SMALL MOLECULE INHIBITORS OF ACID SENSING ION CHANNEL-1

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A DISSERTATION

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Acid Sensing Ion Channel 1 is one of the many proteins in the Epithelial Sodium Channel/Degenerin family. The proteins in this family interact to form cation channels with unique biophysical properties and can all be inhibited by the small molecule amiloride. Their expression in many different cell types underlies their involvement in a large variety of physiological and pathophysiological processes. ASIC-1 containing channels, specifically, are an important therapeutic target for ischemic stroke, nociception, the invasiveness of glioblastoma cells, and many other processes including anxiety and memory formation. Of the members of the ENaC/Deg family, chicken ASIC-1 is the only protein for which crystal structures have been solved. Using homology modeling, structures of the human proteins in the ENaC/Degenerin family were deduced. Further computational methods were applied to examine the interaction of hASIC-1 with known small molecule inhibitors such as amiloride and Psalmotoxin-1, a highly potent peptide toxin. Experimental verification of these computational results was performed using whole-cell and single-channel electrophysiology. Amiloride analogs not known to interact with ASIC-1 were identified by computational studies and verified functionally, showing the strength of this technique for discovering new ASIC ligands. Fully understanding the interactions of these inhibitors with ASIC-1 should permit their manipulation for the benefit of those afflicted with glioblastomas, stroke, anxiety, or pain.

Keywords: Acid Sensing Ion Channel, Epithelial Sodium Channel, Psalmotoxin-1, Homology Modelling, Protein Docking, Drug Discovery
DEDICATION

This work is dedicated to the memory of my grandfather, Professor Dr. M. Afzal Hussain Qadri, a renowned researcher, philosopher, and academician; and also my amazing wife Laura, whose love and support have been like a dream, as she has kept me grounded, fed, and dressed throughout this process.
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INTRODUCTION

This work focuses on describing the interactions of known inhibitors of Acid Sensing Ion Channel-1 using computational and experimental techniques. It is hypothesized that by understanding these interactions, one will be able to find or develop novel small molecule inhibitors of channels formed by the proteins in the Epithelial Sodium Channel / Degenerin family that would be useful in a number of pathological processes.

Epithelial Sodium Channel/Degenerin Proteins

Channel proteins play an important role as pathways for molecules into membrane protected compartments. This simple gateway function belies a complexity that allows them to play a role in a wide range of processes. This work focuses on the Epithelial Sodium Channel (ENaC)/Degenerin (Deg) family of ion channel proteins. Individually, one might expect a single protein unit in this family to be a transmembrane receptor, unable to perform the basic conduction function of a channel, as each subunit contains just two hydrophobic membrane spanning domains, a large extracellular loop containing multiple cysteine rich domains, and relatively small cytoplasmic tails, visualized in Figure 1 (1). However, interactions with other identical proteins, or other proteins within the family, create a conductive pathway for cations, with functional, biophysical, and crystallo-
graphic data arguing for a number of different stoichiometries for the channel assembly (2-16). In humans, there are two subfamilies represented from the ENaC/Deg superfamily, the eponymous Epithelial Sodium Channel proteins and the Acid Sensing Ion Channel (ASIC) proteins, with the Intestinal Sodium Channel (INaC) protein standing alone as an outlier, as visualized by the circular cladogram shown in Figure 2. In addition to the shared topology and sequence identity ranging from 15-60% across the family, the members of this family of proteins are all able to interact with the diuretic amiloride leading to inhibition of the conductive function of the assembled channel (1).

A crystal structure is known for a channel made of homomeric chicken ASIC-1, showing a trimeric arrangement which may be conserved across the ENaC/Degenerin family (15,16). This is of note as the crystal structure of a protein is challenging to obtain. As of November 24th 2009, the UniProtKB/Swiss-Prot protein knowledgebase contained sequence data for 512,994 putative proteins. Of those, only about 3% or 15 thousand unique proteins have some experimentally derived structural data associated with them (http://ca.expasy.org/sprot/relnotes/relstat.html). The majority of these structures are of bacterial proteins and/or soluble proteins. Membrane protein structures are far more challenging to obtain.

*Epithelial Sodium Channels*

Though this thesis will focus mainly on the ASIC proteins, it is informative to appreciate the plethora of data available with regards to the ENaC proteins, as much of our understanding of ASIC function is viewed from the perspective of the ENaC literature. In
humans, there are four well defined members of the ENaC family expressed: α, β, γ, and δ ENaC, with α and δ ENaC showing alternative splicing, as shown in Figure 2. The proteins appear to be expressed in a variety of tissues, but when tested for function in heterologous expression systems with other ENaC subunits, either the α or δ subunit is generally required to form conductive channels (1). The β and γ subunits appear to play a role in the trafficking and surface expression of the channel proteins (17,18), although there is a report of these subunits forming a conducting channel after a prolonged expression period in the *Xenopus* oocyte expression system (19).

 Often, channels made of ENaC proteins are discussed as a singular entity, referred to as the Epithelial Sodium Channel. The idealized or prototypical ENaC is the channel composed of α, β, and γ ENaC proteins found in the distal nephron. This kidney ENaC channel is a highly sodium selective channel $P_{\text{Na}^+}/P_{\text{K}^+} \geq 100$, is constitutively active, and shows an IC$_{50}$ of 100 nM for amiloride (1). Expressed in the apical membrane of renal epithelia, this channel transports $\text{Na}^+$ into the cell, helping control the excretion of sodium in the urine and overall volume regulation in the host (1). Beyond this prototypical channel, other channels proposed to contain ENaC subunits have been noted throughout the body, with a variety of constitutively active cation conductances sensitive to the drug amiloride over a 0.1 – 5 μM range found to be expressed in different cells (1,20).

 This diversity of function and plasticity shown by channels composed of ENaC proteins can be due to differences in the composition of the channel or post-translational modifications to the channels. For example, it is known that ENaCs are methylated, glycosylated, phosphorylated by kinases, and cleaved by proteases, any of which can modify the function of the assembled channels (1). Furthermore, the α or δ subunit can be com-
combined with a combination of the β or γ subunit to form channels with different conductive and amiloride-sensitive profiles (1). The permutations further increase if one allows for the creation of cross-clade channels composed of ENaC and ASIC proteins (21,22).

Acid Sensing Ion Channels

As shown by Figure 2, there are four ASIC genes found in humans, numbered 1 through 4, with a number of splice variants encoding a total of nine unique proteins. The homoclade channels formed by the ASIC proteins are proton-gated cation channels, opening rapidly in the presence of extracellular acid. Their fundamental function appears to be as acid transducers, converting an acidic extracellular environment into a cellular signaling event, most likely by allowing for the conduction of cations into the cell (23). In vitro, these channels activate in the pH range of 3.0 – 7.0, with homomeric ASIC-1 channels having an EC50 at about pH 6.0 while homomeric ASIC-2 channels show an EC50 at about pH 4.0 (1). Although ASIC containing channels are primarily sodium conductors, the PNa+/PCa2+ for homomeric ASIC-1a has been calculated to be 2.5 or 18.5 (24,25).

As much of this work will focus on ASIC-1, a point of confusion in the literature, which revolves around the asic-1 gene, should be clarified. The asic-1 gene has two described splice variants, ASIC-1a and ASIC-1b. In humans, the NCBI designates ASIC-1a as the full length transcript while ASIC-1b is the transcript missing a 46aa insert just before the second transmembrane domain. As ASICs were first studied in rat dorsal root ganglion and rat ASIC-1a (rASIC-1a) is virtually identical to human ASIC-1b, there is
some debate over which human splice variant should be called ASIC-1a. However, rat ASIC-1b, which is also described as ASIC-β, is very divergent from ASIC-1a, possessing a very different 172aa N-terminal tail (26). This confusion can readily be seen by examining the work of Gunthrope et al. where they state in their title that human embryonic kidney (HEK) cells express hASIC-1a (27). Examining their primers and RT-PCR products, it is apparent that HEKs have mRNA for both hASIC-1a and hASIC-1b, but the predominant message is for the smaller hASIC-1b isoform. This confusion is apparent in papers by other groups as well; when the Welsh group published a work on hASIC-1a in 2003, what they called hASIC-1a was once again the NCBI hASIC-1b as they describe their splice variant as being 528aa and not 574aa (28). A thorough examination of each paper referencing hASIC-1a shows no electrophysiological recordings of what can be verified as the 574aa hASIC-1a current. This is an important point as the insertion of 46aa before the second transmembrane domain of this ion channel may have important functional consequences.

Regardless, both in rats and humans, these two ASIC-1 isoforms have been detected throughout the CNS, specifically in areas of high synaptic density, localizing primarily to the soma and processes of neurons (29,30). The other ASIC isoforms are also known to be highly expressed in both the central and peripheral nervous system (1). This expression suggests the channels may play a role in sensory perception and various neural processes (31,32).
**Functional Differences between Homoclade ENaC and ASIC Channels**

Although the ENaC and ASIC proteins are part of the same family and have similar functions, there are considerable differences between the proteins and assembled channels. At the functional level, the primary difference is one of activation; ENaCs are constitutively active or modified by proteases to become active while ASICs are gated by a drop in extracellular pH (1). More precisely, ENaCs exhibit open and closed states, while the ASICs exhibit those states with an additional inactivated or desensitized state where the channel stops or attenuates conduction despite the continued stimulation by the acidity of the extracellular milieu, as shown in Figure 3 (1,22). This difference may have trickle down effects on the regulation of the channel, where control of ENaC function would need to be primarily determined by the number of channels at the surface while ASIC function could be modulated less by trafficking as the channels are not constitutively active. Interestingly, if homoclade ASIC channels are mutated at the degenerin site to become constitutively active, their activity leads to cell death (1). Another important difference is the presence of naturally occurring peptide toxin inhibitors of homoclade ASIC containing channels, which are not found for homoclade ENaC containing channels (33,34), though there is a peptide self inhibitory domain in some ENaC subunits that is released by proteolytic cleavage (35).

**Importance of ASICs in Pathologies**

There are a number of therapeutic opportunities afforded by manipulation of ASIC protein function or expression, including pain states, psychiatric disorders, neurodege-
nerative diseases, and cancer (21,31,36,37). This section serves to discuss the potential clinical impact of inhibitors of ASIC function in these pathologies.

ASICs and Pain

The ASIC proteins as well as acid induced currents have been found in cells of the peripheral nervous system (1,36). It is also known that acidosis accompanies inflammatory and pain states. This suggests that perhaps ASIC containing channels play a role in the sensing of pain or nociception. Verification has been provided by studies in ASIC-3 knockout mice, where the absence of this ASIC led to a reduction in the sensitivity of the animals to high intensity pain stimuli (38). A peptide toxin inhibitor of ASIC-1 injected intrathecally in mice was also able to attenuate pain and activate the enkephalin pathway (39). Moreover, acid induced pain in humans was attenuated by treatment with amiloride (40). This suggests that inhibitors of ASICs may play a role in the treatment of pain, while avoiding the behavioral issues associated with opiates (39).

ASICs and Psychiatric Disorders

Studies of ASIC-1 in mice have implicated it in synaptic plasticity, learning, and memory formation (30,41,42). In the CNS, the protein appears to localize with the post-synaptic density-95 protein (PSD-95), which likely targets ASIC-1 to areas of high synaptic density (41,42). Behavioral tests of mice overexpressing ASIC-1 found increased acquired fear related behavior (30), while ASIC-1 knockout mice showed a deficit in
cued and contextual fear conditioning (41), suggesting that perhaps ASIC-1 could play a role in anxiety or fear learning. However, a case-control twin study showed no association between polymorphisms of ASIC-1 and anxiety spectrum disorders (43). Still, functional tests with ASIC inhibitors in animal models found that ASIC inhibition was able to produce antidepressive, sedative, and anxiolytic effects (44-46). The exact role and mechanism of ASIC in anxiety and fear learning remains to be elucidated, but the data suggest that inhibition of ASICs can play a role in the treatment of anxiety or depression states.

**ASICs and Neurodegenerative Disorders**

The ASIC proteins in the CNS have been shown to play a role in a handful of neurodegenerative disorders (31), including ischemic stroke (47), Parkinson’s disease (48,49), epilepsy (50-52), Huntington’s disease (53), and autoimmune encephalitis (54). The mechanism by which ASIC proteins play a role in these pathologies varies and is still under study.

For example, during ischemic stroke there is a localized reduction in the extracellular pH. This acidosis is hypothesized to activate homomeric ASIC-1 channels, leading to elevations of intracellular calcium and cell death. As noted earlier, the $P_{Na^+}/P_{Ca^{2+}}$ for homotrimeric rat ASIC-1a has been calculated as 2.5 by one group and as 18.5 by another group (24,25). The divergence between these two values is likely due to the difficulties with calculating the permeability for a channel that is allosterically modulated by the divalent cation in question as well as the innate kinetics of the channel making it difficult to
record accurate measurements (55-60). While there is debate over the $P_{Ca^{2+}}$ of homotrimeric ASIC-1 channels, it has been shown by some that cells expressing homotrimeric rat ASIC-1a, natively or heterologously, can respond to an acidic pH pulse with an increase in cytosolic calcium (61). This increase is due to extracellular calcium influx, as it still occurs after the endoplasmic reticulum is emptied by thapsigargin, and is sensitive to amiloride (61). This increase in calcium is postulated to lead to increased cell death secondary to acidosis; cells expressing rat ASIC-1a are more sensitive to cell damage by incubation in pH 5.0 media than cells without the ion channel as measured by an LDH release assay (61). This held true for COS-7 cells exogenously expressing rat ASIC-1a as well as for hippocampal neurons natively expressing rat ASICs (61). However, heteromeric channels formed of ASIC-1 and ASIC-2 are calcium impermeable. In rat models, global ischemia upregulated ASIC-2 expression which would theoretically increase the population of heteromeric ASIC-1/ASIC-2 channels, acting to protect neurons from further ischemic attacks (62).

Further in vitro models, as well as in vivo mouse and rat models, of stroke have shown that inhibition of ASIC-1 effectively protects neurons from acidosis or ischemia (47,63-66). In vivo animal data regarding ASIC-1 suggests that inhibition of this channel will be effective up to 5 hours after the stroke rather than the narrow one hour window of NMDA antagonists (65). However, there is some slight controversy to this calcium based hypothesis, as Samways et al were unable to see a significant increase in intracellular calcium in physiological conditions in a large number of cells natively or exogenously expressing ASIC-1 (67). The authors observed increases in intracellular calcium in a small population of chicken dorsal root ganglia neurons, but the majority of the chicken DRG
cells and the entirety of the HEK293 and COS7 cells did not show increases in [Ca$^{++}$], when expressing chicken ASIC1 or human ASIC-1b (67). Their difficulty in showing significant calcium permeability of ASIC-1 channels, combined with the transient and desensitizing nature of ASIC-1 currents, suggests that there may be more than a calcium conductance playing a role in the neuronal death during stroke.

