE7 and identified NHE7 binding proteins by mass spectroscopy (Kagami et al. 2008). CaM was one of the NHE7 binding partners identified in this study. Cell-based organellar tracer influx assays further showed that CaM inhibitors as well as Ca$^{2+}$ deprivation effectively blocked NHE7 transporter activity.

An interesting finding was that the C terminal tail of NHE7 has no homologous domain for CBDA and CBDB found in NHE1 (Figure 4). Calmodulin Target Database (http://calcium.uhnres.utoronto.ca) suggested a putative CaM binding site at A$^{195}$-A$^{213}$ of NHE7. Although NHE1 and NHE7 share substantial similarity in this region, a cluster of positively-charged amino acid residues are observed only in NHE7 (see Table 2). Moreover, based on the alignment of NHE7 and NHE1, and the membrane topology model by Wakabayashi et al. (2000), this putative CaM binding site falls in the second intracellular loop (IL2). As IL2 has been postulated as part of the ion binding site of NHE1 (Wakabayashi et al. 2000), this finding led us to hypothesize that CaM binding to IL2 of NHE7 causes conformational change(s) in the ion binding site and hence affects transporter activity and possibly ion selectivity (ie. $K^+$ over $Na^+$).
Figure 4. Alignment of C terminus Calmodulin Binding Domain A (CBDA) and second intracellular loop (IL2) for various NHE isoforms. Identical residues are in red, residues that conserve chemical nature are in blue.

<table>
<thead>
<tr>
<th>ISOFORM</th>
<th>CBDA</th>
<th>IL2</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHE1</td>
<td>RNNLQKTQRRLRSYNRHTLVA</td>
<td>LPL RQFTENL</td>
</tr>
<tr>
<td>NHE5</td>
<td>CGGLY KP RR RYKASC RHFIS</td>
<td>MPSRL FF DN L</td>
</tr>
<tr>
<td>NHE7</td>
<td>SPQ VYDNQE PL REED SDFILT</td>
<td>LKK RH FRRN L</td>
</tr>
</tbody>
</table>
2. Materials and Methods

2.1 Materials

All chemicals used were purchased from Fischer (Ottawa, Ontario). DNA oligonucleotides were ordered through Invitrogen (Burlington, Ontario). pET vector was a generous gift from the van Petegem lab (University of British Columbia, Biochemistry and Molecular Biology Department) and pGEX vector was purchased from Amersham (Piscataway, NJ). DNA restriction enzymes were obtained from New England Biolabs (Pickering, Ontario) and DNA sequencing was performed by Macrogen (Seoul, Korea).

Agarose gel electrophoresis supplies were purchased from Bethesda Research Laboratories (Bethesda, MD) and agarose was obtained from Invitrogen (Burlington, Ontario). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) supplies were purchased from Bio-Rad (Hercules, CA) and SDS was obtained from BioShop (Burlington, Ontario). CaM-agarose beads were purchased from Sigma (Oakville, Ontario) and Glutathione 4B Sepharose Beads from GE Healthcare (Piscataway, NJ).

Anti-glutathione-S-transferase (GST) antibody was purchased from StressGen (Victoria, BC) and goat anti-mouse antibody conjugated to horseradish peroxidase (HRP) was obtained from Jackson Laboratories (Bar Harbor, ME). Fully wet transfer supplies were purchased from Bio-Rad (Hercules, CA). Polyvinylidene fluoride (PVDF) membranes were obtained from Pall Corporation (East Hills, NY) and substrate for alkaline phosphatase was purchased from Millipore (Danvers, MA).
2.2 Construct expression and purification

2.2.1 Glutathione-S-transferase (GST) fusion proteins

Glutathione-S-transferase (GST) fusion proteins of NHE peptides were expressed in DH5α Escherichia coli using the pGEX 2T plasmid (Figure 5). Synthetic DNA oligonucleotides corresponding to sense and antisense of the peptides (see Table 1) were annealed together in the presence of buffer containing 10 mM Tris pH 8.0, 50 mM NaCl and 1 mM ethylene diamine tetraacetic acid (EDTA) by boiling for 5 minutes and then letting the solution cool to room temperature. Annealed oligonucleotides were then ligated to the EcoRI and BamHI sites of the pGEX2T vector and sequence was verified by Macrogen (Seoul, Korea). Purified DNA was then transformed into BL21 E. coli, expression was induced by isopropyl β-D-1 thiogalactopyranoside (IPTG) and the GST fusion peptides were purified from bacteria based on a modified version of the procedure of Smith and Johnson (1988). Briefly, the bacteria were grown to approximate density of 0.6 O.D.600 in 2 YT media at 37°C and 0.1 mM IPTG was used to induce expression of the peptides at 32°C for another 4 hours. E. coli were then lysed by incubation with 1% Triton-X-100 and protease inhibitor cocktail (Roche, IN, USA) on ice for 20 minutes, followed by sonication for 5 seconds twice, and a further incubation on ice for 20 minutes. GST fusion peptides were affinity purified with Glutathione 4B Sepharose Beads (GE Healthcare, Piscataway, NJ). 5 mL of lysate was incubated with approximately 50 μL bed volume of Glutathione 4B Sepharose Beads at 4°C for 2 hours with constant rotation. GST fusion peptides were then washed with phosphate buffered saline (PBS) four times and then eluted from the beads using elution buffer (50 mM
Table 1. Oligonucleotides used in this study.
Sequences are listed 5' to 3'. BamHI and EcoRI sequences at ends are in bold and underlined, and internal restriction enzyme sites are underlined.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHE7 IL2sh Sense</td>
<td>GATCCAAAAAACGCCATTTCTTTTGCAACCTGGGGTCCTAAGCTTG</td>
</tr>
<tr>
<td>NHE7 IL2sh Antisense</td>
<td>AATTCAAGCTTGGACCCAGCTTTGGAAGAAATGGGCAATTTCGTCATCAGGCTTTTG</td>
</tr>
<tr>
<td>NHE7 IL2 KKPL Sense</td>
<td>GATCCGGGTATTCCTTGCGCTGCGCCATTCTTTTGCACAACCTGGGTCATTCGGCATGATATCG</td>
</tr>
<tr>
<td>NHE7 IL2 KKPL Antisense</td>
<td>AATTCGATATCATGCCAAGATGGACCCAGCTTTGGAAGAAATGGGCAATTTCGTCATCAGGCTTTTG</td>
</tr>
<tr>
<td>NHE7 IL2sh KKRAAA Sense</td>
<td>GATCCGCCGCCCACTTTTCTTTGCAACCTGGGTCATTTCTGGCATGATATCG</td>
</tr>
<tr>
<td>NHE7 IL2sh KKRAAA Antisense</td>
<td>AATTCGATATCATGCCAAGATGGACCCAGCTTTTTCGTCATCAGGCTTTTG</td>
</tr>
<tr>
<td>NHE7 IL2sh FFAA Sense</td>
<td>GATCCAAAAAACGCCATTTCTTTTGCAACCTGGGTCATTTCTGGCATGATATCG</td>
</tr>
<tr>
<td>NHE7 IL2sh FFAA Antisense</td>
<td>AATTCGATATCATGCCAAGATGGACCCAGCTTTTTCGTCATCAGGCTTTTG</td>
</tr>
<tr>
<td>NHE1 IL2sh Sense</td>
<td>GATCCCGCTCGGCAGTTAACCAGAAACCTTGCCACCCTGATATCG</td>
</tr>
<tr>
<td>NHE1 IL2sh Antisense</td>
<td>AATTCGATATCATGCCAAGATGGGCAATTCTGG GCACCGGGCGGGGGGTCATCAGGCTTTTG</td>
</tr>
<tr>
<td>NHE7 E287I Sense</td>
<td>GATCTTACGCACTTTCTTTTGGAATTAGCGTCTCAAATGATGCTGTTG GCC</td>
</tr>
<tr>
<td>NHE7 E287I Antisense</td>
<td>GGCAACAGCATCATTAGGGACGCTAACTCCAAAAAGAGTGCGTAAGAATGTCGTCATCAGGCTTTTG</td>
</tr>
</tbody>
</table>