Another mechanism of action was postulated for the role of ASICs in Huntington’s disease, where mutations in the *huntingtin* gene lead to the accumulation of a mutant protein with an expanded polyglutamine tract and neuronal damage (68). Studies using in vitro and in vivo models of Huntington’s disease showed that the use of the amiloride or shRNA to ASIC genes was able to reduce the impact of Huntington’s disease (53). The authors showed that reducing ASIC-1 or ASIC-2 function or expression led to an increase in the activity of the ubiquitin-proteasome system and showed a decrease in the toxic aggregation of huntingtin-polyglutamine (53). The chemical inhibitor and ASIC-1 knockdown might suggest this could be due solely to ASIC-1’s calcium conductance capabilities, but similar results were found with knockdown of ASIC-2 which is known to form calcium impermeable channels (1,53). Still, this work is promising and suggests a role for ASICs in this disease and possibly other CNS diseases where protein aggregation leads to neuronal death.

**ASICs and Cancer**

The involvement of ASICs in cancer is a bit more complicated than their role in the earlier pathologies. An initial report noted the presence of ASIC and ENaC subunits
and, more importantly, that a constitutively active amiloride sensitive current was expressed in human malignant glioblastoma primary cultures as well as human glioma cell lines. This conductance was not present in lower grade gliomas or in noncancerous astrocytes (69). The ability of this amiloride sensitive glioma conductance showed some selectivity for cations, was inhibited by a potent and specific peptide inhibitor of ASIC-1, but was not acid gated like a homoclade ASIC containing channel (70). It was observed transfection of dominant negative constructs, which decreased expression of the ENaC subunits, was able to disrupt the glioma conductance, suggesting that perhaps this is a heteroclade channel is created by interactions between ENaC and ASIC proteins (21).

Another group noted the presence of a pH activated, heteromeric ASIC channel in cultured adenoid cystic carcinoma cells which was not present in normal salivary gland epithelia (37). The presence of these amiloride sensitive channels in malignant cells and absence in benign cells suggests the possibility that these conductances may be useful therapeutic targets. Indeed, the use of interventions that inhibit the amiloride sensitive current in glioma cells appears to decrease the migration and volume regulatory capabilities of the cancerous cells and a similar role has also been postulated for K⁺ and Cl⁻ channels (71-74).

Inhibitors of ENaCs and ASICs

The prior section elaborated the usefulness of inhibitors of the ASIC and ENaC proteins in numerous pathologies. As noted earlier, the ENaC and ASIC proteins form channels that are all inhibited by the small molecule drug amiloride. There are also other
reagents known to inhibit ENaC or ASIC protein function. A brief overview is provided on the history and function of the known inhibitors.

**Amiloride and Other Small Molecule Inhibitors**

Amiloride was first described as a K⁺ sparing diuretic in 1965 (75). The drug is thought to inhibit ENaC and ASIC containing channels by mimicking a cation, showing a positive charge at physiological pH, and physically occluding the conductive pathway or pore (76). Derivatives of amiloride have been developed and used to dissect sodium transport pathways in a wide variety of systems (77). Although amiloride shows some specificity for ENaC and ASIC proteins, amiloride and related analogs are known to interact with many other molecules. It has been shown to interact with sodium transporters (77), viral ion channels (78), DNA (79,80), and the urokinase-type plasminogen activator (81). This pleiotropy in function makes understanding results based solely on inhibitor data challenging, but by varying the concentration of amiloride it is generally accepted that ~ 1 μM doses inhibit typical ENaC channels, while concentrations in the 10-100 μM range also inhibit ASIC or atypical ENaC channels (1), such as those formed by αβ-ENaC or δ-ENaC containing channels (82,83). In terms of clinical use, amiloride has been safely used for the treatment of hypertension since the early 1970s (84). However, the dose of amiloride needed to inhibit ASIC containing channels is too high to retain the specificity of the relatively low dose therapy used for management of hypertension.

There are some other small molecules that have been shown to inhibit ASIC containing channels. Some of the non-steroidal anti-inflammatory drugs (NSAIDs) such as
aspirin, diclofenac, and the 2-arylpropionic acid (2-APA) derived drugs ibuprofen and flurbiprofen have been shown to inhibit the function of ASIC-1 and/or ASIC-3 at doses much higher than what is needed for the anti-inflammatory effects (85). The $K_i$ for these effects is in the 100-500 μM range, and a mechanism is unclear, though it does appear to be distinct from the anti-inflammatory role of the drugs (85). There is also a novel drug, A-317567, which appears capable of inhibiting ASIC currents, showing some selectivity for ASIC-1 and ASIC-3 over ASIC-2, with an IC$_{50}$ ranging from 2-30 μM (86). This small molecule has shown some usefulness but it is not commercially available and is not specific enough to differentiate between ASIC currents (45,46).

Peptide Toxins

ASIC containing channels are also inhibited by peptide toxins found in nature. For ASIC-1 channels, a peptide found in the venom of Trinidad Chevron tarantula *Psal-mopoeus cambridgei*, PcTx-1, is commonly described as a potent and specific inhibitor of homomeric ASIC-1 channels (34,87). The IC$_{50}$ for PcTx-1 with ASIC-1 is ~1 nM (1). The interaction of this peptide with ASIC-1 is examined in Chapter 2. A peptide found in the sea anemone *Anthopleura elegantissima*, APETx2, is capable of interacting with ASIC-3 containing channels, inhibiting homomeric channels with an IC$_{50}$ of ~ 65 nM while inhibiting some heteromeric ASIC-3 containing channels with an IC$_{50}$ between 100 – 2000 nM (33,88,89). Although these peptides are extremely potent, there are difficulties associated with synthesis and delivery of peptides into the CNS. For example, PcTx-1 is
known to not readily cross the blood brain barrier when given intravenously or orally, though retrograde transport can occur through intranasal administration (90).

Furthermore, there are some concerns with PcTx-1 due to the method by which it inhibits ASIC-1 function. Rather than blocking the pore or conductive pathway, this toxin appears to bind the desensitized state of the homomeric ASIC-1 channel (91) and/or alter the pH sensitivity of the channel (92). At first glance this may seem trivial, but under certain circumstances, such as when the channel is not likely to desensitize, PcTx-1 alone is able to activate the channel (67) or it may make the channel more sensitive to activation by protons(92). In theory, this shift in the pH sensitivity, combined with activation of the calcium conductance of ASIC-1, could lead to certain ischemic stroke situations where treatment with PcTx-1 leads to more damage than not treating due to these modulatory effects of the toxin. Furthermore, the specificity of PcTx-1 for ASIC-1 homomers is under debate, as there is some evidence it interacts with heterocline ASIC and ENaC channels (22).

The Search for ASIC Inhibitors

As this Chapter has thus far established the role of ENaC and ASIC proteins in pathologies as common as pain and depression to rare genetic disorders such as Huntington’s disease, and also explained the problems with current ASIC inhibitors for clinical treatments, it should be evident that small molecule drugs capable of specifically and potently inhibiting specific channels composed of ASIC subunits could be of great clinical utility. This work began as a project leveraging the discovery that NSAIDs inhibited AS-
IC currents to search for drugs in the 2-arylp ropionic acid family of proteins that were capable of inhibiting ASIC-1 currents (93). As the project progressed, the low potency of the NSAIDs and difficulty with consistent observation of the effect in different expression systems led to repeated setbacks. Fortunately in 2007, the first crystal structure of an ASIC was published (16). Thus, the search for inhibitors was able to use well described computational and structural tools rather than time consuming electrophysiological assays to isolate novel inhibitors.

There are advantages and disadvantages to the computational approach as compared with a functional experimental approach. The primary advantage is an increase in the number of drugs that can be screened and examined, while the primary disadvantage lies with the post-computational validations of the target proteins. Electrophysiology is normally used to experimentally screen putative ion channel inhibitors. However, electrophysiology based assays are generally time consuming and challenging, and although high throughput automated electrophysiology technology is commercially available, the equipment and consumables are generally very expensive and the applications relatively inflexible as compared to a conventional electrophysiology system (94). Although fluorescent dye based assays can also be designed for examining channel function (95,96), the specificity of the dye signal for the specific ion channels and a model system must be established, and for ASICs, the absence of interference between the dye and pH changes examined. With ENaC and ASICs, this is possible to do, and has been utilized in HEK293 cells, which endogenously express ASIC channels (27), using pH-insensitive, voltage-sensitive dyes (97). Still, the capability to screen a large number of novel chemicals in silico exceeds that of most in vivo assays.
The validity of computational data are often questioned, as most computational protocols require approximations and simplifications, partially due to limitations of either hardware, software, or the mathematical representations used. At the simplest level, a protein or molecule is reduced to a set of spheres and springs parameterized with mathematical values based on theoretical models or empirical data, which at first seems to be far removed from the reality of the molecular world, but is capable of reproducing many basic and complex effects (98). A problem with much of the computational work at the level of protein and small molecule docking performed in this work, is the inability to capture the full flexibility of the target or ligand, explicitly consider water or solvent molecules, and also the inability to capture chemical reactions that could occur upon interactions between two molecules, in terms of not only induced conformational changes but also possible chemical modifications made. Though there are computational techniques that in some way can account for these effects, the computational workload increases dramatically when considering these events.

The power of computational techniques is limited by the availability of high quality structures of the target proteins, especially for channel or membrane proteins (99). As noted earlier, structures are challenging to obtain. For channel proteins this is especially challenging due to the difficulty of expressing, purifying, and crystallizing these membrane spanning proteins (100). To date, 554 crystal structures of membrane proteins have been described, though only 204 of those are unique (Membrane Proteins of Known 3D Structure, http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html), as compared to the approximately 49,000 different x-ray structures of proteins deposited in the RCSB protein data banks (www.rcsb.org). For comparison, it is thought that somewhere be-
tween 15-39% of the human proteome is comprised of membrane proteins (101). Thus, the description of the crystal structure of a homomeric chicken ASIC-1 channel was an unexpected but greatly appreciated discovery (16).

*The Chicken ASIC-1 Crystal Structure*

Eric Gouaux’s group was able to crystallize a chicken ASIC-1 channel, resolving the structure to 1.9 Å (16). The structure showed that a homoclade ASIC channel was composed of three subunits, a finding inconsistent with the predominating tetramer view for homoclade ENaC channels (1,16). The initial structure, 2QTS, was of a construct lacking N- and C- tails and showed no channel activity (16), the group revisited the problem using a minimal functional channel protein, describing another crystal structure 3HGC of the channel at a resolution of 3.0 Å (15). The 2QTS structure contains 6 sub-units arranged into two channels while the 3HGC structure is of one single subunit, with a symmetry operation required to generate the trimeric biologically relevant structure. There are some issues with the structures, for example the 2QTS structure showed an odd kink in the transmembrane domains that could have been caused by crystal lattice contacts which were not apparent in the 3HGC structure (15,16). Furthermore, despite identical descriptions of the expression and purification procedures, the 3HGC structure did not show any of the glycosylations present in the 2QTS structure (15,16). Neither structure contained explicit data regarding the conductive pathway, as they both appear to be in a desensitized state, likely due to the low pH during crystallization, however soaking crystals of the minimal functional channel construct in cesium ions led to the generation
of 3IJ4 which shows some possible cation binding sites (15,16). A clear pathway for cations however was not observed. While the lack of an open structure and/or a completely defined conductive route was disappointing, the structure itself allowed for a flurry of work using both computational and experimental techniques to dissect the ENaC and ASIC proteins (102-108). Chapters 2 and 3 of this work show how one can use homology modeling, protein docking, and small molecule docking, with the goal of understanding the interactions of inhibitors with the channel proteins.

**Homology Modeling**

The difficulties associated with crystallizing membrane proteins necessitate that one leverage a single crystal structure heavily, rather than patiently waiting for the crystallization of the exact protein in the perfect conditions to answer our questions. As Jasti et al had crystallized a chicken ASIC channel (16), a species whose ASICs have been poorly studied (67,109), and although with some proteins there are very few differences between species, it has been noted that possibly clinically relevant differences do exist even between the closely related rASIC-1 and hASIC-1 proteins (110). Using homology modeling it is possible to account for these differences in the primary structure, assuming that there is still some conservation of general structure between related proteins. The basic protocol involves using a primary sequence alignment, a template for structural data, and an algorithm to apply the restraints of the sequence alignments to create the structure of a target protein (111).

The challenges with homology modeling relates to these underlying assumptions. There can be structural differences between proteins with related structures, although
there are relatively few ways to generate a transmembrane spanning peptide as compared to soluble proteins (112). Depending on the method of modeling and amount of intervention, the validity of a homology model can be debated. Studies of the predictive ability of homology modeling of membrane proteins has shown that this technique can predict structures based on as little as 25% identity between the target and template proteins (113). However, when entire domains are absent and major functional differences are seen, the validity of a homology modeling approach can be debated, though some insights can be made (107).

Protein and Small Molecule Docking

One of the insights this work attempts to make is with regards to the interaction sites of known inhibitors of the ASIC containing channels. The solution structure of the two peptide toxins PcTx-1(87) and APETx2(88), which interact specifically with the ASIC proteins, have been solved using NMR. Combined with homology models, it is possible to attempt to define where these toxins interact with the ASIC containing channels, described in Chapter 2 for PcTx-1. It is also possible to perform this for small molecules, as their 3D coordinates can be extracted from experimentally determined structures or generated de novo. An example of this is shown in Chapter 3 for the interaction of amiloride and related drugs with ASIC containing channels. There some considerations regarding in silico docking techniques that merit discussion.

The software used in these studies, as mentioned earlier, hold the receptor molecule rigid. This is primarily for computational considerations, as it minimizes the number
of calculations required per receptor-ligand combination. It is possible for the small molecule docking program, Autodock Vina (114), to create flexibility in the receptor or ligand, using a stochastic method to randomly set rotation angles for flexible bonds. This simple method, using random quantized angles rather than searching through the entire rotational space, captures the tradeoffs made by computational methods. Rather than create a fully flexible situation, requiring a large number of calculations for each slightly different conformation of either the ligand or the receptor, the software gets close by taking random steps, and in the case of Autodock (114), is capable of doing a more realistic local search where the ligand is actually fully flexible rather than constrained to quantized values.

Another challenge with in silico docking methods revolves around the scoring of docked poses. Unlike with experimental methods, even if mistakes are made with regards to the setup or execution of computational methods, as long as the software can execute, results will be returned. This can lead to results that have little or no basis in reality, but are theoretically valid. In docking, this issue of stratifying results is generally handled by a scoring algorithm which attempts to rank the poses (115). For example, with the protein-protein docking conducted in Chapter 2, a second program was used to rerank the initial docked poses (116). This is a larger issue with blind docking, where no binding pocket is explicitly defined, as there is a larger search area leading to possibly unrealistic docking positions (117). It is important to realize that despite these difficulties, computational docking has been successful in finding novel drugs and therapeutics, exemplified perhaps by the HIV integrase inhibitor Isentress™ (raltegravir) (118).
Electrophysiology as Validation

As the validity of computational techniques is always under debate, a description of the functional techniques used to validate the computational results is provided. Ideally one would be able to directly visualize the movement of ions in the channel protein, watching as conduction was affected in some manner by a reagent. Though we, as a field, have the capabilities to directly visualize the conduction of a single channel using dyes and microscopy (119), the direct visualization of residues moving and ions flowing is not experimentally feasible. Aided by the relatively few structures which show ions within a conductive pathway, such as with the potassium channel (120), one can make very strong inferences regarding the structural changes and conduction. With the ENaC and ASIC proteins, similar work has been done using electrophysiology and mutagenesis to make deductions regarding the channel structure.

The basic tools of electrophysiology are glass electrodes filled with a conductive solution and a thin wire. Simplistically, current and voltage changes at the tips of the glass electrode can be sensed, controlled, and recorded. For example, the representative recording in Figure 3 was recorded from a system with two glass electrodes that are impaling a *Xenopus* oocyte, a single cell which is basically a ~1.0 mm sphere. In these experiments, the electrodes are used independently, with one sensing the voltage and the other creating a current to maintain a set voltage. The amount of current changes as channels are activated or inhibited or the membrane’s conductivity changes through some other process. For smaller cells, a single glass electrode is used and rapidly alternates between sensing the voltage and applying a current. Also, in smaller cells, rather than impaling and possibly destroying the cell, the patch-clamp technique, pioneered by Neher
and Sakmann (121), is used. In this challenging method, the 1-2 micron glass tip of the electrode is gently abutted to the cell membrane and slight suction is applied to create an \( \Omega \)-like seal of the membrane invaginating into the electrode. At this stage, recordings can examine just the channels within that small patch of membrane, or further suction can be applied in an attempt to rupture that patch and gain electrical access to the entire cell. At this point, signals recorded are representative of the whole cell. For an excellent review, consult the Axon Guide, which covers the practical and theoretical considerations involved with electrophysiology experiments (122).

While these electrophysiology techniques can allow for the recording of single channel events, they are limited as they only measure a functional endpoint. Combined with other techniques, inferences can be made regarding the stoichiometry, the conductive pathway, residues involved in cation or inhibitor binding but, especially when lacking an actual physical structure, the deductions made can easily be wrong. All techniques and deductions made from those techniques are limited by the lack of comprehension of the variables involved in the experiment. This is exemplified by the plethora of data regarding the stoichiometry of the assembled ASIC or ENaC channel, which trended to believe that although at least 3 different subunits were required for ENaC function, the stoichiometry was 2 \( \alpha:1 \beta:1 \gamma \) ENaC subunit (1) and a similar tetrameric arrangement was expected for ASICs just a few months before the publishing of the crystal structure (4). Although the debate in the field appears to have settled on a trimer model for ENaC structure (107), until a crystal structure is available, or the discordance with experimental data is addressed, it is unlikely this specific issue will be resolved.
For our purposes, ASIC channel activity is measured in the presence and absence of various drugs. The channels are activated by rapidly changing pH, a variable which in and of itself is known to affect the interaction of amiloride (76) and PcTx-1 (123) with the channel. An important point is the model system used to express the channel, as generally an amenable cell type is used to express large amounts of the protein. For example, one reason the 2-APA derived NSAID based inhibitor work was abandoned was due to an absence of the effect in Chinese Hamster Ovary cells as compared to the *Xenopus* oocyte expression system (93). As the reagents used may have effects on proteins endogenously expressed in the system, different systems are often tested to assure similar effects, as in Chapter 3 where two different CHO cell lines are examined. In this author’s experience, time scale can also be useful to show the specificity of an interaction, especially where the inhibitory effect of some drugs can be tested with rapid exposure and washout, rather than with long incubations where the reagent can interact with multiple partners or trigger unknown signaling events. Combining a short (1 – 10 seconds for ASICs) incubation with a long washout (30 - 60 s), and assaying for recovery of the channel currents can be indicative of a specific interaction. This isn’t always possible as some drugs bind slowly to their targets, while others bind irreversibly, but it is helpful when off target effects are a concern.

It is hoped that with a combination of computational and experimental techniques, fully understanding and appreciating that there are significant flaws in either approach, the interaction of the ASIC containing channels with known inhibitors is examined. Specifically, Chapter 2 examines the interaction of the peptide inhibitor PcTx-1 with the hASIC-1b channel while Chapter 3 focuses in on the interaction of amiloride and at-
tempts to show the capabilities of a virtual screening approach to finding new inhibitors of ASICs.
Figure 1 – The overall topology of an individual ENaC or ASIC protein is conserved across the family and is diagrammed in Figure 1. An ENaC or ASIC protein subunit has two short cytoplasmic domains, two transmembrane spanning domains, and a relatively large extracellular domain.
Figure 2 – The NCBI reference sequences for the human ENaC/Deg proteins were aligned in ClustalX v2.09 and plotted using Dendroscope v2.4 as a circular cladogram to allow one to appreciate the relationships between members of the hENaC and hASIC protein family. Of note is the INaC protein which appears to be distinct from the two families.
Figure 3 - Representative two-electrode voltage clamp recording of *Xenopus* oocytes expressing hASIC-1b. These oocytes were bathed in ND96 pH 7.4 for 25s and pulsed with a ND96 pH 5.0 for 5s. For brevity, only acid induced peaks are shown. In response to acid, there is rapid activation of the channel, followed by inactivation despite the presence of low pH. Repeated pulses show desensitization or tachyphylaxis of the channel, where there is a reduced response to the same stimulus. The degree of desensitization depends upon factors such as the conditioning pH, the stimulating pH, extracellular calcium, intracellular pH, and the time allowed between stimulation.
PSALMOTOXIN-1 DOCKING TO HUMAN ACID-SENSING ION CHANNEL-1

by

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Abstract

Acid-sensing ion channel-1 (ASIC-1) is a proton-gated ion channel implicated in nociception and neuronal death during ischemia. Recently the first crystal structure of a chicken ASIC was obtained. Expanding upon this work, homology models of the human ASICs were constructed and evaluated. Energy-minimized structures were tested for validity by in silico docking of the models to psalmotoxin-1, which potently inhibits ASIC-1 and not other members of the family. The data are consistent with prior radioligand binding and functional assays while also explaining the selectivity of PcTX-1 for homomeric hASIC-1a. Binding energy calculations suggest that the toxin and channel create a complex that is more stable than the channel alone. The binding is dominated by the coulombic contributions, which account for why the toxin-channel interaction is not observed at low pH. The computational data were experimentally verified with single channel and whole-cell electrophysiological studies. These validated models should allow for the rational design of specific and potent peptidomimetic compounds that may be useful for the treatment of pain or ischemic stroke.
INTRODUCTION

Acid-sensing ion channels are a subfamily of the epithelial sodium channel/degenerin family of proteins (29). The proteins in this family share a general topology; each member has two transmembrane-spanning domains, relatively short intracellular N and C termini, and a large extracellular loop containing multiple cysteine-rich domains. These proteins interact with themselves and other family members to form ion channels with unique properties (29, 34). The channels formed are all functionally linked by sensitivity to the small molecule inhibitor amiloride and a general selectivity for conducting sodium despite a sequence identity ranging from 15 to 60% across the family. The epithelial sodium channel/degenerin proteins are important for many physiological and pathophysiological processes. For example, αβγ-epithelial sodium channel channels expressed in the kidney are important in blood pressure homeostasis, whereas homomeric ASIC-1 channels found in neurons are implicated in nociception and neuronal death during ischemia (29, 48).

Using fluorescence detection size exclusion chromatography, Jasti et al. (27) found that homomeric chicken acid-sensing ion channel-1 (ASIC-1) could be crystallized and described the structure of a homomeric cASIC-1 channel lacking intracellular domains and in a nonfunctional state. Unfortunately chicken ASICs are very poorly characterized either pharmacologically or functionally. As crystallization of integral membrane proteins is a challenging technique, obtaining crystal structures of the human members of this family may not be feasible. Despite species difference of just a few amino acids, there may be clinically relevant variations between ASIC homologs (41).
This work used homology modeling to deduce structures of the human ASICs. These models were evaluated using structural verification suites and validated using in silico inhibitor docking to recapitulate functional results. Binding predictions were experimentally validated with single channel and whole-cell electrophysiology experiments. Together these models and the docked complex create structures that may be used for the rational design or virtual screening of new inhibitors or peptidomimetic compounds.

**EXPERIMENTAL PROCEDURES**

*Template Structures*

The work of Gouaux and co-workers (27) described the structure of *Gallus gallus* ASIC-1 arranged to form a homomeric channel (available as Protein Data Bank code 2QTS). This structure contains six cASIC-1 subunits arranged to form two channels. Chains A, B, and C of the structure were of higher quality and thus were used for modeling (27). Heteroatoms other than the chloride ions were removed.

*Target Sequences*

The amino acid sequences for full-length chicken ASIC-1, human ASIC-1b, hASIC-IC-2b, hASIC-3a, and hASIC-4a were obtained from the NCBI Protein Database. Pairwise alignments were performed using AlignX in VectorNTI Advance 10.3 (Invitrogen). Results of the alignments, as well as accession numbers, are shown in Table 1. Splice va-
riants were chosen to minimize gaps in the alignments. It should be noted that there is considerable confusion in the field regarding human ASIC-1 as compared with the rodent ASIC-1. To clarify, there are two known variants of hASIC-1, which are both very similar to the rodent ASIC-1a. The NCBI recognizes hASIC-1 variant 1 or “isoform a” as the splice variant containing a 46-amino acid insertion, whereas “isoform b” is lacking this insertion. However, as isoform b is most identical to rASIC-1a or mASIC-1a, it has often been labeled as hASIC-1a in prior publications. To our knowledge, no experimental work has been done with the actual hASIC-1a, and it is unclear whether it is of biological importance.

**Homology Modeling**

MODELLER 9v2 was used to perform automatic homology modeling of templates from cASIC-1(17). N and C termini were removed from target sequences as no data are available for them within the crystal structure of cASIC-1. Support for the chloride ions was enabled in MODELLER, and they were considered during the modeling of the channels. The scripts used for modeling, in addition to adding support for the chloride ions, increased the thoroughness of the default optimization protocol. The variable target function method optimization was set to “slow” with the maximum iterations set at 300. The molecular dynamics with simulated annealing optimization was also set to slow, and the entire process was repeated three times to generate one high quality model. Models for homomeric hASIC-1b, hASIC-2b, hASIC-3a, and hASIC-4a channels were created. As an internal control, the template was submitted to MODELLLER as a target for mod-
eling against itself. Following modeling, each structure was parameterized with the GROMOS96 43a1 force field and solvated with a simple point charge water model using genbox with GROMACS 3.3.1(43). The system was energy-minimized using the steepest descents algorithm with no position restraints until the system converged to machine precision on the Coosa computer cluster at the University of Alabama at Birmingham.

**Peptide Docking**

NMR structures for the peptide psalmotoxin-1 were obtained from Protein Data Bank code 1LMM. As this was a solution-derived NMR structure, the top 20 poses of the toxin were separated into individual files, and the hydrogen atoms were removed for inputting into ZDOCK 3.0.1(7). The transmembrane portions of the models were blocked as they are known to not participate in the interaction of psalmotoxin-1 (PcTX-1) and ASIC-1(39). Docking was performed between the channel structures and each of the top 20 NMR solution structures of PcTX-1 (15). Further docking of PcTX-1 to ASIC-1 was performed using the “dense” flag that uses a smaller rotational step to obtain more refined docking results at the cost of computational cycles. These results were reranked using ZRANK to obtain the best docked pose (35). Structures of the docked state at pH 6, 7, and 8 were calculated using PDB2PQR 1.3.0 and PROPKA 2.0 (3, 13, 32).
**Structural Visualization**

Models were visualized using Visual Molecular Dynamics from the University of Illinois. Figures were rendered using Tachyon (24). Calculations of interaction energies were performed on the best docked pose using the Adaptive Poisson-Boltzmann Solver 1.0.0 (2, 19-22).

**Planar Lipid Bilayer Recordings**

Planar lipid bilayer recordings were conducted as described previously(26). Briefly hASIC-1b was expressed in *Xenopus* oocytes, and vesicles were isolated. These ASIC-1-containing vesicles were fused to a planar lipid bilayer bathed with symmetrical 100 mM NaCl and 10 mM MOPS, pH 6.2. The holding potential was +100 mV referred to the virtually grounded *trans* chamber. Synthetic toxin was produced and purified by Pneumosite LLC (Shreveport, LA). Dwell time histograms were constructed following the analyses of events performed using pCLAMP software (Axon Instruments) on single channel recordings of 10 min in duration filtered at 300 Hz with an eight-pole Bessel filter before acquisition at 1 ms per point using pCLAMP software and hardware. The event detection thresholds were 50% in amplitude of the transition between closed and open states and 3 ms in duration. Closed and open time constants shown were determined by fitting the closed and open time histograms to the probability density function

\[ g(x) = \sum_{j=1}^{k} a_j g_o(x - s_j), \]

where \( s_j \) is the logarithm of the \( j^{th} \) time constant and \( a_j \) is the fraction of total events represented by the \( j^{th} \) component (42) and using the Simplex least
square routine of pSTAT. The number of bins per decade in all histograms was 16. For illustration purposes records shown were digitally filtered at 100 Hz using pCLAMP subsequent to acquisition of the analog signal.

**Plasmids and Mutant Construct Generation**

The constructs used in this study include the bicistronic pBi-eGFP:hASIC-1b and pBi-eGFP:hASIC-2b plasmids, the parent pBi-eGFP vector, pcDNA3.1-hASIC-2b, and a mutant hASIC-2b (mut_hASIC-2b) construct containing mutations E344D, G348D, L349F, and A351V rendering residues 340–355 of hASIC-2b identical to residues 343–358 of hASIC-1b. PAGE-purified oligonucleotides encoding for the mutated domain were purchased from Invitrogen and used in Stratagene's QuikChange II XL Site-Directed Mutagenesis kit according to the manufacturer's protocol. The mutations were verified by sequencing the domain in question at the Genomic Core Facility of University of Alabama, Heflin Center for Human Genetics.

**Cell Culture and Transfection**

CHO-K1 cells were maintained in 1:1 Dulbecco's modified Eagle's medium/F-12 (Hyclone) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin (Invitrogen). Cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol using 4 μg of total
DNA and 12 μL of lipid. Cells were replated onto glass coverslips and used 24–96 h later. For homomeric hASIC-1b or hASIC-2b channels, the bicistronic constructs were transfected individually allowing for patching of visually green cells expressing channel protein. For patching homomeric mutASIC-2b channels, a ratio of 1 μg of pBi-eGFP parent vector to 3 μg of hASIC-2b chimera DNA was cotransfected. For heteromeric channels, a bicistronic construct with a fluorescent reporter was cotransfected in a 1:3 ratio with another plasmid. The 1:3 ratio ensures that cells expressing the fluorescent tag are very likely to be expressing the cotransfected plasmid as well.