26
Figure 5. pGEX vector.
glutathione, 50 mM unpHed Tris-HCl, 0.1% Tritox-X-100 and 150 mM NaCl) at room temperature for 10 minutes. Purified GST fusion peptides were kept as small aliquots at -20°C and the protein concentration was determined by means of a bovine serum albumin (BSA) standard (see 2.3 below).

2.2.2 Maltose Binding Protein (MBP) fusion proteins

Maltose Binding Protein (MBP) fusion proteins of CaM possessing a histidine tag after MBP were expressed in Escherichia coli using the pET 28b TEV plasmid (Figure 6), a kind gift from Dr. van Petegem, and then purified based on a modified version of the procedure of Franke and Hruby (1993). BL21 E. coli were grown to approximate density of 0.6 O.D.600 in 2YT media at 37°C and 0.1 mM IPTG was used to induce expression of the constructs at 32°C for an additional 4 hours. E. coli were then lysed by incubation with 1% Triton-X-100 and protease inhibitor cocktail (Roche, Mississauga, Ontario) on ice for 20 minutes, followed by sonication for 5 seconds twice, and a further incubation on ice for 20 minutes. Next, 5 mL of precleared lysate was incubated with approximately 50 μL bed volume of Invitrogen Pro-Bond Beads (Burlington, Ontario), which are nickel beads, at 4°C for 2 hours with constant rotation. MBP fusion proteins coupled to the beads were then washed with PBS four times and MBP fusion proteins were eluted from the beads using elution buffer (500 mM imidazole pH 7.4, 1 mM CaCl2 and 250 mM KCl) at room temperature for 10 minutes. Purified MBP fusion proteins were kept as small aliquots at -20°C and the protein concentration was determined by means of a BSA standard (see 2.3 below).
Figure 6. pET vector.
2.3 Determination of protein concentration

SDS-PAGE was performed using 10% (for MBP fusion proteins) or 12% (for GST fusion peptides) acrylamide gels. Purified samples had SDS sample buffer (see 2.4 below for recipe) added to them and were boiled for 3 minutes and then loaded onto the acrylamide gels. Along with the purified samples, BSA samples of known concentrations were loaded. Purified samples were resolved in 10% (MBP fusion proteins) or 12% (GST fusion peptides) SDS-PAGE gels. Gels were then stained with Coomassie Blue dye for 30 minutes to overnight and destained for at least 2 hours using a destain solution containing 100 ml acetic acid and 900 ml ddH2O:Methanol (1:1). Protein concentration was determined by comparing the degree of staining of the purified samples to the staining seen with the BSA samples.

2.4 Pulldown assay

CaM binding was assayed using approximately 20 µL bed volume CaM-agarose beads. These were incubated with purified GST fusion peptides at 4°C for 2 hours in the presence of Buffer A or Buffer B. Buffer A contained 140 mM KCl, 10 mM HEPES pH 7.3 and 1 mM CaCl₂ while Buffer B contained 140 mM KCl, 10 mM HEPES pH 7.3 and 1 mM ethylene glycol tetraacetic acid (EGTA). Glutathione 4B Sepharose Beads were then washed with appropriate buffer (Buffer A for Ca²⁺ condition and Buffer B for EGTA condition) four times and then eluted with SDS sample buffer. SDS sample buffer contained 100 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 0.2% bромothемол blue and 100 mM dithiothreitol (DTT). For some experiments, the flowthrough fraction, consisting of the first wash solution, was kept and Western
blot was performed on it (see 2.5 below for details). Eluted samples were resolved in a 12% SDS-PAGE gel and visualized by Coomassie Blue staining (see 2.3).

For testing pH-dependence of CaM binding to GST fusion peptides, the standard protocol outlined above was modified. Two additional buffers were used to dilute GST fusion peptides and wash the beads. Buffer C contained 140 mM KCl, 10 mM MES pH 5.8 and 1 mM CaCl₂ and Buffer D contained 140 mM KCl, 10 mM MES pH 5.8 and 1 mM EGTA. A total of four conditions were tested, representing Buffers A-D. Otherwise, the pulldowns proceeded as outlined above.

2.5 Western blot

Western blot was performed on the flowthrough fractions from the pulldown assay (see 2.4). Five µL of the flowthrough fractions were resolved in a 12% SDS-PAGE gel (see 2.3) and transferred onto a PVDF membrane using a fully wet procedure. PVDF membrane was then blocked in 5% skim milk for 1 hour and then incubated with a 1:5000 dilution of mouse anti-GST antibody overnight at 4°C. Following extensive washing with 1X Tris-buffered saline with 0.075% Tween 20 (TBST), the blot was incubated with a 1:10000 dilution of goat anti-mouse antibody conjugated to HRP for 1 hour at room temperature. Finally, membrane was washed with 1X TBST for 1 hour and signal was detected by enhanced chemiluminescence (Millipore, Danvers, MA).

2.6 Isothermal Titration Calorimetry (ITC)

ITC is a biophysical technique used to determine the thermodynamic parameters of biochemical interactions. It is most commonly used to measure
binding of two proteins (Protein A and Protein B for example). It requires an
isothermal titration calorimeter, which is composed of two identical cells (see
Figure 7 for schematic). Sensitive circuits are used to detect temperature differences
between the reference cell (filled with buffer or water) and the sample cell
containing Protein A. Protein B, the ligand, is then titrated into the sample cell in
precisely known aliquots, causing heat to be either consumed or evolved, depending
on the nature of the reaction between Protein A and Protein B. The reaction may be
either exothermic, in which case heat is evolved, or endothermic, in which case heat
is consumed. Measurements consist of the time-dependent input of power required
to maintain equal temperatures between the sample and reference cells.
Observations are plotted as the power in μcal/sec needed to maintain the reference
and the sample cell at an identical temperature. This power is given as a function of
time in seconds (Ream et al. 1992). Through MicroCal Origin software, this data can
then be used to calculate various parameters, including $k_d$, of the interaction.

In this project, purified NHE7 IL2, NHE1 CBDA and CaM were dialyzed against
150 mM KCl, 1 mM CaCl$_2$, 10 mM HEPES pH 7.4 and 5 mM beta-mercaptoethanol.
Protein concentrations were determined by absorbance (Edelhoch 1967). Titrations
were then performed on an ITC-200 calorimeter (MicroCal, NJ, USA) at 25°C. CaM at
a concentration of 500 μM was titrated, separately, into NHE7 IL2 peptide at a
concentration of 50 μM and NHE1 CBDA peptide at a concentration of 50 μM using
one 4 μl injection followed by 29 injections of 10 μl titrant (CaM). No interaction was
detected for NHE7 IL2 and CaM. The results for NHE1 CBDA and CaM were
processed with MicroCal Origin 7.0 using a single binding site model.
Figure 7. *Isothermal Titration Calorimetry (ITC) setup.*
2.7 Assay for binding of CaM lobes

Binding of the lobes of CaM to NHE7 IL2 was assayed using three MBP fusion proteins: full-length CaM, N-CaM and C-CaM. 10 μg of each of these were incubated with 5 μg of NHE7 IL2 GST fusion peptide at 4°C for 1 hour and then approximately 20 μL bed volume of Glutathione 4B Sepharose Beads were added. The reaction was then incubated at room temperature for 30 minutes with constant rotation. Beads were then washed with Buffer A or Buffer B, depending on the condition (see 2.4 for recipes and details), and eluted with SDS sample buffer (again see 2.4 for recipe). Eluted samples were resolved in 10% SDS-PAGE gels and stained with Coomassie Blue dye (see 2.3) to test for MBP fusion proteins pulled down along with NHE7 IL2.

2.8 NHE7 IL2 versions used

Two different versions of NHE7 IL2 were used during the course of this project, differing in their size. Originally, NHE7 IL2 was identified as a putative CaM binding domain using online prediction software (Kagami et al. 2008). The prediction software predicted a very short CaM binding domain, of approximately 10 amino acids. This short version of NHE7 IL2 was termed NHE7 IL2sh. This NHE7 IL2sh was used in the testing of NHE1 IL2 CaM binding in comparison to NHE7 IL2 (where NHE1 IL2 was similarly 10 amino acids long) and as a basis for making the KKRAA and FFAA mutant versions of NHE7 IL2 to test for CaM binding of these mutants.

We were concerned, however, with how short NHE7 IL2sh was in comparison to known CaM binding sites, which are approximately 20 amino acids long.
Therefore, we also created a longer version of NHE7 IL2, which contained several hydrophobic amino acids putatively in TMs surrounding NHE7 IL2sh in addition to the original NHE7 IL2sh predicted by the online prediction software. This longer version of NHE7 IL2 was termed NHE7 IL2 and was approximately 20 amino acids long. It was used in the experiments looking at pH-dependence of CaM binding to NHE7 IL2 and the experiments verifying the validity of the CaM binding to NHE7 IL2 by comparison with NHE1 CBDA. In addition, the KKPL NHE7 IL2 mutant was based on this longer version of NHE7 IL2 and the ITC experiments were done with this longer version.
3. Results

3.1 NHE7 IL2, but not NHE1 IL2, binds to CaM

To test out the Ca^{2+}-dependent interaction between CaM and NHE1 IL2 or NHE7 IL2, we set out pulldown experiments in the presence of Ca^{2+} or EGTA (in order to chelate any Ca^{2+} present and so prevent the binding of Ca^{2+} to CaM) (see 2.4 for details). As a positive control, the CaM binding of NHE1 CBDA was also tested. Binding was assessed by the amount of GST fusion peptides pulled down in the presence of Ca^{2+} or EGTA, as assessed by the approximately 30 kiloDalton (kDa) band corresponding to the GST fusion peptides. Experiments were repeated at least three times and reproducible results were obtained.

NHE1 CBDA and NHE7 IL2 GST fusion peptide bands appeared significantly higher in intensity in the presence of Ca^{2+} than in the presence of EGTA (Figure 8). This suggests that both NHE1 CBDA-CaM binding and NHE7 IL2-CaM binding are Ca^{2+}-dependent. This is in good agreement with previous observations (Bertrand et al. 1994, Kagami et al. 2008).

Interestingly, no NHE1 IL2 GST fusion peptide was co-eluted with immobilized CaM, either in the presence of Ca^{2+} or EGTA (Figure 8). This suggests that NHE1 IL2 is entirely incapable of binding to CaM. Therefore, it appears that NHE7 IL2 but not NHE1 IL2 is capable of binding CaM. This is particularly interesting in view of the fact that the two regions (NHE7 IL2 and NHE1 IL2) are quite similar, with only a few amino acid residues not conserved (see Figure 4). However, there is evidence that even small amino acid changes between NHE isoforms have functional
Figure 8. **CaM binds to NHE7 IL2 and NHE1 CBDA but not to NHE1 IL2.**
Glutathione-S-transferase (GST) fusion peptides of GST-NHE7 second intracellular loop (IL2), GST-NHE1 IL2, and GST-NHE1 C terminal calmodulin binding domain A (CBDA) were incubated with calmodulin (CaM)-agarose beads in the presence of either 1 mM Ca\(^{2+}\) or 1 mM EGTA for 2 hours and then eluted off with sodium dodecyl sulfate (SDS) sample buffer (see 2.4 for recipe). Eluted samples were resolved in 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) gels and stained with Coomassie blue dye. A representative set of experiments is shown.
consequences (Mukherjee *et al.* 2006). Our results suggest that the amino acid residues uniquely found in NHE7 are of particular importance in binding to CaM.

### 3.2 Identification of critical residues for CaM binding

To better characterize the interaction between NHE7 IL2 and CaM, and to identify critical residues in NHE7 IL2 for CaM binding, we next conducted mutagenesis experiments. We paid special attention to basic, positively-charged amino acids (*Bagchi et al.* 1992, *Afshar et al.* 1994) and hydrophobic amino acids (*Bagchi et al.* 1992, *Afshar et al.* 1994, *van Petegem et al.* 2005, *Kim et al.* 2008) that may mediate the binding to CaM.

#### 3.2.1 NHE7 IL2 KKPL

The first mutant we tested was a KKPL mutant where the first two lysines of NHE7 IL2 were mutated to proline and leucine respectively in order to mimic NHE1 IL2, which has those amino acids in the equivalent positions (see Table 2) and was shown to be unable to bind to CaM (Figure 8).