**Patch Clamp**

Micropipettes with an electrical resistance of 3–5 megaohms were prepared using a Narashigi PP-83 two-stage micropipette puller and filled with 120 mM KCl, 5 mM NaCl, 10 mM HEPES, 0.4 mM CaCl2, 2 mM MgCl2, 1 mM EGTA, and 2 mM MgATP (pH 7.4). The whole-cell configuration was achieved by abutting the pipette tip with a cell, applying suction, forming a >1-gigaohm seal, and rupturing the membrane to achieve cytoplasmic access. The barrels of a VC-77MCS perfusion system (Warner Instruments) were moved adjacent to the cell for rapid, local perfusion. Signals were recorded with pCLAMP 9 using an Axopatch 200B patch clamp amplifier and a DigiData 1320 digitizer (Molecular Devices). The signal was sampled at 5 kHz and low pass filtered at 5 kHz with the four-pole Bessel filter of the Axopatch 200B patch clamp amplifier. Currents were recorded by holding the membrane voltage at −60 mV and perfusing with a modified Krebs buffer (130 mM NaCl, 2 mM CaCl2, 10 mM d-glucose, 10
mM HEPES, and 10 mM MES, pH 7.4 with HCl). For calcium permeability mea-
ments, NaCl was replaced with N-methyl-d-glucamine. Acid pulses of 10-s duration were
applied every 20 s, and little to no desensitization was seen in the absence of access resis-
tance changes. Thus, data were normalized to the pulse immediately prior to initiation of
the experimental protocol. Although data suggest that PcTX-1 binding is decreased at pH
5, hASIC-2 containing channels are not significantly activated until pH 5(34).

Statistics

Data were analyzed using Clampfit (Molecular Devices), Excel 2007 (Microsoft),
and SAS 9.1 (SAS Institute Inc.). Data are presented as averages ± 95% confidence inter-
vals (CIs) as calculated by Excel 2007. One-way ANOVAs performed in SAS 9.1 with α
= 0.05 were used to assay for differences between groups. Scheffe’s post hoc test was
used to define different groups as it is better suited for unequal sample sizes and is a very
conservative measure.
RESULTS

\textit{Computational Results}

\textit{Homology Modeling}

MODELLER uses a peptide sequence alignment to create a model structure from a template. Pairwise alignments of the human ASIC proteins against the crystallized portion of cASIC-1 show that there is significant identity between the human and chicken ASIC proteins with greater than 50% identity and 60% similarity at the peptide level (Table 1). Studies of homology modeling of membrane proteins have shown that MODELLER is able to produce valid results with as little as 25% identity; thus it is expected that valid models can be obtained for the ASIC proteins (38). Furthermore the functional similarities between these proteins, as they all conduct cations and are sensitive to the small molecule amiloride, favors a conserved structure.

An initial visual inspection, shown in Figure 1, of the energy-minimized structures shows a general conservation of the template structure and allows for visualization of the residues that are altered as compared with the template. Using the NIH Structure Analysis and Verification Server and Matching Molecular Models Obtained from

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Name & NCBI Accession & Percent Consensus & Percent Identical & Length Aligned / Total Length \\
\hline
\textit{cASIC-1} & NP_001035557.1 & 100.0 & 100.0 & 420/527 \\
\textit{hASIC-1b} & NP_001086.2 & 94.1 & 90.1 & 424/528 \\
\textit{hASIC-2b} & NP_001085.2 & 82.7 & 72.5 & 422/512 \\
\textit{hASIC-3a} & NP_004760.1 & 66.7 & 53.9 & 438/531 \\
\textit{hASIC-4a} & NP_061144.2 & 62.8 & 51.3 & 427/531 \\
\hline
\end{tabular}
\caption{Protein alignments of the human ASIC family members to the chicken ASIC-1 portion available from crystallization. Alignments were performed pairwise, comparing each individual to the crystal sequence, using AlignX in VectorNTI Advance v10.3.0 from Invitrogen.}
\end{table}
Theory-mult server, structures were compared against theoretical and observed parameters for other protein structures as well as the template structure (Tables 1 and 2) (11, 23, 30, 31, 33). Based solely on structure, the hASIC-1b and hASIC-2b structures are most similar to the template cASIC-1, whereas the hASIC-3a and hASIC-4a diverge more from the template as expected based on the sequence alignment. All the models scored similarly to the template as would be expected for valid structures.

**Inhibitor Docking**

The examinations of the models showed that the hASIC models appear valid; however, they have yet to be tested. Ideally one would be able to crystallize the proteins in question and compare the model against the experimental structure. However, crystallization of integral membrane proteins is an arduous task. Fortunately one can still examine whether these models can recapitulate functional data that are readily observable and have already been well studied with the ASICs. For example, homomeric ASIC-1 channels are known to be inhibited by PcTX-1, a peptide toxin found in the venom of the Trinidad chevron tarantula (9, 10, 39). The NMR solution structure for this 40-amino acid peptide has been solved (15). The peptide appears to be specific for ASIC-1, showing no functional effect on other ASICs (16), and does not appear to bind to the other ASIC proteins (39). Domains involved in binding (9, 39) as well as a putative functional surface on the toxin (15) have been established. This allows for a clear cut in silico docking test: will PcTX-1 dock in silico to the hASIC-1b model and not to the other hASIC models? If so, is the docking site consistent with the published literature?
Using ZDOCK 3.0.1 at low resolution, the top 20 NMR structures of PcTX-1 were docked to the human ASIC models. The low resolution docking is much less computationally intensive allowing relatively rapid screening of all the models. Transmembrane domains were blocked from docking as these domains have been shown to have no effect on PcTX-1 binding (39). ZDOCK uses shape complementarity and electrostatics to perform rigid body docking (8). In this case, by using 20 NMR structures of PcTX-1, some biologically observed flexibility was introduced into the ligand. ZDOCK outputs the top 2000 docked poses for each NMR structure and grades the quality of the docking with a larger score signifying a stronger interaction. Figure 2, panel A, shows the average scoring of the toxin docking with the models with docking to hASIC-1b scoring higher on average than the other models.

Using denser docking results and reranking with ZRANK produced a best docked pose for PcTX-1 and hASIC-1b as shown in Figure 3. The orientation of PcTX-1 docking with the hASIC-1b channel is consistent with the predictions for an inhibitor cysteine knot fold toxin as noted by Escoubas et al. (15). The residues involved in the docking appear to electrostatically orient the toxin with residues Arg-27 and Arg-28, shown in purple, leading a positively charged region into the docking site created at the interface of the channel subunits (Figure 3 or Supplemental Figure 1). Arg-26 and Arg-27 of the toxin are capable of hydrogen bonding with Glu-235 of hASIC-1b, and Phe-30 can hydrogen bond with His-173. The docking site in the channel also agrees with the domains noted by Salinas et al. (39) in the mapping of the PcTX-1 binding site on rat ASIC-1 as well as the smaller high affinity domain defined by Chen et al. (9). However, unlike the model proffered by Salinas et al. (39), the docking site appears to be created by the interaction
of domains on adjacent subunits. A complete list of residues involved within the docking site, defined as within 6 Å of the toxin or channel, is available as supplemental Table 3.

This new insight from the computational models may explain the remarkable selectivity of PcTX-1 for homomeric ASIC-1 channels. To examine this further, the PcTX-1 structure that interacted best with hASIC-1b from the dense docking results was docked to models of heteromeric hASIC-1b and hASIC-2b channels. Models containing two ASIC-1 subunits docked similarly to the homomeric hASIC-1a model, whereas those containing two ASIC-2 subunits scored similarly to homomeric hASIC-2b models (Figure 2, panel B). This suggests that there may be preferential construction of ASIC heteromers such that they contain only one ASIC-1 subunit as coexpression of ASIC-1 with other ASIC subunits has been shown to abolish inhibition by PcTX-1 (16). A similar phenomenon of preferential heteromerization and assembly has been noted for the related epithelial sodium channel proteins (18, 25).

Closer computational analysis of the best docked pose also explains lingering questions regarding the binding of PcTX-1 to ASIC-1. Calculations of energies of interactions between the ASIC-1 model and PcTX-1 show that there is a large contribution to the binding energy, ΔG, by the coulombic interactions of the positively charged toxin with the negatively charged docking pocket created by the channel. This finding partially explains the bell-shaped binding curve observed by Salinas et al. (39) where binding peaks at ~pH 7.0 and falls off to about 25% or less at pH 6.0 and 8.0. By calculating the protonation state and electrostatic potentials of the docked structure at pH 6.0, 7.0, and 8.0, one can appreciate that the coulombic contribution drops as the pocket in hASIC-1b
becomes more positively charged at the lower pH (Figure 4). Furthermore based on the electrostatic and solvation components of the binding energy, there appears to be a stabilization of the toxin-channel complex as evidenced by the highly negative $\Delta G$.

**Experimental Tests of the Model**

**Planar Lipid Bilayer Studies**

There is evidence in the literature that PcTX-1 binds to and stabilizes the desensitized state of ASIC-1; this is consistent with the computational data. However, these data are limited to extrapolations from whole-cell currents (9, 10, 41). In the present study, the effect of the synthetic PcTX-1 on hASIC-1b channels was assayed using the planar lipid bilayer technique. We have previously described the effects of Ca$^{2+}$ and protein kinase C on hASIC-1b using this technique (4, 5). With this technique, hASIC-1b channel transitions can be observed continuously at pH 6.2 in nominally calcium-free solutions with the channel spending as much as 90% of the time in an open state with an apparent unitary conductance of $\sim 19$ picosiemens (Figure 5, panel A). Addition of 25 nM PcTX-1 to the external solution resulted in an obvious change of the single channel behavior with a relatively long lived closed state becoming more evident and a decrease in the duration of time spent by hASIC-1b in the open state (Figure 5, panel A). At this concentration, the open probability of the channel decreased from 0.91 to 0.33. No change in unitary conductance was apparent. Also PcTX-1 was only effective when added to the presumptive extracellular face of hASIC-1b as expected by the binding site defined by the work above
and previously in the literature (9, 39), Figure 5, panel B, shows that increasing the
PcTX-1 concentration resulted in a dose-dependent decrease of open probability of the
hASIC-1b.

Examining the kinetics more closely in the absence and in the presence of PcTX-
1, a double exponential function described the closed time distributions (Figure 6, panel
A): the presence of PcTX-1 caused no change in a short lived closed state ($\tau_{c'}$), but the
time spent by channel in its relatively long lived closed state ($\tau_{c''}$) was lengthened. A sin-
gle exponential function described fairly well the open time distributions of hASIC-1b in
the absence and in the presence of PcTX-1 (Figure 6, panel A). The presence of PcTX-1
decreased the duration of time spent by hASIC-1b in the open state ($\tau_{o}$). This is also illu-
strated in the reciprocal plots of open and closed states of hASIC-1b as a function of
PcTX-1 concentration (Figure 6, panel B). These findings demonstrate that $\tau_{o}$ of hASIC-
1b is inversely proportional to PcTX-1 concentration. The short lived closed state, $\tau_{c'}$,
appeared to be independent whereas the relatively long lived closed state, $\tau_{c''}$, was linear-
ly proportional to PcTX-1 concentration. It should be noted that because of our sampling
and filtering rates the presence of a change in the short lived closed state may not have
been observed.

Thus, the pattern observed is likely that of a toxin that reduces the open time and
increases the time spent in the long lived closed state while not altering the conductance
of the channel. This is consistent with an allosteric modulator where the channel is being
stabilized in the desensitized state as proposed by Chen et al (9, 10). These data also rein-
force the binding energy calculations that show a stabilization of the toxin-channel complex as compared with the toxin and channel alone.

**Patch Clamp Studies**

As the model for PcTX-1 interaction previously suggested that the docking site was self-contained within one subunit and our computational findings disagreed with this, we tested the finding using a chimeric hASIC-2b subunit. As Salinas et al. (39) and Chen et al. (9) focused on showing a direct involvement of residues identical to 157–185 of hASIC-1b, our work focused on the less well defined domain also implicated in binding of PcTX-1 to ASIC-1 defined as domain 5 by Salinas et al. (39) that contains residues 271–371 of hASIC-1b. Using the best docked pose of PcTX-1 with hASIC-1b, a small section of hASIC-2b that was similar to domain 5 was targeted for mutagenesis to render it identical to hASIC-1b, narrowing the 100-amino acid domain down to just 10 residues. Only four point mutations were required to make residues 340–355 of hASIC-2b identical to residues 343–358 of hASIC-1b; however, it was postulated that alteration of these residues would create a PcTX-1 docking site within heteromeric hASIC-1b/muthASIC-2b channels.

CHO-K1 cells were first transfected with hASIC-1b, hASIC-2b, or muthASIC-2b alone or in combinations thereof. To establish that heteromeric transfected cells expressed heteromers, the well described calcium permeability of homomeric ASIC-1 channels was exploited (1, 45, 46, 49). In our hands, when external sodium was replaced
with the impermeant cation \(N\)-methyl-d-glucamine and the only major cation gradients were for calcium influx and potassium efflux, homomeric hASIC-1b-transfected cells showed a significant acid-induced inward current of \(\sim 63.3\%\) of the peak current as compared with conditions with sodium at pH 5.0 (Figure 7). Conversely hASIC-2b- or \textit{mut}hASIC-2b-transfected cells showed on average an inward current of 4.7 or 6.5\%, respectively, showing significantly decreased permeability to calcium relative to hASIC-1b (Figure 7). Moreover as the calcium permeability is reported to be half that of the potassium permeability for hASIC-2b, after a short influx of calcium, an outward current was detected, signifying the efflux of potassium (Figure 7, inset) (1). Combined with a fluorescent reporter and hASIC-1b in a bicistronic vector, this allowed us to differentiate cells that had both hASIC-1b and hASIC-2b or \textit{mut}hASIC-2b from those with solely hASIC-1b based on a significantly decreased calcium influx and the presence of an acid-induced outward current.

When synthetic toxin was applied to these cells at 25 nM in both pH 7.4 and the acid pulse, there was a significant decrease in acid-induced currents after 2 pulses or \(\sim 60\) s of exposure to PcTX-1 in cells transfected solely with hASIC-1b and no effect in cells transfected with hASIC-2b or \textit{mut}hASIC-2b (Figure 8, panel A). In cells expressing heteromers of hASIC-1b and hASIC-2b or \textit{mut}hASIC-2b, there was no significant effect of 25 nM PcTX-1 (Figure 8, panel B). However, a consistent but slight increase in acid-induced currents was observed in cells transfected with hASIC-1b and \textit{mut}hASIC-2b. Using 100 nM PcTX-1, a significant increase in acid-induced current was elucidated from cells expressing the hASIC-1b/\textit{mut}hASIC-2b heteromers, whereas cells expressing hAS-
IC-1b and wild type hASIC-2b showed no effect (Figure 8, panel B). Although an increase in current was not the initial expectation, similar PcTX-1-induced increases in current for chimeric channels have been shown by Salinas et al. (39) and Chen et al. (9). For example, Chen et al. (9) show that PcTX-1 activates rASIC-1b while inhibiting rASIC-1a. Although the N terminus of rASIC-1b is very different from that of rASIC-1a, the rest of the protein is nearly identical, and much like our muthASIC-2b it contains half of the PcTX-1 docking site we defined, residues 343–358 of hASIC-1b. This result strongly suggests that the muthASIC-2b is able to interact with hASIC-1b to create an interaction site for PcTX-1, affirming the hypothesis that the PcTX-1 docking site is created at subunit interfaces.