We tested out the binding of the KKPL mutant to CaM by a pulldown assay of the GST fusion peptide of the KKPL mutant using CaM-agarose beads (see 2.4). Pulldowns were performed in the presence of Ca$^{2+}$ or EGTA and wild type NHE7 IL2 (*i.e.* NHE7 IL2 without any mutations) was used as a positive control. Binding was assessed by the amount of GST fusion peptide pulled down in the presence of Ca$^{2+}$ or EGTA, as judged by the intensity of the approximately 30 kDa band, corresponding to the GST fusion peptide. Experiments were repeated at least three times and reproducible results were obtained.
It was found that the KKPL GST fusion peptide band was of about equivalent intensity in the presence of Ca\(^{2+}\) or EGTA (Figure 8), although it was of lesser intensity than the wild type NHE7 IL2 GST fusion peptide band used as a positive control (Figure 9). This was confirmed by Western blot using an anti-GST antibody on the flowthrough fractions (see 2.5 for details), which represent the unbound fraction and showed a mirror image pattern to the bound fraction (Figure 9). Again, approximately equivalent amounts of KKPL GST fusion peptide were seen in the presence of Ca\(^{2+}\) or EGTA, while less unbound, wild type NHE7 IL2 GST fusion peptide was seen in the presence of Ca\(^{2+}\) than in the presence of EGTA (Figure 9). This suggests that the KKPL mutant binds to CaM in a Ca\(^{2+}\)-independent manner. We postulated that the mutations to hydrophobic proline and leucine in the KKPL mutant caused non-specific hydrophobic interactions. Therefore, in subsequent experiments we converted amino acids to alanines instead and examined their capability to bind to CaM.

3.2.2 NHE7 IL2 KKRAAA

The next mutant we tested was the KKRAAA mutant where the first two lysines and the first arginine of NHE7 IL2 were mutated to alanines. This mutant tested the contribution of positively-charged amino acids of NHE7 IL2 to binding of CaM.

We examined whether a GST fusion peptide of the KKRAAA mutant binds to CaM by pulldown assay using CaM-agarose beads (see 2.4). Pulldowns were performed in the presence of Ca\(^{2+}\) or EGTA and wild type NHE7 IL2 was used as a
Figure 9. **NHE7 IL2 KKPL binds to CaM in a Ca^{2+}-independent manner.**

Glutathione-S-transferase (GST) fusion peptides of GST-NHE7 second intracellular loop (IL2), and GST-NHE7 IL2 KKPL were incubated with calmodulin (CaM)-agarose beads in the presence of either 1 mM Ca^{2+} or 1 mM EGTA for 2 hours and then eluted off with sodium dodecyl sulfate (SDS) sample buffer (see 2.4 for recipe). Eluted samples were resolved in 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) gels and stained with Coomassie blue dye to obtain the bound fraction. The unbound fraction was generated by keeping the flowthrough and performing a Western blot on it using an anti-GST antibody (see 2.5 for details). A representative set of experiments is shown.
positive control, while NHE1 IL2 was used as a negative control (it was established to be unable to bind to CaM in Figure 8). Binding was measured by the amount of KKRAAA mutant GST fusion peptide pulled down in the presence of Ca$^{2+}$ or EGTA, as judged by the approximately 30 kDa band corresponding to the GST fusion peptide. Experiments were repeated at least three times and reproducible results were obtained.

Either in the presence of Ca$^{2+}$ or EGTA, GST fusion peptide of the KKRAAA mutant was not co-eluted with immobilized CaM (Figure 10). This suggests that the KKRAAA mutant is entirely incapable of binding to CaM. This furthermore suggests that positively-charged amino acids are important for binding of NHE7 IL2 to CaM, as their mutation led to loss of CaM binding. Finally, this suggests the importance of the KKR motif in the NHE7 IL2-CaM interaction and is consistent with previous reports indicating the importance of basic amino acids in CaM binding sites (Bagchi et al. 1992, Afshar et al. 1994).

3.2.3 NHE7 IL2 FFAA

In addition to the stretch of positively-charged amino acids, NHE7 IL2 uniquely contains two phenylalanines. Since the involvement of bulky hydrophobic amino acids has been implicated in CaM binding (Bagchi et al. 1992, Afshar et al. 1994, van Petegem et al. 2005, Kim et al. 2008), we next tested the binding of the FFAA mutant where the two phenylalanines of NHE7 IL2 were mutated to alanines.

To examine the binding of the FFAA mutant to CaM, we carried out a pulldown assay of GST fusion peptide of the FFAA mutant using CaM-agarose beads (see 2.4).
Figure 10. **NHE7 IL2 KKRAAA** and **NHE7 IL2 FFAA** are incapable of binding to CaM.

Glutathione-S-transferase (GST) fusion peptides of GST-NHE7 second intracellular loop (IL2), GST-NHE1 IL2, GST-NHE7 KKRAAA and GST-NHE7 IL2 FFAA were incubated with calmodulin (CaM)-agarose beads in the presence of either 1 mM Ca\(^{2+}\) or 1 mM EGTA for 2 hours and then eluted off with sodium dodecyl sulfate (SDS) sample buffer (see 2.4 for recipe). Eluted samples were resolved in 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) gels and stained with Coomassie blue dye. A representative result is shown.
Pulldowns were performed in the presence of Ca\(^{2+}\) or EGTA and wild type NHE7 IL2 was used as a positive control, while NHE1 IL2 was used as a negative control. Binding was assessed by the amount of FFAA mutant GST fusion peptide pulled down in the presence of Ca\(^{2+}\) or EGTA, as judged by the intensity of the approximately 30 kDa band corresponding to GST fusion peptide. Experiments were repeated at least three times and reproducible results were obtained.

No GST fusion peptide was co-eluted with immobilized CaM, either in the presence of Ca\(^{2+}\) or EGTA (Figure 10). This suggests that the FFAA mutant is entirely incapable of binding to CaM. This furthermore suggests the importance of the two phenylalanines for binding of NHE7 IL2 to CaM. Our results reiterate the importance of hydrophobic interactions in the binding of CaM to its target proteins.

### 3.3 pH-Independence of NHE1 CBDA and NHE7 IL2 Binding to CaM

We hypothesized that there could be a pH-dependent interaction between NHE7 IL2 and CaM because NHE7 IL2 contains a histidine residue missing in NHE1 CBDA (see Figure 4), which could become protonated upon change of pH in the physiological range. Such reasoning is based on the expected pK\(_a\) of the pyrole NH proton of histidine, which is 6.0 and hence close to neutral. Furthermore, it has been shown that in the plant *Arabidopsis thaliana* NHX1, a homologue of human NHE6-9, can bind to a CaM-like protein in a pH-dependent manner (Yamaguchi *et al.* 2005). This may be true for NHE7 as well. For all these reasons, we were interested in seeing whether pH can affect NHE7 IL2 binding to CaM.
To define pH-dependence of CaM binding to NHE7 IL2 and NHE1 CBDA, a pulldown assay was conducted by incubating GST fusion peptides of NHE7 IL2 and NHE1 CBDA with CaM-agarose beads (see 2.4 for details). Pulldowns were performed in the presence of Ca\(^{2+}\) or EGTA and under two different pH conditions: pH 7.3 and pH 5.8. These pHs were chosen because they lie on either side of the pK\(_a\) of histidine's pyrole NH, which is expected to be 6.0. Hence, these pHs should represent the unprotonated (pH 7.3) and protonated (pH 5.8) forms of histidine. These pHs are also close to the pH values of the cytosol and acidic organelar lumens. GST alone, coded by pGEX, was used as a negative control. We then compared the amount of GST fusion peptides pulled down in the presence of Ca\(^{2+}\) and EGTA, at pH 7.3 and pH 5.8, as judged by the intensity of the GST fusion peptide band, which ran at approximately 30 kDa. Experiments were repeated at least three times and reproducible results were obtained.

NHE7 IL2, and to some extent NHE1 CBDA GST fusion peptide bands were more intense at pH 5.8 than 7.3, although this was much less pronounced than the difference between Ca\(^{2+}\) and EGTA conditions (Figure 11). However, normalization of the obtained data with the amount of background binding in the presence of EGTA at pH 7.2 and pH 5.8 resulted in no significant difference in binding between the two pHs in the presence of Ca\(^{2+}\) for both NHE7 IL2 and NHE1 CBDA GST fusion peptides. This suggests that both NHE7 IL2 and NHE1 CBDA may bind to CaM in a pH-independent manner. The protonation status of the histidine in NHE7 IL2 appears to not be crucial for CaM binding.
Figure 11. **pH-independence of CaM binding to NHE7 IL2 and NHE1 CBDA.** Glutathione-S-transferase (GST) fusion peptides of GST-NHE1 C terminal calmodulin binding domain A (CBDA) and GST-NHE7 second intracellular loop (IL2), or just GST (pGEX), were incubated with calmodulin (CaM)-agarose beads at pH 7.3 and pH 5.8, in the presence of either 1 mM Ca\(^{2+}\) or 1 mM EGTA, for 2 hours and then eluted off with sodium dodecyl sulfate (SDS) sample buffer (see 2.4 for recipe). Eluted samples were resolved in 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) gels and stained with Coomassie blue dye. A representative result is shown.
3.4 Isothermal Titration Calorimetry (ITC)

ITC experiments were performed with affinity-purified CaM and GST fusion peptides of NHE7 IL2 and NHE1 CBDA (see 2.2 for details) and the quality of the purified proteins were evaluated by resolving in SDS-PAGE gels prior to ITC experiments. CaM was titrated, separately, into NHE7 IL2 and NHE1 CBDA GST fusion peptides at 25°C. ITC was used to characterize these interactions because this method allows the direct measurement of the thermodynamic parameters of the binding reaction, gives an unmatched level of accuracy and resolution (Velazquez Campoy and Freire 2005), and permits one to obtain an accurate value for $k_d$.

No interaction was detected between the NHE7 IL2 GST fusion peptide and CaM. By contrast, an interaction was observed with moderate binding affinity ($k_d = 5.52 \mu M$) between the NHE1 CBDA GST fusion peptide and CaM (Figure 12 and Table 2). The reaction caused a relatively moderate change in enthalpy ($\Delta H$) (Table 2), although the change was negative, suggesting enthalpy may drive the reaction. The change in entropy ($\Delta S$) was also negative (Table 2), suggesting the reaction was not driven by entropy.

The interaction between NHE1 CBDA and CaM has been previously published (Bertrand et al. 1994). However, we obtained a $k_d$ of 5.52 $\mu M$ while in the literature the value is 20 nM (Bertrand et al. 1994). Bertrand et al. (1994) used a recombinant protein corresponding to the entire NHE1 C terminus deleted specifically in CBDB (to obtain the value for CBDA) while we used a GST fusion peptide of just CBDA.
Figure 12. Isothermal Titration Calorimetry (ITC) results of CaM Titration into NHE1 CBDA.
Purified calmodulin (CaM) at a concentration of 500 μM was titrated into NHE1 C terminal Calmodulin Binding Domain A (CBDA) peptide at a concentration of 50 μM using one 4 μl injection followed by 29 injections of 10 μl titrant (CaM) using an ITC-200 calorimeter (MicroCal, NJ, USA) at 25°C.
Table 2. *Isothermal Titration Calorimetry (ITC) results of CaM Titration into NHE1 CBDA.*

Purified calmodulin (CaM) at a concentration of 500 μM was titrated into NHE1 C-terminal Calmodulin Binding Domain A (CBDA) peptide at a concentration of 50 μM using one 4 μl injection followed by 29 injections of 10 μl titrant (CaM) using an ITC-200 calorimeter (MicroCal, NJ, USA) at 25°C. Origin 7.0 software (MicroCal, NJ, USA) was then used to calculate thermodynamic parameters using a single binding site model.

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<td>N</td>
<td>$0.934 \pm 0.00503$</td>
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<td>ΔH (kcal/mol)</td>
<td>$-1.00 \times 10^{-7} \pm 0.07612$</td>
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<td>ΔS (cal/mol/deg)</td>
<td>$-9.61$</td>
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<td>Kd (μM)</td>
<td>$5.52 \pm 0.00572$</td>
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Hence, it is possible that residues surrounding CBDA are important for increasing the affinity for CaM and we were simply missing those in our GST fusion peptide.

In addition, Bertrand et al. (1994) used a different method from ours. Instead of ITC, Bertrand et al. (1994) used titration of fluorescence of dansyl-CaM to measure $k_d$. This could account for our different findings, especially if the binding of CaM to NHEs is primarily driven by entropy as opposed to enthalpy. This would make detection of the binding by ITC more difficult, although it should not affect the results obtained using fluorescence of dansyl-CaM. Such an entropy-driven interaction between NHEs and CaM could account not only for the smaller $k_d$ value obtained by Bertrand et al. (1994) for the interaction between NHE1 CBDA and CaM, but could also account for our inability to detect an interaction between NHE7 IL2 and CaM using ITC.
4. Discussion

4.1 Characterization of NHE7 IL2 binding to CaM

We tested out the binding of NHE7 IL2 and NHE1 IL2 to CaM. It was found that although NHE7 IL2 bound to CaM, NHE1 IL2 was not able to bind to CaM (Figure 8). This is particularly interesting in view of the fact that the two regions (NHE7 IL2 and NHE1 IL2) are quite similar, with only a few amino acid residues not conserved (see Figure 4). The functional consequences of such a difference in CaM binding between NHE7 IL2 and NHE1 IL2 could be very interesting. As CaM binding to NHE7 IL2 is specific, this interaction could have specific physiological effects.