**DISCUSSION**

**Relevance**

The ASIC family of proteins is an important therapeutic target for pathologies such as pain, cancer, stroke, epilepsy, or anxiety (47). However, finding potent and specific modulators of these channels has required careful screening of venoms and small molecule libraries using time-consuming functional assays (12, 14, 16, 44). Many of the agents found either lack specificity or are clinically difficult to administer, such as amiloride or PcTX-1(37). A structure of the target permits the rational design or the virtual screening of molecules that will interact specifically with the protein rather than blindly screening drug libraries in vitro. As crystal structures of membrane proteins are still diffi-
cult to obtain, this work leverages the finding of Jasti et al. (27) to define structures of the human ASIC proteins. Using a classic homology modeling approach, we produced valid models of the four human ASIC proteins. These models of homomeric ASICs were validated in silico to conform both to observed and theoretical structural parameters as well as to the template cASIC-1 structure as shown in the supplemental data and to recapitulate in silico the interactions of the peptide toxin PcTX-1 as shown in Figure 2. Moreover these models were tested experimentally in single channel and whole-cell electrophysiological studies.

**Computational Observations**

The result of docking PcTX-1 to the models confirms that the hASIC-1b binding site is consistent with expectations (15, 39). Both domains 3 and 5, as described by Salinas et al. (39), form the docking site. However, there are additional residues contributed from domains 2, 4, and 6 as well. Similar conclusions have been reached by the computational studies of Pietra (36) who uses similar techniques but limits his study to only hASIC-1b. Of note, the docking site is created by the interaction of these domains within two separate subunits, not in one single subunit as the initial models suggested (39). Moreover our docking studies clarify some confused points in the field.

For example, the crystal structure of cASIC-1 was thought to be in a closed or desensitized state (29). Combined with the functional data from other studies, our ability to dock PcTX-1 to the structure strongly argues that the molecule is in a desensitized state
or that there is no significant structural difference between the closed or desensitized states (9, 10, 39, 41). The latter idea that the structure of the desensitized state is indistinct is contradicted by functional and binding data that both show that the interaction of PcTX-1 with the ASIC-1 channel correlates strongly to the desensitization state of the channel and not the closed state (9, 10, 39, 41). For example, Salinas et al. (39) show with radiolabeled PcTX-1 that binding increases as the pH is lowered from 8 to 7, which correlates with the channel becoming more desensitized. Sherwood and Askwith (41) also show that point mutations, distinct from our binding site, appear to affect the desensitization of hASIC-1b allowing it to be inhibited by PcTX-1 at a resting pH 7.4 in the manner of rASIC-1a. An interesting aspect of the binding curve generated by Salinas et al. (39) is a decrease in binding as the pH is lowered from 7 to 5. As discussed above, this can be explained by alterations in the coulombic interactions, which as Escoubas et al. (15) correctly predicted appear to steer the toxin into a pocket on the channel.

Furthermore this decrease in the coulombic contributions to binding explains the reduced effectiveness of PcTX-1 when applied concomitantly at acidic pH. The charged pocket that nestles the positively charged toxin in the docked state becomes less inviting as the pocket becomes more positive with a drop in pH. This is also visible in the functional data.
Experimental Observations

The dose-response curve in the bilayer studies (Figure 5, panel B) was right shifted by approximately a log as compared with prior results (9, 10, 39). Prior experiments with synthetic toxin have measured the interaction at pH 7–8 with rASIC-1a, whereas these single channel experiments measured the interaction at a pH of 6.2. This right shift is then consistent with the computational data regarding a reduced interaction at lower pH due to a reduction in the coulombic interaction.

From the bilayer data, PcTX-1 appears to cause a reduction in the time spent in the open state and an increase in time spent in the long closed state of hASIC-1b. This is compatible with the model proposed by Gründer and co-workers (9, 10) that suggests that the toxin binds to the open or desensitized state of rASIC-1a and inhibits the channel by shifting the pH activation curve toward more alkaline values, leading it to become desensitized in the presence of toxin. However, their paradigm is based on extrapolations from whole-cell currents in the *Xenopus* oocyte system. Our single channel bilayer data showed a similar effect where the desensitized or long closed state is stabilized whereas the open state is destabilized for rASIC-1a or hASIC-1b.

To verify that the PcTX-1 binding site was located at the subunit interfaces, an ASIC-1/ASIC-2 chimera was created where half of the predicted docking site was created in ASIC-2. This would be predicted to lead to an interaction of ASIC-1/mutASIC-2 heteromers with PcTX-1, whereas wild type ASIC-1/ASIC-2 heteromers would be unaffected as is reported in the literature. This model prediction was borne out in our patch clamp experiments (Figure 8). However, although an interaction of PcTX-1 was observed in the
heteromeric ASIC-1/mutASIC-2 and not in the wild type ASIC-1/ASIC-2, PcTX-1 increased the peak acid-induced current rather than inhibiting the channel. Although this does confirm the docking site and is similar to observations made with PcTX-1 and other chimeric channels (9, 39), it also reinforces that PcTX-1 does not necessarily just inhibit ASIC-1.

**PcTX-1 as a Modulator**

Although PcTX-1 is considered a highly potent inhibitor of ASIC-1, there are multiple reports of PcTX-1 potentiating or activating ASIC-1 in various situations (9, 10, 40). For example, applying 30 nM PcTX-1 concomitantly with a pH pulse of 7.1 or in low calcium activates rASIC-1a overexpressed in oocytes, whereas 30 nM PcTX-1 alone can activate cASIC-1 expressed in COS-7 cells or endogenous cASIC-1 in chicken dorsal root ganglion cells. Whether this ability to potentiate ASIC-1 could be detrimental in clinically relevant scenarios has yet to be closely studied.

**Extrapolations to Gating**

Although the present study was limited by the rigidity of the structures and may have missed a binding site present when the channel was in a closed or open state, it suggests that the stabilization of the open or desensitized state of ASIC-1 by PcTX-1 occurs at the same domains. This is also noted by Salinas et al. (39) who found that placing do-
domains 1, 2, 3, and 5 of rASIC-1a into rASIC-2a created a chimeric construct that was signifi-
cantly activated by PcTX-1. However, when domain 4 or 6 of rASIC-1a was also present, no activation of the chimeric rASIC-2a was observed (39). If both domains 4 and 6 were present, inhibition similar to that of rASIC-1a was found (39). Salinas et al. (39) concluded that although domains 3 and 5 were the main mediators of the interactions domains 1, 2, 4, and 6 were needed for positioning the docking site or for transmitting the interaction into inhibition or activation.

Our results showed that residues in domains 2, 4, and 6 could also play a role in the docking of hASIC-1b with the toxin and may mediate structural changes upon bind-
ing. One could postulate that perhaps the main docking site domains 3 and 5 are part of a hinge location that is relatively unchanged between the open and desensitized state. Interactions of the toxin or the channel domains 3 and 5 with domains 2, 4, and 6 could then lead to either opening of the channel or desensitization. This is similar to the model suggested by Jasti et al. (27) with the toxin interacting at or near the pH sensor between the finger and thumb. However, as PcTX-1 interacts at this location to cause either activation or inactivation, this model would suggest that the interaction site would be relatively static whereas binding would cause conformational changes elsewhere in the structure, such as in domains 2, 4, and 6, which roughly correspond to the finger, ball, and palm regions, rather than a slight flick through the wrist domain as suggested by Jasti et al. (27).

The availability of these validated structures and the docking site of hASIC-1b with PcTX-1 will spur the isolation and design of novel therapeutics aimed at this family.
Future work should leverage molecular dynamics studies to assay for conformational changes in the structural models in the presence and absence of toxin and as a function of pH as well as expand to APETx2, the sea anemone toxin that inhibits ASIC-3-containing channels (6, 12, 28).

ACKNOWLEDGMENTS

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SUPPLEMENTARY DATA

All the supplementary data associated with the article can be found in the online version, at doi:10.1074/jbc.M109.003913, but except for the embedded 3D figured is, the data are also available in the appendix.

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18. Harris M, Garcia-Caballero A, Stutts MJ, Firsov D, and Rossier BC. Preferential assembly of epithelial sodium channel (ENaC) subunits in Xenopus oocytes:


Figure 1: The structures of the template cASIC-1 and models are shown as ribbon diagrams, colored by subunit. One subunit from each template has overlays of the vdW surfaces of residues which are conserved (yellow), nonconserved (purple), or insertions (green) as compared to the template. Identical residues are not overlayed, allowing one to visually appreciate the conservation in the peptide sequence across these proteins. Not shown are deletions, of which are there 16 aa missing in hASIC-3a and 5aa in hASIC-4a as compared to the template.
Figure 2: Shown in Panel A are the average and median ZDOCK scores for the top 40000 docked poses of the 20 solution structures of PcTX-1 to the human ASIC models. The docking scores are best for the PcTX-1 to hASIC-1b docking, consistent with expectations from functional and binding studies (9, 39) (n=40000 per model, ± 95% CI). The docking scores of the toxin with hASIC-1b are higher than with the other models, (1-way ANOVA, Scheffe post hoc, α=0.05.) Panel B shows the average and median for the best docked solution structure of PcTX-1 with homomeric and heteromeric hASIC-1b and hASIC-2b structures (n=2000 per model, ± 95% CI). The docking scores of models containing two hASIC-1b subunits is similar to that of a hASIC-1b homomeric channel while models containing one hASIC-1b subunit score similarly to hASIC-2b homomeric channels, (1-way ANOVA, Scheffe post hoc, α=0.05.).
Figure 3: Shown in panel A and B is the top docked pose of PcTX-1 (green) to hASIC-1b. In panel A, the domains shown in red and blue are mediators of toxin binding as defined by Salinas et al (39). The domains shown in cyan, a subset of the domains in blue, are part of the high affinity binding domain which was refined by Chen et al (9). Shown in purple licorice are residues R27 and R28 of PcTX-1 which appear to lead a positively charged peg into a negatively charged pocket in the channel, consistent with the expectations of Escoubas et al (15). Panel B shows the surface of the binding pocket, colored by residue type: basic (red), acidic (blue), polar (green), nonpolar (white). Supplemental figure 1 consists of this figure as embedded 3D models.
Figure 4: Using APBS and PROPKA, the electrostatic potentials were calculated for the docked structures at various pH values. As can be visually appreciated, the pocket on the surface of the channel shifts from being negatively charged to being neutral/positive as the pH drops. Not shown is the electrostatic potential of the toxin, which is relatively unaltered with the pH drop. This is recapitulated in the binding energy calculations which show that the coulombic contribution becomes more positive as the pH is reduced, making the interaction less favorable.

<table>
<thead>
<tr>
<th>Binding Energy</th>
<th>pH 6.0</th>
<th>pH 7.0</th>
<th>pH 8.0</th>
</tr>
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<tbody>
<tr>
<td>Coulombic Contribution</td>
<td>-2125.68 kJ/mol</td>
<td>-3072.41 kJ/mol</td>
<td>-3996.57 kJ/mol</td>
</tr>
<tr>
<td>Solvation Contribution</td>
<td>415.96 kJ/mol</td>
<td>339.43 kJ/mol</td>
<td>348.57 kJ/mol</td>
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Figure 5: Shown in Panel A are representative single channel recordings of hASIC-1b reconstituted into planar lipid bilayers in the absence (top) and presence (bottom) of 25 nM PcTX-1. In Panel B is the summary of the open probability of hASIC-1b as a function of PcTX-1 concentration. Bilayers were bathed with symmetrical 100 mM NaCl, 10 mM MOPS, pH 6.2. The holding potential was +100 mV referred to the virtually grounded trans chamber. For illustration purposes records shown were digitally filtered at 100 Hz using pCLAMP software (Molecular Devices) subsequent to acquisition of the analog signal filtered at 300 Hz with an 8-pole Bessel filter before acquisition at 1 ms per point. Data are the average of at least 3 separate bilayers.
Figure 6: In panel A, the effect of 10 nM PcTX-1 on the kinetic properties of hASIC-1b in planar lipid bilayers. Shown in dark grey is the overall population fit and, where applicable, in light grey and lighter grey are the individual states within the population. PcTX-1 appears to increase the time spent in the longer closed state and decrease the time spent in the open state. Numbers of events used for construction of the closed and open time histograms shown were: 811 and 812 in the absence of PcTX-1 and 989 and 988 in the presence of 10 nM PcTX-1. Panel B shows the reciprocal plots of the open time, the short closed state, and the long closed state of hASIC-1b as a function of PcTX-1 concentration.
Figure 7: Shown are the average peak inward acid induced whole-cell currents for CHO-K1 cells transfected with hASIC-1b, hASIC-2b, muthASIC-2b, and combinations thereof. Acid induced currents in the Na⁺-free NMDG solutions were normalized to the acid pulse prior in Na⁺ containing solutions. Values for the hASIC-1b homomers were used as the calibrator. Cells transfected with hASIC-2b or muthASIC-2b only showed a significantly lower whole-cell current in the absence of Na⁺ as compared to cells transfected only with hASIC-1b. Cells transfected with both hASIC-1b and either hASIC-2b or muthASIC-2b showed statistically similar currents to cells expressing only hASIC-2b or muthASIC-2b. Shown above the bars are representative traces of the acid induced peaks. (n ≥ 3 per group, ±95% CI, ANOVA, Scheffe’s posthoc, α=0.05)
Figure 8: Shown in Panel A and B are the average peak inward acid induced whole-cell currents for CHO-K1 cells transfected with hASIC-1b, hASIC-2b, muthASIC-2b, and combinations thereof in the presence of 25 nM (A) or 100 nM (B) PcTX-1. Currents were normalized to the peak prior to PcTX-1 treatment began. Cells transfected with hASIC-1b were significantly inhibited by PcTX-1 application after the second pulse, or after approximately 60s of toxin application. Cells expressing heteromeric hASIC-1b and either hASIC-2b or muthASIC-2b showed no effect of 25 nM PcTX-1 treatment, but a significant increase in peak-acid induced currents was noted in cells expressing hASIC-1b:muthASIC-2b with 100 nM PcTX-1 as compared to cells expressing hASIC-1b:hASIC-2b channels. Together, these data strongly suggest that the mutations in hASIC-2b create an interaction site for PcTX-1 at the interface with hASIC-1b. (n > 3 per group, ±95% CI, ANOVA, Scheffe’s posthoc, α=0.05)
Abstract

Amiloride is a small molecule diuretic which has been used to dissect sodium transport pathways in many different systems. This drug is known to interact with the Epithelial Sodium Channel and Acid Sensing Ion Channel proteins, as well as Sodium/Hydrogen antiporters and Sodium/Calcium exchangers. The exact structural basis for these interactions has not been elucidated as crystal structures of these proteins have been challenging to obtain, though some involved residues and domains have been mapped. This work examines the interaction of amiloride with Acid Sensing Ion Channel-1, a protein whose structure is available, using computational and experimental techniques. Using molecular docking software, amiloride and related molecules were docked to model structures of homomeric human ASIC-1 to generate potential interaction sites and predict which analogs would be more or less potent than amiloride. The predictions made were experimentally tested using whole-cell patch clamp. Drugs previously classified as NCX or NHE inhibitors are shown to also inhibit hASIC-1. Potential docking sites were re-examined against experimental data to remove spurious interaction sites. The voltage sensitivity of inhibitors was also examined. Using the aggregated data from these computational and experimental experiments, putative interaction sites for amiloride and hASIC-1 have been defined. Future work will experimentally verify these interaction sites, but at present this should allow for virtual screening of drug libraries at these putative interaction sites.
Introduction

Amiloride is a small molecule best known for its ability to inhibit channels formed by the Epithelial Sodium Channel (ENaC)/Degenerin (Deg) family of proteins (1). Amiloride was discovered in 1965, a product of the concerted effort to find diuretics that conserved potassium while still leading to overall volume reduction and blood pressure reduction in hypertensive patients (2). Using a rodent model of hypertension, Bicking et al. screened over 300 compounds to find that amiloride and some structurally related molecules were able to increase sodium excretion in deoxycorticosterone acetate-induced hypertensive rats, while maintaining potassium levels (2). Clinical trials found amiloride to be safe for the long-term treatment of hypertension, as it reduced the risk of hypokalemia which was associated with other diuretics in use at the time, such as furosemide and ethacrynic acid (3).

Although the exact molecular targets of amiloride were not identified until the 1990s with the cloning of the ENaC proteins, it was apparent much earlier that this molecule could inhibit both sodium conducting and sodium exchange proteins, albeit with different affinities (1). However, even after identification of the ENaC/Deg proteins as high affinity binders for amiloride, showing an IC₅₀ of ~ 0.1 μM for the prototypical channel composed of αβγ-ENaCs (1), the mechanism of amiloride’s inhibition and the residues involved remained elusive. Using mutagenesis and chimeric proteins coupled to electrophysiological and radioligand binding data, model emerged where amiloride physically occluded the channel pore from the external aspect (1,4), though there are data implicating sites in the extracellular loops (5-9). As inhibitors of ENaC/Deg proteins could be useful in the treatment of pathologies ranging from cardiovascular disease and
hypertension (10), cystic fibrosis (11), neurodegenerative diseases (12), and various cancers (13-15), in this study we have used computational techniques to examine the structural basis of the amiloride and ENaC/Deg relationship.

This is possible thanks to recent work by the Gouaux group which has been able to define the crystal structure of channels made up of homomeric chicken Acid Sensing Ion Channel 1 (ASIC-1) (16,17). As galline ENaC/Deg channels are scantily described, a homology modeling approach has been used previously to examine the interactions of human ASIC models with the peptide inhibitor Psalmotoxin-1 (PcTX-1) (18,19). This study focuses on the interactions of hASIC-1 and hASIC-3 with amiloride in order to define binding pockets for future inhibitor studies. Using a small molecule docking program, Autodock Vina (20), amiloride and 29 other related molecules were docked to channel models, created with MODELLER (21), based on the crystal structure of the nonfunctional cASIC-1, 2QTS, and that of the functional cASIC-1, 3HGC. Whole-cell patch clamp recordings were used to experimentally verify the predictions of the docking software with ASIC-1, while the findings of Kuduk et al. were leveraged to validate the results of docking to ASIC-3 (22). Comparisons of the docking sites against putative cation binding sites described in the crystal 3IJ4 (16) suggested sodium competition experiments with amiloride. By focusing the findings from these different studies on the ENaC/Deg interaction with amiloride (5-9,22-26), and adding the computational and experimental results in this current study, putative interaction sites for amiloride with the ENaC/Deg proteins are described. This work also validates these sites for future virtual screening of larger drug libraries beyond the handful of amiloride derivatives used in this work.
Experimental Procedures

Template structures

The work of Gouaux’s group described the structure of *Gallus gallus* Acid-Sensing Ion Channel 1 (ASIC-1) arranged to form a homomeric channel, available as PDB #2QTS and 3HGC (16,17). There are differences between the structures, both in resolution and constructs used, and thus both are used in this study as templates. Heteroatoms/residues other than the chloride ion were removed from both. For 2QTS, chains A, B, and C were used, as these chains were of higher resolution than D, E, and F, while the biological unit was used for 3HGC to create a similar trimeric channel structure.

Target sequences

The amino acid sequences for full length chicken ASIC-1, human ASIC-1b, hASIC-2b, hASIC-3a, hASIC-4a, α-hENaC, β-hENaC, and γ-hENaC were obtained from the NCBI Protein Database. Alignments were performed using ClustalX 2.0.9 (27). Care was taken to realign regions as initial alignments did not conserve cysteines across the family, leading to better conservation of disulfide bonding order between the template and models. Results of the alignments, as well as accession numbers, are shown in Table 1, while the alignment itself is presented as Supplemental Figure 1. Identity and similarity statistics were calculated and formatted using the Sequence Manipulation Suite (28). Splice variants were chosen to minimize gaps in the alignments.
Homology Modeling

MODELLER 9v7 was used to perform automatic homology modeling of the target sequences using templates from cASIC-1 (21). N- and C- termini were removed from target sequences as no data are available for them within either of the crystal structures of cASIC-1 (16,17). Support for the chloride ions was enabled in MODELLER and they were considered during the modeling of the channels. The scripts used for modeling, in addition to adding support for the chloride ions, increased the thoroughness of the default optimization protocol. The variable target function method optimization was set to ‘slow’ with the maximum iterations set at 100. The molecular dynamics with simulated annealing optimization was also set to ‘slow’, and the entire process was repeated three times to generate 128 models. The model with the best average rank from both the discrete optimized protein energy score (29) and molecular probability density function value (30) were selected for energy minimization in Gromacs 4, using the GROMOS96 43a1 force field and solvated with simple point charge water model (31,32). The system was energy minimized using the steepest descents algorithm with no position restraints until the system converged to machine precision on the Cheaha computer cluster at the University of Alabama at Birmingham. Models were created using either the 2QTS or 3HGC template leading to a total of four new models, two of hASIC-1b and two of hASIC-3a. Attempts to create models based on both templates were problematic as there is divergence in the transmembrane domains of the two templates (16,17). Models were validated against their templates using the NIH Structural Analysis and VErification Server at http://nihserver.mbi.ucla.edu/SAVES/.
Small molecule docking

Structures for amiloride and related molecules were retrieved from PubChem, at [http://pubchem.ncbi.nlm.nih.gov/](http://pubchem.ncbi.nlm.nih.gov/), as 3D SDF files. The drugs chosen for this study were either readily available or were hits in a search for the string “amiloride” in the NCBI PubChem webserver. OpenBabel 2.2.3 was used to create MOL2 files. For comparison with the work of Kuduk et al. (22), small molecule PDBQT files were generated using the Dundee PRODRG web server at [http://davapc1.bioch.dundee.ac.uk/prodrg/](http://davapc1.bioch.dundee.ac.uk/prodrg/) (33). The receptor and ligands were prepared for docking with Vina (20). Up to 1000 binding poses within 10 kcal/mol of the lowest binding energy were outputted and analyzed, with an exhaustiveness setting of 256. For each pose, a center of mass (COM) was computed and the COMs were clustered using a quality threshold clustering algorithm (34) defining a 2Å distance for clusters, with those clusters containing one or two poses considered as outliers and removed from further analyses.

Structural Visualization

Models were visualized using Visual Molecular Dynamics (VMD) from University of Illinois. Figures are rendered using Snapshot or Tachyon (35).

Cell culture and Transfection

CHO-K1 cells were maintained in 1:1 DMEM/F12 (Hyclone) supplemented with 10% FBS (Hyclone) and 1% pen/strep (Invitrogen). CHO-AP1 cells were a generous gift of Sergio Grinstein (Hospital for Sick Children, Toronto, ON) and were maintained in αMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 26 mM NaHCO₃.
Cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol at a ratio of 1:2.5 μg: μL of the bicistronic pBi-eGFP/hASIC-1b plasmid DNA to lipid. Transfected cells were replated onto sterile glass coverslips and patched.

**Patch clamp**

Micropipettes were prepared using a Narashigi PP-83 two-stage micropipette puller with an electrical resistance of 3-5 MΩ when filled with 120 mM KCl, 5 mM NaCl, 10 mM HEPES, 0.4 mM CaCl2, 2 mM MgCl2, 1 mM EGTA, and 2 mM MgATP (pH 7.4). The whole-cell configuration was achieved by abutting the pipette tip with a cell, applying suction, forming a > 1 GΩ seal, and rupturing the membrane to achieve cytoplasmic access using either suction or the Zap function of the Axopatch 200B patch-clamp amplifier. The barrels of a VC-77MCS perfusion system (Warner Instruments) were moved adjacent to the cell for rapid, local perfusion. Signals were recorded with pCLAMP 9 using a DigiData 1320 digitizer (Molecular Devices). The signal was sampled at 5 kHz and low pass filtered at 5 kHz with the 200B’s four-pole Bessel filter. Cells were perfused with a modified Krebs buffer containing 130 mM NaCl, 2 mM CaCl2, 10 mM D-glucose, 10 mM HEPES, and 10 mM MES; pH 7.4 with HCl. For sodium competition measurements, NaCl was replaced with NMDG and calcium was omitted to create a high Na⁺ solution with 10 mM NMDG/120 mM NaCl and a low Na⁺ solution with 120 mM NMDG/10 mM NaCl. Cells were held at -60 mV and pH 6.0 pulses of 3s duration were applied every 30s, with 200 ms (-100 to +100 mV) applied once before the acid pulse and once during the latter part of the acid pulse. Little to no
desensitization was seen in the absence of access resistance changes. Thus, data were normalized to the pulse immediately prior to initiation of the experimental pulse.

**Statistics**

Data were analyzed using Clampfit (Molecular Devices), Excel 2007 (Microsoft), SAS 9.1 (SAS Institute Inc.), and R 2.9.2 (www.r-project.org/) (36). Data are presented as averages ± SD as calculated by Excel 2007. One-way ANOVAs were performed in SAS or R, with α=0.05, to assay for differences between groups. Tukey’s HSD post-hoc test was used to define different groups.

**RESULTS**

**Homology Modeling**

Models were created for hASIC-1b and hASIC-3a against the template structures of 2QTS (17) and 3HGC (16). The best model for human ASIC-1b and human ASIC-3a were created and analyzed using the NIH SAVES web server. The models scored well as compared to the initial templates, as would be expected. The protein alignments of the human ASIC/ENaC proteins against chicken ASIC-1 are shown in Table 1. Multiple protein sequence alignment was performed using ClustalX2, with care being taken to realign cysteine residues to better conserve disulfide bonding. Supplemental Figure 1 provides the exact alignment.

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Table 1: Protein alignments of the human ASIC/ENaC proteins against chicken ASIC-1.
expected by the level of identity shown in Table 1, similar to what was found for prior ASIC models(18). NIH SAVES results are shown in Supplemental Table 1 for the individual models and the template molecules.

_Small Molecule Docking to hASIC-1b Models_

The small molecules were blind docked, screening the flexible ligands against the entirety of the rigid protein models. The top 1000 best docked poses were computed for each drug/model combination. For each docking position, a COM for the molecule was computed. Using a quality threshold clustering approach, poses whose COMs were less than 2.0 Å from at least one other pose were removed to reduce outliers. A visual example of this data reduction protocol is shown in Figure 1. The average binding energy for the remaining poses for each drug was computed and is shown in Figure 2. Using a one-way ANOVA followed by a Tukey-HSD post-hoc test, many statistically significant differences were observed. First, examining differences between ligands binding to either 2QTS based hASIC-1b or 3HGC based hASIC-1b shows that at least 16 of the ligands score statistically differently between the two templates. However, the statistically significant differences in binding energies for the same ligand between different templates were minimal, averaging 0.29 kcal/mol. The largest error was for 5-(N-2'-{(4''-Azidosalicylamidino)ethyl-N'-isopropyl})amiloride which showed a differences of 0.99 kcal/mol between the two models, and was also the largest molecule tested with 33 non-hydrogen atoms, suggesting that this may be an issue of sampling the conformational space of the molecule rather true discordance between the models. Further, supporting this hypothesis is correlation of the molecular mass of the ligands with the average
difference between the two templates, with a Pearson correlation coefficient of 0.56, which is statistically significant at a $p < 0.01$ for $n = 30$. This suggests that there is no significant difference between models based on the two structures, at least from an inhibitor docking standpoint.

Five of the 30 drugs were chosen for further examination: Amiloride, the serine protease inhibitor benzamidine, the amiloride analog benzamil, the NHE inhibitor 5-(N,N-hexamethylene) amiloride, and the NCX inhibitor 3',4'-dichlorobenzamil. Amiloride was chosen as it is a well described inhibitor of ENaC/Deg channels (1,37). Benzamil is known to be a more potent inhibitor of ENaC channels (37), and there is evidence for it being a more potent ASIC inhibitor (38). Though the other 3 drugs have known biological functions, their functional effects on ASICs are undefined and allow for functional testing of the screening system.

**Experimental Testing of Docking**

Using the whole-cell patch clamp technique in CHO-K1 cells transiently transfected with hASIC-1b, the effects of 25 μM of the selected drugs were tested on the pH 6.0 induced current. As the chosen drugs have a variety of known functions, a quick testing protocol was chosen where the drug was present only during the acid pulse. The reported $K_i$ for amiloride with ASIC-1 is in the 1-10 μM range (1), thus 25 μM with this rapid protocol was chosen as it was expected not to be maximally inhibitory. As suggested by the computational docking, the protease inhibitor benzamidine had no statistically significant effect on the acid induced currents, as compared with vehicle alone (Figure 3). With this protocol, amiloride reduced currents by about 40% and benzamil was
slightly more effective, inhibiting ASIC-1 currents by about 50%. More interestingly, 5- (N,N-hexamethylene) amiloride, and the 3',4'-dichlorobenzamil also both were able to inhibit the ASIC-1 currents, as predicted by the computational screening. 5-(N,N-hexamethylene) amiloride reduced currents by approximately 25% while 3',4'-dichlorobenzamil proved to be most potent, inhibiting the ASIC current by about 70%. These results are generally consistent with the computational data, although it was expected that 5-(N,N-hexamethylene) amiloride would be much more effective than amiloride.

As it was noted that some of these drugs have described biological functions, it is possible that their off target effects are leading to apparent inhibition of the channel. This should be relatively unlikely as the drugs are present only during the acid pulse, but it is a possibility. For example, CHO-K1 cells are described as having endogenous NHE activity (39,40), the inhibition of which by 5-(N,N-hexamethylene) amiloride would perturb cytosolic pH. This could lead to alterations in ASIC-1 function, as intracellular pH has been shown to modulate ASIC currents in murine primary cortical neurons (41). To test for this, a CHO cell line devoid of endogenous NHE activity, CHO-AP1 cells (42), were transiently transfected with hASIC-1b and the drugs were rescreened in these cells (Figure 4). Similar to the CHO-K1 cells, amiloride, 5-(N,N-hexamethylene) amiloride, benzamil, and 3',4'-dichlorobenzamil drugs were able to inhibit the acid induced currents while benzamidine showed no statistically significant effect. These data further rule out a secondary mechanism of action, suggesting that the ligands are acting directly on hASIC-1b.
**Defining a Docking Site for Amiloride**

With functional data verifying the computational data of this blind docking, work was done to define a docking site for these drugs. Initial examinations of the docking clusters for these drugs show that there are a large number of putative interaction sites, with 69-104 clusters found per docking of the five chosen ligands to the hASIC-1b models. This is to be expected with the unbiased blind docking approach used, the large number of docking runs for each set, and the flexibility of the ligand allowing for many possible energetic minimums to be found.