The NHE7 IL2 region contains two lysines and one arginine. Mutation of the two lysines to proline and leucine resulted in residual CaM binding (Figure 9), whereas the mutation of the three basic amino acids to alanines completely abolished Ca\textsuperscript{2+}-dependent CaM binding (Figure 10). Likewise, mutation of the two phenylalanines in the middle of NHE7 IL2 to alanines completely abolished CaM binding (Figure 10). These data indicate that NHE7 IL2 binds to CaM via basic and hydrophobic amino acids, as previously reported in other CaM-binding proteins. To test whether IL2 is the sole CaM binding domain in NHE7, it is important to test binding ability of various mutants of full-length NHE7 in the future. This will be readily done by transfecting different mutants into cultured cells and conducting CaM pulldowns.

We also tested the pH-dependence of the CaM and NHE7 IL2 interaction, using the CaM and NHE1 CBDA interaction as a negative control. Some pH-dependency of
NHE7 IL2 binding to CaM was indeed observed (Figure 11), but normalization with the amount of background binding in the presence of EGTA resulted in no significant difference between the two pHs tested. Hence, our data suggest that NHE7 IL2 binding to CaM is likely pH independent. However, there is a possibility that pH-dependent CaM binding is context-dependent and the sensitivity and/or specificity of our pulldown assay was insufficient to detect such a pH-dependency. Indeed, our initial attempt of ITC experiments did not detect any binding between NHE7 IL2 and CaM. Alternative approaches such as band-shift assays under non-denaturing conditions should be considered. For example, non-denaturing PAGE could be used to directly detect the CaM-NHE7 IL2 complex. Since non-denaturing PAGE does not disrupt protein complexes, the CaM-NHE7 IL2 complex could be visualized with Coomassie Blue dye and the $k_d$ could be calculated from a series of experiments with known amounts of the CaM and NHE7 IL2, based on the intensity of the band for the CaM-NHE7 IL2 complex. In addition, use of recombinant proteins covering larger segments of NHE7 and/or untagged purified synthetic peptides should be considered in the future for further characterization of the NHE7 IL2-CaM interaction.

4.2 CaM lobes responsible for binding to NHE7 IL2

A novel finding in this project is the presence of an unconventional CaM-binding site that is significantly shorter than the previously characterized CaM-binding sites. A question remains: how the large CaM molecule can bind to such a site. Perhaps with only one lobe? Perhaps only one lobe at a time? Indeed, the ability of one lobe of CaM to bind with higher affinity than another to CaM binding proteins
is well documented. Several CaM binding partners, including CaM-dependent kinase II (Forest et al. 2008) and myosin light chain kinase (Persechini et al. 1994), have a higher affinity for C-CaM than N-CaM. Yet other CaM binding partners, like nitric oxide synthase (Spratt et al. 2007), have a higher affinity for N-CaM, but this appears to be more rare. In all cases, it is postulated that the lobe with the higher affinity for the target protein will bind first, followed by the binding of the second lobe.

The significance of this sequential binding of the lobes of CaM ties in to the different Ca$^{2+}$ binding properties of C-CaM and N-CaM. C-CaM has the higher affinity for Ca$^{2+}$ but N-CaM has the faster kinetics of Ca$^{2+}$ binding (Bayley et al. 1984, Teleman et al. 1986). Therefore, having C-CaM bind to a target protein first will result in CaM binding the target protein when intracellular Ca$^{2+}$ is only moderately elevated but has been elevated for awhile, as C-CaM becomes activated at lower concentrations of Ca$^{2+}$ than N-CaM but with slower kinetics than N-CaM. Conversely, having N-CaM bind to a target protein first will result in CaM binding the target protein when intracellular Ca$^{2+}$ is highly elevated but immediately after such elevation occurs, allowing for more transient and intense intracellular Ca$^{2+}$ elevation. When CaM binding partners are expected to respond to more transient Ca$^{2+}$ fluxes, as for example in the synapse during neuronal firing, N-CaM can then be expected to bind with greater affinity to these binding partners. On the other hand, when CaM binding partners are expected to respond to less intense yet stable Ca$^{2+}$ fluxes, as for example during NaCl reabsorption in the digestive tract, than C-CaM can be expected to bind with greater affinity to these binding partners.
To determine which CaM lobe(s) bind(s) to NHE7 IL2, we began to optimize a pulldown assay of the binding of N-CaM and C-CaM to NHE7 IL2 using Glutathione 4B Sepharose Beads (see 2.7 for details). Our initial attempts at this experiment were unsuccessful because of unexpected rapid degradation of these recombinant proteins. It would be important to re-optimize these experiments in the future. One could also test whether N-CaM and C-CaM together can mimic the binding of full-length CaM even though the lobes are not connected as in full-length CaM. There is evidence in the literature that this can occur with other CaM binding proteins that contain conventional CaM binding sites (Vetter and Leclerc 2003, Forest et al. 2008).

Finally, it is an interesting finding that the unconventional CaM-binding site of NHE7 IL2 still makes use of the same forces for binding to CaM as previously characterized, conventional CaM-binding sites. Namely, it still uses electrostatic and hydrophobic interactions to dock onto the CaM molecule. Further research on the exact details of how NHE7 IL2 binds to CaM, such as from an X-ray crystallographic study of the NHE7 peptide bound to CaM, will help establish how this interaction works and may help identify other proteins that bind to CaM in a similar manner. This is yet another avenue of research that would be interesting to pursue in the future.

### 4.3 Functional significance of the CaM-NHE7 interaction

The results delineated here are an important first step in understanding the interaction between Ca$^{2+}$ signaling and pH regulation. Two molecules that have been shown to be important for, individually, Ca$^{2+}$ signaling (CaM) and pH regulation
(NHE7) have now also been shown to interact together. To define the functional significance of the CaM-NHE7 interaction would be an important next step.

Our previous cell-based organellar tracer influx assays showed that various CaM inhibitor drugs as well as Ca\textsuperscript{2+} depletion markedly reduced pH-gradient dependent \textsuperscript{86}RbCl influx by NHE7 (Kagami et al. 2008). To further refine the previous findings, it is important to establish liposome-based \textit{in vitro} assays. Previous studies have successfully used yeast \textit{Saccharomyces cerevisiae} as an expression system and reconstituted the affinity-purified NHEs in liposomes (Nakamura et al. 2005). By mutagenizing several crucial amino acid residues responsible for CaM binding, direct effects of CaM-dependence on NHE7 transporter activity could be measured. Although such an \textit{in vitro} system may not adequately represent the complex \textit{in vivo} reality, it does allow one to study NHE7 and its transporter activity in greater detail and without the problem of interference from other ion transporters or channels.