Examination of amiloride binding to the ENaC/Deg proteins has shown that there may be multiple binding regions of differing affinities. This would be consistent with the multiple clusters retained from the computational data. Specifically, there are data in support of binding to a pore region (5,24,25) and in support of amiloride binding domains further from the pore (5-9). The electrophysiological examination of amiloride inhibition of ENaC (43) and ASIC (5) currents shows a voltage dependence to the block, increasing at more negative potentials, likely due to the active form of amiloride being positively charged at physiological pH (44). This voltage sensitivity was observed for the tested inhibitors, except for benzamidine, in the whole-cell patch clamp experiments performed (data not shown). This functional data would suggest that the inhibition of ENaC by amiloride occurs either in a manner so as to expose the drug to the effects of transmembrane voltage, either by a voltage induced conformational change or by mimicking the effects membrane voltage on cations. The concept of voltage induced conformational changes has been examined in ENaCs by the use of the electroneutral amiloride analog 6-chloro-3,5-diamino-pyrazine-2-carboxamide, also known as CDPC,
which showed no voltage dependence to inhibition suggesting the charge of amiloride is
directly sensing the voltage rather than a charge on the channel protein (45). Taken
together, these observations suggest that an additional constraint of being within the
conductive pathway be applied to the possible docking sites.

Unfortunately, the conductive pathway of the ENaC/Deg channels is poorly defined.
However, using the cesium binding sites in the crystal structure (16), a conduction route
can be deduced from the multiple vestibules found in the models. The 3IJ4 structure
gives a starting point, one of three symmetrically distributed Cs$^+$ ions binding in the
extracellular vestibule, and an ending point with 2 Cs$^+$ ions binding in the channel pore
(Figure 5, panel A). There is also a large central vestibule in the channel where no cations
have been found, but as it is directly in the path between the Cs$^+$ ions, it could be
expected to be involved in transit from the extracellular sites to the pore region. An
additional Cs$^+$ ion binding site is located on the external aspect of the channel but does
not appear to be involved in a conductive pathway.

Using these locations as additional criteria, it can be observed that there is overlap
between the computed docking clusters and Cs$^+$ ions (Figure 5, panel A). This would
suggest that cations and amiloride may compete for binding to hASIC-1b, an observation
that has been noted (3,43,45) and disputed (46) for amiloride sensitive ENaC channels in
epithelia or heterologous expression systems. To test this predicted competition between
sodium for amiloride inhibition of hASIC-1b, the relative inhibition of pH 6.0 induced
currents was computed for CHO-K1 cells transiently transfected with hASIC-1b in 10
mM and 130 mM Na$^+$ solutions. The impermeant cation NMDG was used to maintain
osmotic balance and thus Na$^+$ and H$^+$ were the only conductive cations. At high sodium
concentrations, 10 μM amiloride was able to inhibit only ~20% of the high sodium acid induced current while at low sodium 10 μM amiloride was able to inhibit ~40% of the low sodium acid induced currents. The observation that sodium and amiloride appear to compete, confirm that the clusters docking near the Cs⁺ cations are likely valid.

Isolating the COMs which are within 10 angstroms of Cs⁺ ions, based on 3HGC model returns approximately 532 of the 947 non-outlier docked poses while the 2QTS based model returned 470 of 982 non-outlier poses. Isolating the poses with the most negative calculated energies in these clusters and defining residues with atoms within 5 Å of the docking sites returns 42/33 residues for the 2QTS/3HGC based models, shown visually in Figure 6 and described in Supplemental Table 3. For 2QTS, multiple minimal poses returned the same binding energy for some Cs⁺ pockets, with one binding pocket located at a very different location. Of these, 24 residues are shared between the two models. These residues are not identical to residues implicated by functional mutagenesis data for ENaC or ASIC proteins (5-9,24,25); however they are within similar regions. For example, in murine αENaC S583, and the equivalent glycine residues in β- and γ-ENaC, have been implicated in interacting with amiloride (1). Though this residue wasn’t implicated by our docking, a 5 amino acid tract (GDIGG) located two residues before this residue and the L residue just after this site were implicated (Supplemental Figure 2 or Supplemental Table 3). Using this information, it is now possible to define these regions within the channel as sites for larger scale virtual screening experiments and better guided experimental verification.
Small Molecule Docking to hASIC-3a models

To test the validity of small molecule screening for novel drugs based on amiloride, the experimental results of Kuduk et al. (22) were compared with the computational results found for hASIC-3 models based on 2QTS or 3HGC. Although 60 drugs were synthesized and screened using an automated patch clamp technique with HEK293 cells stably expressing ASIC-3a, only 4 were chosen for in-depth dose response calculations (22). The correlation between the experimental and computed binding energies for these four drugs were examined in Figure 7, finding that the Pearson correlation for the lowest binding energy correlated best with the experimental data for the 2QTS based models (0.96) while the average binding energy correlated best with the experimental data for the 3HGC based data (0.93). This recapitulation of the physical data based on the computational work further validates the strength of this methodology for finding novel small molecule inhibitors of ASICs.

DISCUSSION

The interaction of amiloride with the ENaC/Deg proteins has been studied extensively since they were elucidated as the target of this sodium channel inhibitor (1). However, until the description of the chicken ASIC1 structure (16,17), there was little known about the structure of the target proteins beyond the topology and inferences made from mutagenesis and functional experiments (1). Using computational methods, we have docked amiloride against the channel models. Although this is computationally expensive, the methodology used allows for unbiased determination of docking regions and appears robust enough to help define novel small molecule inhibitors of the channels.
However, there are important considerations that can affect the interpretation of these results.

*The Validity of a Homology Modeling and Docking Approach*

At the core of homology modeling is an assumption that there is structural similarity between proteins related at the primary sequence level, but the technique is capable of recapitulating valid structures based on as little as 25% identity between target and template (47). However, this requires that other criteria are met such as a shared function between the target and template. As this work attempts to leverage functional data from both ENaC and ASIC proteins, it should be appreciated that there are significant functional differences between the prototypical ENaC and the prototypical ASIC containing channels (1). For examples, ENaCs are generally considered to be constitutively active or more recently, modified by proteases to become active, while ASICs are gated by a drop in extracellular pH (1). They are still, however, cation channels and share in inhibition by amiloride (1), so they are likely structurally similar enough to allow for some functional data from ENaCs to be applied to ASICs and for some homology modeling attempts (48).

The crystal structure templates were considered separately during the modeling and docking process. This is due to the structural differences between the two and the resolution difference between the two template models (16,17). Although the 2QTS structure was of higher quality, the 3HGC structure was of a construct which showed the ability to conduct cations unlike the 2QTS structure. Models based on the individual
subunits could have been created, but this would have lost data regarding the subunit-subunit interactions that may affect docking of amiloride analogs.

There are valid issues raised by the approaches used in this work. For example, the selectivity filter in ENaCs/ASICs has been described as being a conserved G/SxS motif in the transmembrane helices (1). The structural data suggests this corresponds to an area within the cytoplasmic vestibule of the channel (16), which is inconsistent with some pre-structural expectations (1). This could be due to the desensitized state of the ASIC structures (16-18), a functional state which is not well described for ENaC containing channels, and it highlights the importance of not expecting a one-to-one translation of functional data to structural data.

Another important point is the assumption of amiloride as interacting with the channel only in an open state. There is generally an absence of amiloride interaction data for nonfunctioning or closed channels, though it should be possible to perform these experiments with radioligand binding assays for ENaCs resistant to protease cleavage (49), but it has been shown for ASIC-2 that amiloride is capable of interacting with the channel when it is not conducting at the whole-cell level (5). This suggests that although the channel structures are closed and desensitized, valid binding data or sites may still be deduced.

**Amiloride docking to ASIC-1**

Examining amiloride binding to the model, at least two distinct regions were found where amiloride can bind to hASIC-1 channels, corresponding to the cation binding sites in the crystal structures deduced by Gonzales et al. (16). This agrees with experimental
ENaC and ASIC data suggesting amiloride directly competes with sodium cations and is in the conducting pathway (5,43). Another putative site was detected in the acidic central midline vestibule created by the beta sheets of the palm domain (16), however with the absence of a defined cation within this chamber, we refrain from defining this as another possible amiloride binding pocket due to the lack of experimental validation.

In terms of known locations affecting amiloride binding, the two best described motifs are the WYRFHY tract found in ENaCs and residues in the pore and pre-M2 region (6-9,23,25,26,45,46). Unfortunately, the WYRFHY domain itself is not directly conserved in ASICs (Supplemental Figure 1), although there is a QYYFHY tract near the pore region of which Y67 in the hASIC-1b model based on 3HGC was found to be near the best docked pose (Supplemental Figure 1 or Supplemental Table 3). As for residues within the pore region and pre-transmembrane region 2, inferences from ENaC experiments implicate that multiple residues are involved. Functional data are clouded by the role of these residues in conductance and permeability of the cations. As noted earlier, although the docking results don’t directly recapitulate residues implicated by functional data, this could be due to the desensitized state of the crystal structure. There are some expectations that were recapitulated and tested functionally, such as the competition of amiloride with cations in the channel (Figure 6) (1). Residues implicated in binding cations by the Cs+ cocrystallized structure 3IJ4 (16), such as G432 and D433 at the pore and T237-T240 in the extracellular domain of cASIC-1, are also implicated by the docking results.

Although we have defined residues that may mediate the interaction, we are better able to define pockets where amiloride binds and interacts with the channel than specific
residues for mutagenesis. Because of the flexibility introduced to the ligand, the rigidity of the receptor, the absence of explicit water molecules, and the lack of a true open conformation of the channel, this work is somewhat limited. However, the goal of defining binding pockets makes the ability to virtually screen or rationally design small molecule inhibitors more computationally feasible.

**Virtual screening of amiloride analogs**

To show the feasibility of computational determination of analog affinity to ASIC models, a group of 30 drugs related to amiloride were examined for their ability to inhibit ASIC-1 models. Furthermore, models of ASIC-3 were computationally screened against a group of 58 amiloride derivatives to examine whether our in silico approach could recapitulate the functional observations of Kuduk et al. (22).

For the first part, four drugs were compared against amiloride for their ability to interact with ASIC-1 function in whole-cell patch clamp. The functional results repeated the trends saw in computational results, with benzamil, 5-(N,N-hexamethylene) amiloride, and 3',4'-dichlorobenzamil all found to inhibit the channel, while benzamidine was found to have no effect (Figure 3). An important point often overlooked is the pleiotropic effects of amiloride and its analogs. Although it is well known that amiloride affects ENaC (1-100 nM) and ASIC (1-100 μM) at differing doses (1), its capabilities to interact with the endogenously expressed NHE1 (5-50 μM) and NCX (1 mM) transport proteins is less well appreciated (10). Importantly for this work and others, intracellular pH (41), which could be perturbed by NHE inhibition, has been shown to modulate ASIC function. As at least one of the drugs is characterized as an NHE inhibitor, the functional
results were recapitulated and validated once more with a cell line lacking endogenous NHE activity (Figure 4). It is important to consider that some of the functional effects of amiloride seen in experiments on ASICs are due to proteins other than those in the ENaC/Deg family. For example, the paradoxical stimulation of ASIC-2 by 0.1 – 1 mM amiloride (5) could be due to effects on the NHE rather than direct interactions with the channel. Our results do not preclude a stimulatory site for amiloride binding, as the computational results are currently incapable of differentiating between stimulatory and inhibitory interactions.

To further examine the capabilities of this technique, models of ASIC-3 were created and were tested in silico. The 58 ligands created using medicinal chemistry by Kuduk et al. (22) were recreated in silico using PRODRG (33), which allows for the de novo generation of a 3D structure of small molecules. These ligands were blindly docked to the ASIC-3 models and a similar analysis protocol to that of the ASIC-1 models was applied. Dose response data for only 3 of the drugs and amiloride was available, and a significant correlation between experimental and computational results was found for the best docked pose of the 2QTS based models (Figure 7). It should be noted, these experiments were performed using an automated patch clamp protocol where cells stably expressing ASIC3 were pretreated with drugs for 120s and thus it is possible that there may be off target effects of these analogs leading to the functional inhibition of currents (22). The use of this methodology to find novel small molecules that interact with specific ENaC/ASIC containing channels could greatly speed the search for therapeutics involving these channels.
Although this study defines amiloride binding residues/domains, describes the competition of sodium and amiloride for ASIC-1, exhibits the ability of two unexpected amiloride analogs to inhibit ASIC-1, and shows the capabilities of the blind docking approach to recapitulate high throughput electrophysiological data, it is still limited by the initial crystal structures and the rigidity of the models. By defining smaller pockets, it is possible that future studies can use molecular dynamics to better explore localized flexibility in the ligand/model interaction as well as accounting for water molecules. This should better define residues involved in the amiloride interaction with the ASIC proteins. Docking to smaller regions of the channel, such as the pore or the Cs\(^+\) binding sites in the extracellular domains, should also reduce the computational load from weeks to days and remove many outliers, increasing the number of drugs that can be screened and the validity of the results.

The experiments to test these results functionally with mutagenesis should be performed, but is fraught with the complexities of a system where mutations affecting amiloride binding also alter conductance and permeability (1). Ideally one would be able to cocrystallize amiloride and cASIC-1 or soak cASIC-1 crystals with amiloride to obtain a structure (50), which would allow for verification of docking data without the variables introduced by the complex conduction and function of the channel.

Perhaps the most promising aspect of this work is the predictive capability of this technique to calculate the ability of small molecule drugs to interact with the channel models. Using this homology modeling and docking technique, it may be feasible to design inhibitors of specific ASIC containing channels, such as the homomeric ASIC-1b channel whose inhibition appears to be neuroprotective (12) or the putative heteromeric
ENaC/ASIC channel whose inhibition could be useful in the treatment of malignant gliomas (13). It could also be used to create novel inhibitors of mutants of ASICs as a protective mechanism if the channels are used as tumor therapeutics (51). These possibilities show the impact of this work, and also the large step that the crystal structures have allowed in the understanding of and search for inhibitors of the ASIC/ENaC proteins.