Alternatively, functional implications of CaM binding to NHE7 could be tested in a more \textit{in vivo} system with a pH-sensitive Green Fluorescent Protein (GFP). This is an established method for measuring the pH within living cells (Robey et al. 1998) and could be used to measure the pH of intracellular organelles, specifically the Golgi in the context of NHE7. Then the function of CaM binding to NHE7 in maintaining organellar pH could be tested by means of several constructs of NHE7 fused to this pH-sensitive GFP and appropriate targeting of the pH-sensitive GFP to the Golgi lumen (\textit{i.e.} the luminal side of NHE7). A fusion with wild type NHE7 could
be used as a positive control, a fusion with mutant NHE7 unable to bind CaM (for example, the FFAA mutant) could be used as the experimental condition and a fusion with mutant NHE7 that is transport deficient could be used as a negative control. Indeed, some expression constructs as well Chinese hamster ovary cells stably expressing these constructs already made in our laboratory. One could test for differences in Golgi pH between these conditions and hence test for the effect of NHE7 binding to CaM on Golgi pH.

This research will help delineate the contribution of NHE7 to cellular functioning, particularly if some defect is seen in cells expressing the CaM-binding deficient NHE7 or the transport deficient NHE7. Little is known about the role of NHE7 and indeed any of the organellar NHEs. Because organellar NHEs are highly conserved from yeast to man, it is conceivable that they perform some essential function for the organism, but as yet the exact nature of that function is still unknown. Reverse genetics approaches using model organisms are required to address these fundamental scientific questions.

4.4 Possible roles of organellar NHEs and CaM in neuronal function

It has long been known that Ca$^{2+}$ signaling is vital for many physiological effects, especially in the nervous system. Not only is intracellular Ca$^{2+}$ the trigger for release of neurotransmitters (Morris et al. 1987), but Ca$^{2+}$ signaling is also vital for overall proper functioning of neurons. For example, Ca$^{2+}$ signaling has been implicated in schizophrenia. Schizophrenia is a mental disorder characterized by abnormalities in the perception or expression of reality and one of the most serious
kinds of mental disorder (Miyakawa et al. 2003). The Ca\textsuperscript{2+}-CaM dependent phosphatase calcineurin has been shown to be abnormally downregulated in schizophrenic patients (Gerber et al. 2003, Eastwood et al. 2005) and mice lacking calcineurin show symptoms similar to those of schizophrenic patients (Miyakawa et al. 2003). Furthermore, administration of antipsychotic medication has been shown to upregulate calcineurin (Rushlow et al. 2005), as well as other components of Ca\textsuperscript{2+} signaling, including CaM (Rushlow et al. 2009) in the rat. Indeed, it has long been known that some antipsychotic medications directly bind to CaM (Weiss and Greenberg 1980, Weiss et al. 1980). Therefore, Ca\textsuperscript{2+} signaling is clearly a key process disrupted in schizophrenia, suggesting normal Ca\textsuperscript{2+} signaling is vital for proper functioning of the nervous system.

More recently, the contribution of pH regulation to the nervous system has been shown to be important as well. It has been shown that pH regulation can be especially vital in neurons, and that NHEs are specifically implicated in various neuropsychiatric disorders. For example, NHE7 is an X-linked gene and its locus of Xp11 has been implicated in X-linked Mental Retardation (XLMR) (Claes et al. 1997). XLMR is a class of disorders characterized by some degree of mental retardation and some degree of mutation on the X chromosome. XLMR includes various specific disorders, including Angelman Syndrome. NHE7 may be directly involved in XLMR, much like NHE6, which has been directly implicated in an Angelman-Syndrome-like phenotype (Gilfillan et al. 2008) and whose locus of Xq26 has also been linked to XLMR (Shashi et al. 2000). Most recently, NHE9 has been implicated in autism.
(Morrow et al. 2008) and an attention deficit hyperactivity disorder (ADHD)-like phenotype (de Silva et al. 2003).

Autism is a severe neuropsychiatric disorder characterized by impaired social interaction and communication and by repetitive and stereotyped interests and behavior. It has long been known to have a genetic component, as it is highly heritable, but the extreme genetic heterogeneity of autism, and the high de novo mutation rate seen in autism, have both impaired the identification of genes associated with autism. Morrow et al. (2008) overcome this problem by studying related families with the same, heritable, genetic cause of autism. Specifically, they looked at large, heritable deletions in related families. One of the genes found to be partially deleted in a specific family was NHE9.

Furthermore, Morrow et al. (2008) specifically relate this deletion of NHE9, which encompasses the C terminus of the protein and starts from the last extracellular loop, to the mutation in NHE6, also a deletion starting with the last extracellular loop, seen to cause an Angelman Syndrome-like phenotype by Gilfillan et al. (2008) and to the mutation in NHE1, yet again a deletion starting with the last extracellular loop, seen in the SWE mutant mouse that is deficient for NHE1 (Cox et al. 1997) (see Figure 13). Especially in the case of the NHE6 mutation, the phenotype seen is remarkably similar to that of autism. Indeed, Angelman Syndrome is considered an autism-like condition, in addition to being an XLMR. It is often characterized by impairment in social interaction and communication.
Figure 13. Location of start of deletion that in NHE1 causes slow wave epilepsy, in NHE6 causes Angelman Syndrome, and in NHE9 may cause autism.
Such a widespread association between NHEs (NHE1, NHE6, NHE7, NHE9) and neuropsychiatric disorders clearly implies pH regulation to be vital for neuronal function. Indeed, such findings that pH regulation may be important in the nervous system is not surprising. After all, it has long been known that changes in extracellular and intracellular pH have been associated with changes in neuronal excitability (Chesler 2003).

Furthermore, normal and abnormal functioning of neurons has been associated with changes in pH. Specifically, depolarization of neurons has been associated with a Ca\(^{2+}\) dependent acidification of intracellular pH (Chesler 2003). In addition, large, stable changes in intracellular and extracellular pH have been associated with neuropsychiatric disorders. For example, a prolonged intracellular and extracellular acidification is seen both during and after seizures (Chesler 2003). Changes in pH are also associated with spreading depression, which is a slowly moving depression of electrical activity in the cerebral cortex that seems to be related to migraine, although it has been observed to accompany ischemia in the brain as well (Ruscak 1962). Ischemia is a loss of blood flow and most often occurs in the brain during stroke. Whether it is associated with migraine or ischemia, spreading depression is associated with a transient intracellular alkalinization followed by a prolonged acidification, both intracellular and extracellular. The acidification is thought to be metabolically mediated, as neurons produce excessive lactic acid during this phenomenon (Mutch and Hansen 1984). By contrast, the preceding alkalinization is thought to be mediated by overcompensation by HCO\(_3^-\).
/Cl− exchangers on nearby glial cells for the acidification of the extracellular space associated with neuronal activity (Mutch and Hansen 1984).

The brain possesses multiple way of regulating pH by means of several families of plasmalemmal ion transporters. The NHEs are just one such family, but electrogenic Na+/HCO3− transporters (especially in glial cells) and Na+ driven Cl−/HCO3− antiporters also exist and can regulate pH in the nervous system (Chesler 2003). Clearly, such extensive regulation of pH could not have evolved in the nervous system without a reason. Perhaps the reason is to have multiple levels of regulation of pH in case one breaks down and fails to act. The evolution of such a backup mechanism clearly indicates that pH regulation is important in the nervous system.

In addition to its direct importance to the nervous system, pH regulation may also be indirectly important through its effects on Ca2+ signaling. In this context, the connection to Ca2+ signaling shown in this project may explain some of the far-reaching effects of pH regulation in the brain. For example, it may be that the neurological symptoms in the NHE1 deficient mice (Cox et al. 1997, Bell et al. 1999) are at least partially due to indirect effects of intracellular pH on Ca2+ signaling, which has been long known to affect cell survival (Benveniste et al. 1988) as well as other important cellular processes. For example, the process of regulated secretion is dependent on Ca2+ signaling. Regulated secretion is the process by which proteins to be secreted are packaged ahead of time but are only secreted upon the receipt of a specific Ca2+ signal and it is of key important to the nervous system. Intriguingly,
regulated secretion is also known to be affected by pH, making it a good candidate to be regulated by the CaM-NHE7 interaction.

It should be emphasized that the interaction of NHE7 with CaM is not the only mechanism that links NHE7 to Ca\textsuperscript{2+} signaling. In general, the whole family of NHEs is responsive to Ca\textsuperscript{2+} in several ways. For example, there is the contribution of PKC, which requires Ca\textsuperscript{2+} for activity and, once active, can increase the transporter activity of NHE1 (Borgese \textit{et al.} 1992) and NHE2 (Alrefai \textit{et al.} 2001) while it inhibits the transporter activity of NHE3 (Alrefai \textit{et al.} 2001) and NHE5 (Attaphitaya \textit{et al.} 2001). Furthermore, this PKC-mediated regulation has been shown to involve NHE binding proteins, much like CaM-mediated regulation of NHEs, in which case the NHE binding protein is CaM. For example, the PKC-mediated inhibition of NHE3 is mediated through NHERF2 (Lee-Kwon \textit{et al.} 2003), which is a multi-PDZ domain containing protein that connects transmembrane molecules to the actin cytoskeleton and thereby regulates their localization. Specifically, NHERF2 stimulates the endocytosis of NHE3 upon PKC stimulation, thereby inhibiting NHE3 transporter activity (Lee-Kwon \textit{et al.} 2003).

The Na\textsuperscript{+}/H\textsuperscript{+} exchanger regulatory factor (NHERF) family of which NHERF2 is a member contains four members altogether (NHERF1-4) and has been shown to be expressed in various tissues, including the digestive tract (Yun \textit{et al.} 1997), lungs (Raghuram \textit{et al.} 2001), and the nervous system (Gatto \textit{et al.} 2003). The localization of NHERF1 in the nervous system (Gatto \textit{et al.} 2003) is of particular interest, as this implies a colocalization with NHE7 and other organellar NHEs enriched in the brain.
Although a direct interaction between NHERF1 and NHE7 has yet to be shown, there is potential for NHE7 to be regulated by NHERF1 in neurons, both due to the implied colocalization and due to the fact that NHERF1 has been shown to bind and regulate other NHE isoforms (Kurashima et al. 1999, Cardone et al. 2007). Such potential regulation of NHE7 by NHERF1 may be dependent on PKC and Ca^{2+}, similar to the regulation of NHE3 by NHERF 1 (Kurashima et al. 1999). Indeed, NHE7 is known to respond to PKC activation. Specifically, NHE7 has been shown to colocalize with CD44 in the plasma membrane upon PKC activation (Kagami et al. 2008). Such a promotion of localization to the plasma membrane has been shown to be mediated by NHERF proteins for the ROMK channel complex (Yoo et al. 2004). Therefore, it is possible that the localization of NHE7 to the plasma membrane in response to PKC activation may be similarly regulated by NHERF proteins. This would be an interesting hypothesis to test.

Changes in Ca^{2+} levels would clearly be expected to impact NHE7 in more than one way. For example, the localization of NHE7 at the plasma membrane after PKC activation may reflect a unique role of NHE7 in the plasma membrane, perhaps as a scaffold protein. Meanwhile, intracellular Ca^{2+} would also result in CaM binding to NHE7 and increasing its transporter activity. Such complications at the molecular level may correspond to complications at the physiological level in the phenotype of neuropsychiatric disorders.

The same may hold true for other tissues where NHEs have been shown to be important, for example in the heart. There, too, NHEs may be important for their
indirect effect on Ca\textsuperscript{2+} signaling as well as for their transporter function. In either case, this interesting connection between Ca\textsuperscript{2+} signaling and pH regulation is certainly worth further exploration and this project should be seen as only the beginning of the understanding (and research) still to come.

For example, after characterizing the interaction between NHE7 IL2 and CaM it would be worthwhile to expand the research into other NHEs and CaM. For example, it would be interesting to see which lobe(s) of CaM are important for its interaction with NHE1 CBDA and how NHE1 responds to CaM inhibitors on a functional level. The first could be elucidated by pulldown experiments using N-CaM and C-CaM and the second could be elucidated by re-constituting heterologously expressed NHE1 (for example, expressed in yeast) in an \textit{in vitro} liposome system. This system could then be used to measure transporter activity via a Na\textsuperscript{+} uptake assay. This assay relies on radioactive ions being transported inside the liposome by NHEs. Radioactive Na\textsuperscript{+} is used and the transport of Na\textsuperscript{+} into the liposome by NHE1 is used as a proxy for transporter activity. One would make sure the Na\textsuperscript{+} influx was due to NHE1 transporter activity by use of an NHE-specific inhibitor such as 5-(N-ethyl-N-isopropyl) amiloride (EIPA) to block this Na\textsuperscript{+} influx and by determining that this Na\textsuperscript{+} influx is pH dependent, as one would expect for NHE1 transporter activity. Although an \textit{in vitro} system may not adequately represent the complex \textit{in vivo} reality, it does allow one to study NHE1 and its transporter activity in greater detail and without the problem of interference from other NHE isoforms.
4.5 Conclusion

We have shown that NHE7 and CaM interact by means of an unconventional, extremely short CaM-binding site comprising NHE7 IL2. This interaction is likely to be pH-independent and is mediated by positively charged and hydrophobic amino acids of NHE7 IL2. This interaction is expected to affect the functionality of NHE7 and hence to have potential for great relevance to physiology and, specifically, the nervous system. Such potential for relevance to the nervous system is highlighted by the numerous NHE isoforms that have been implicated in neuronal function: NHE1, NHE6 and NHE9.

Future research needs to be done to elucidate the exact nature of the contribution to physiology and the nervous system of the NHE7-CaM interaction, however. There is much potential for more research to be done under this theme of intersection between pH and Ca$^{2+}$ signaling, as this is still a relatively new and unexplored area of research. In particular, the intersection of pH and Ca$^{2+}$ signaling could be tested in the context of the nervous system, as there is much potential for physiological relevance there.
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