ACKNOWLEDGMENTS

We would like to thank Drs Eric Gonzales and Eric Gouaux for supplying the coordinates of cesium cations bound to ASIC-1, Drs Jonathan Plumb and Sergio Grinstein for the gift of the CHO-AP1 cell line, and Melissa McCarthy for cell culture assistance. We also thank Niren Kapoor, Edlira Bashari, and Dr. Bakhrom Berdiev for helpful discussions. This work was supported by NIH Grant DK37206 and the AMA Foundation.
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Figure 1: This visualization shows the data processing performed to remove outliers using amiloride interacting with the model of hASIC-1b based on 2QTS as an example. In Panel A are the one thousand poses computed for a flexible amiloride molecule docking to the rigid model, with each non-hydrogen atom of amiloride shown as a sphere using the VMD default coloring according to the name of the atom (Carbon and Chloride in cyan, Oxygen in red, Nitrogen in blue.) In Panel B, shown by green beads are the computed COMs for each pose. Panel C shows how by using a simple QT clustering algorithm, these 1000 COMs can be replaced by a smaller number of red beads helping to remove outlier poses and also to visualize areas where the poses cluster.
Figure 2: The average binding energies ± SD are shown for the 30 ligands docked to hASIC-1b models based on either the 2QTS or 3HGC. Solid white bars are the maximal or worst energies computed for the dockings, while the dark black or grey bars represent the minimum or best binding energy for the docking results. Though there are significant differences for docking between the two models (One-way ANOVA, α=0.05, Tukey HSD), the discordance between models is at most 1 kcal/mol, averaging 0.29 kcal/mol.
Figure 3: Using whole-cell electrophysiology in CHO-K1 cells transiently transfected with hASIC-1b, the effect of five of the 30 drugs were tested for interaction with the channel. The peak acid induced currents in the presence of 25 μM drug were normalized to the peak prior. All drugs were dissolved in DMSO, and compared to the pH 6.0 + vehicle, amiloride, 5-(N,N-hexamethylene) amiloride, benzamil, and 3',4'-dichlorobenzamil were able to significantly inhibit the peak acid induced current as predicted by the computational docking. Benzamidine was not statistically different from vehicle. (n >= 4, ± SD, * - significant vs. DMSO, ANOVA w/ Tukey-HSD, α = 0.05)
Figure 4: Using whole-cell electrophysiology in CHO-AP1 cells transiently transfected with hASIC-1b, the effect of the drugs were tested for interaction with the channel in the absence of a possible interaction with endogenous NHE in CHO-K1 cells. The peak acid induced currents in the presence of 25 μM drug were normalized to the peak prior. As compared to the DMSO vehicle control, amiloride, 5-(N,N-hexamethylene) amiloride, benzamil, and 3',4'-dichlorobenzamil were able to significantly inhibit the peak acid induced current as predicted by the computational docking. Benzamidine was again not statistically different from vehicle. (n >= 4, ± SD, * - significant vs. DMSO, ANOVA w/ Tukey-HSD, α = 0.05)
Figure 5: Following data reduction and clustering, many putative docking pockets remained. To narrow down the scope, examination of the putative conductive pathway was conducted. In Panel A, the hASIC-1b model based on 3HGC is shown as a transparent grey surface diagram. The red sphere represents the clustering results for amiloride. The yellow van Der Waal surfaces represent cesium cations cocrystallized with cASIC-1 aligned to the model structure. Visually, overlap between some of the putative binding sites and the cesium cations can be observed in both the extracellular and pore region. This suggests sodium cations may directly compete with amiloride when interacting with hASIC-1b. In Panel B, this prediction is verified by testing the effects of sodium concentration on the effect of 10 μM amiloride on hASIC-1b expressed in CHO-K1 cells. There is ~200% more amiloride inhibition seen in 10 mM Na⁺ (39.5 %) as compared to 120 mM Na⁺ (19.7 %). (n=9, ± SD, 2-tailed unpaired Student’s t-test, * = p < 0.01)
Figure 6: The lowest docked poses within 10 Å of the Cs⁺ binding pockets were extracted and are shown as red licorice models. Residues with atoms within 5 Å of the amiloride molecules are shown as solid grey colored ribbon diagrams, with the solid blue ribbon diagram representing the residues shared between the 3HGC (Panel A) and 2QTS (Panel B) models. The transparent grey outlines show the overall ribbon structure of the molecules. Individual residues are stated in Supplemental Table 3 as well as diagramed in the ENaC/ASIC protein alignment in Supplemental Figure 1.
Figure 7: Using data generated by Kuduk et al. (22), the capabilities of the docking algorithm to recapitulate experimental amiloride-based ligand binding to ASIC-3 were examined. Computing the experimental binding energy as $RT \ln(10^{pK_i})$, the average and best minimal computationally calculated energies were correlated to experimental data for the 4 thoroughly examined drugs. In this small set, the Pearson correlation coefficient for the average energies was 0.88 for the 2QTS based model and 0.93 for the 3HGC based model. For the best or minimum binding energy, the correlation was 0.96 and 0.87 for the 2QTS and 3HGC based models. The critical value for the significance of the Pearson correlation coefficient at $p < 0.05$ for $n=4$ is 0.95, and thus only the correlation of the minimum binding energy of the 2QTS based models is statistically significant. However, the other values also trend to significance and this further validates the virtual screening methodology applied, suggesting it can be used to predict small molecule binding to ASIC models.
SUMMARY AND CONCLUSIONS

This study has examined the interactions of known inhibitors with models of ASIC containing channels, both a peptide based inhibitor and small molecule inhibitors. This section summarizes the important findings of the work, discusses the strengths and weaknesses of the data, and delineates future directions for following up this work.

Homology Modeling of ASIC and ENaC Channels

The foundation for all the computational work is the homology modeling approach used to create models of ASIC channels. The software program MODELLER (111) was used, combined with alignments of the target sequences against two template structures 2QTS and 3HGC (15,16). The models were validated against known theoretical and observed structural values and then also validated for usefulness and coherence with functional data by docking data. However, there are some interesting points to discuss with the approach itself and future modeling questions.

When performing homology modeling with MODELLER, one has the option of modeling just a single subunit, or modeling the entire channel. When only the 2QTS structure was available, the choice was relatively simple to make, as the subunits within the crystal showed considerable structural differences between each other (16). With the
solution of the 3HGC structure, it is apparent that the second subunit in 2QTS is most similar to the single subunit found in 3HGC (15,16). The problems areas were generally in the transmembrane and wrist area, where two of the three subunits looked similar while one subunit was rather different. Thus, rather than forcing MODELLER to deal with the discrepancy between the subunits, and to maintain the subunit interface data in the crystal rather than trying to use symmetry to create the channel from a subunit, the models were designed considering the entire channel rather than individual subunits.

However, in the future, one could obtain interesting results by using the individual subunits. For an electrophysiologist, the initial interpretation of why the subunits were structurally divergent from each other would likely not be based on crystal contacts or other artifacts of the technique. This is because in a simple channel gating scheme where there are multiple subunits, one would postulate that each subunit can coordinate with other subunits to open or close the channel, and thus each subunit can exist in slightly different conformations (124). Thus perhaps by using these subunits as individual entities, one could recreate three different structures representing different conformations of the channel. From a computational standpoint this can be done in two ways, by applying a simple symmetry operation to the coordinates to create a trimer or a more complicated protein-protein docking algorithm. A protocol for multimer docking is implemented in a program called m-ZDOCK. This software is designed to perform protein-protein docking of cyclical symmetrical multimers based on a single protein structure (125). Of course, this could also be used in order to determine if a tetrameric or higher order assembly is possible for these proteins, but it may be specious to expect that a subunit from a trimeric assembly would allow for another stable multimeric assembly.
Another future goal would be to attempt to create structures of the ENaC protein channels based on the ASIC structures. This has been attempted by Stockand et al., but they take pains to state that their inferences are independent of the homology modeling approach used (107). This is likely due to the need to remove large portions of ENaC to create a valid appearing model, as there are many portions of the protein that are not similar to ASIC-1 template. Although modeling algorithms are capable of handling small gaps in alignments, large domains can be complicated and are perhaps better tackled by an approach using fold recognition or secondary structure prediction as the sequence homology diverges (112). This would require one to assume that the functional differences between the channels do not preclude a shared structure. If this assumption is accepted, it is also possible to focus on obtaining experimental structure data on the domains of ENaC that are poorly conserved in ASICs, which is primarily the nonmembranous portions; it may be much easier than attempting to crystallize the ENaC protein in its entirety. This might be simplest to do for the peptide domain that is cleaved from α-ENaC and γ-ENaC to generate a fully activated ENaC containing channel (126).

Psalmotoxin-1 Docks at Subunit Interfaces

Docking of PcTx-1 to the homology models was able to recapitulate results from functional experiments. The docking scores for models based on hASIC-1b were greater than the scores based on hASIC-2b, hASIC-3a, or hASIC-4a, as expected based on functional assays which showed that PcTx-1 primarily affects homomeric hASIC-1b channels (1,127). The residues in the channel found to interact with PcTx-1 were within domains
mapped out by prior functional assays (91,123). The most interesting aspect, in this author’s opinion, was that the binding site for the peptide was created by the interaction of two ASIC-1 subunits. This suggested that PcTx-1 interacted specifically with homomeric hASIC-1b because in heteromers of hASIC-1b with other ASICs, there was no docking site created. That would suggest a nonrandom association between subunits, an observation that has been noted in the ENaC literature (14,18) and indirectly in the ASIC literature where coexpression of two subunits at similar levels leads to one population of channels, rather than the three populations which would be expected based on random association. The mechanism of subunit assembly would be an interesting question to pursue, as cells expressing endogenous ASIC subunits are known to have discrete populations of homomeric channels, as well as some channels which exhibit properties of heteromeric channels (128). One possibility is that the endogenous heterogeneity is due to a temporal effect of gene expression with the composition of the channel being due to whichever subunits are expressed during production. This would not explain how similar ratios of subunit cRNA expressed in *Xenopus* oocytes leads to a single channel population (129). It might be useful to attempt to assess whether an equal amount of subunits are expressed at the total oocyte level, while a different ratio is expressed at the plasma membrane, suggesting that perhaps there is some control of assembly at the ER level mediated by chaperone interactions. A more challenging, but also more definitive, approach would be to use native gel electrophoresis in an attempt to define the channel assembly found with homo- and heteromeric expression of various ASIC subunits.

However, this all assumes that the docking site is truly created by subunit interactions. To test this, we exploited the binary difference between the interactions of PcTx-1
with ASIC-1 as compared with ASIC-2. The toxin had been well described as being highly selective for ASIC-1 as compared to ASIC-2, showing no discernable effect on channels that contained ASIC-2 (34,87). Thus, by mutating half of the binding site from ASIC-1 into ASIC-2, we attempted to create a situation where if the two subunits interacted, a channel sensitive to PcTx-1 would be made. This was assessed with the patch clamp technique in CHO cells transfected with hASIC-1b and either the wildtype hASIC-2b or the mutated hASIC-2b. To ensure that the channels assayed were truly heteromeric channels, the presence of GFP fluorescence which was associated with hASIC-1b by the use of a bicistronic DNA plasmid and the absence of Ca²⁺ permeability combined with the presence of K⁺ permeability which is associated with hASIC-2b containing channels (1) was used to discern heteromeric channels from hASIC-1b homomers. Mutating the sites allowed PcTx-1 to interact with the heteromeric channel but the interaction led to an increased in the current rather than inhibition. This was unexpected, but not without precedent, as prior work with chimeric channel constructs had created chimeras where the PcTx-1 increased the current (91,123), and PcTx-1 has been known to activate wild-type ASIC-1 under certain conditions (67). This validates the computational docking results of the peptide, and allows us to consider future applications of this result.

With the docked pose of the toxin in hand, the next step towards generating peptidomimetic compounds would be to discern which portions of the interaction are most important (130). This could be approached from a computational standpoint, performing virtual mutagenesis along the protein-protein interface (130), and then generating synthetic versions of mutant PcTx-1 that could be tested with electrophysiology or radioligand binding assays to show increases or decreases in the interaction. Although most
would expect that mutations in PcTx-1 could weaken the interaction with hASIC-1b, one could argue that the toxin-channel interface has evolved to be as highly potent as physically possible, but there is no reason to suspect that a human or even mammalian ASIC is the original target of a toxin found in tarantula venom, as the natural diet of the most tarantulas is predominately insects, and although they may be preyed upon by mammals, they likely have not felt much evolutionary selection pressure due to human predation.

Once the interface peptides have been delineated, one can attempt to mimic their interactions with derivatized peptides or novel small molecules that mimic the interface peptides (130). Another approach would be to use the binding pocket defined by the toxin docking as a site for virtually screening large drug libraries, functionally testing those that show strong interactions.

Another question that can be raised is how does PcTx-1 inhibit the glioma channel (69,70)? This channel is putatively a heteroclade ASIC/ENaC containing channel (21), constitutively active as with homoclade ENaC channels but also potently inhibited by PcTx-1 like homomeric ASIC-1 channels. It was noted by Meltzer et al. that when ASIC-1 was coexpressed with either α-, β-, γ-, or δ-ENaC, there was an acid activated current that was almost a log-fold more sensitive to the venom of the Psalmopoeus cambridgei tarantula, but no constitutively active channel (22). There was also a change in the Na+/K+ selectivity for ASIC-1 with α-, β-, or δ-ENaC, but not for γ-ENaC, that was interpreted as further evidence for the interaction of the heteroclade subunits. Again, it would possible to use protein blotting techniques to see whether these heteroclade ASIC/ENaC channels contain one or two ASIC-1 subunits in the assembled state. This could also be pursued by a computational approach, if valid homology models could be de-
duced for the ENaC subunits. Based on the data of Kapoor et al. (21), the glioma channel is made of ASIC-1, α-ENaC, and γ-ENaC, and thus the PcTx-1 binding site would have to be created by heteroclade subunit interactions if the channel is still a trimer. This might be possible but it is dubious based on the lack of similarity seen in the protein alignments for the regions implicated in binding. Another possibility, which is compatible with the data, could invoke the idea that a heteroclade channel is tetrameric rather than trimeric or perhaps more plausibly that there are two populations of glioma channels, some containing ASIC-1 and α-ENaC and others with ASIC-1 and γ-ENaC. However, for the two populations hypothesis to be possible there would have to be other factor(s) in the gliomas as compared to the *Xenopus* oocyte system that leads to constitutive activity of the channel (22). For example, this could be mediated by interactions with trafficking proteins such as syntaxin or kinasases such as protein kinase C (69,131,132). Interestingly, when manipulations are made that bring ASIC-2 to the plasma membrane of the gliomas, the conductance disappears, which could tie into the interactions with syntaxin (72,74).

Another future direction for this work is to examine the interaction of other known peptide inhibitors, such as examining the interaction of the sea anemone toxin APETx-2 with ASIC-3 containing channels (33,88,89). It may also be clinically relevant and useful to examine the interactions of peptides that modulate the channels in other ways, such as the endogenous Phe-Met-Arg-Phe-NH2 (FMRMamide) peptides that appear to increase ASIC activity (133,134).
The Interactions of Amiloride with ASIC-1

Although the interaction of PcTx-1 is far more potent and specific, understanding the interaction of amiloride may be more useful in the search for clinically relevant small molecule inhibitors. Using the small molecule docking program Autodock Vina (114), a blind docking algorithm was followed to dock amiloride and structurally related drugs to the channel models based. Due to the relatively large size of the search space and the flexibility of the molecule, 1000 docked poses were calculated with an exhaustiveness setting of 256, as compared with the default settings of 10 docked poses with an exhaustiveness setting of 8. This increases the computational load, but also allows for a more comprehensive search of the docking space, with Vina taking 1-2 hours per ligand/model combination on a 3 GHz Xeon processor. The large amount of data generated required the creation of a rational, unbiased method for data analysis. We decided to simplify the data, reducing each docked pose to a single center of mass (COM), rather than a multi-atom representation. A clustering algorithm was then applied, pulling together poses within 2 Å of each other, which allows for simpler visualization and also removes outlier poses whose COMs are more than 2 Å apart. The remaining docked poses were used in the data analysis of where amiloride might bind and for selection of ligands for functional screening experiments.

Prior to our work, the location of amiloride binding to the ASIC and ENaC proteins was thought to involve residues near the pore and, in the ENaCs, a small 6-aa tract located distally in the large extracellular domain (1,135-137). An initial visual analysis of the docking clusters suggested that there was considerable overlap of the docking sites with cesium cation binding sites located in three symmetrically spaced pockets found in
the extracellular domains of the channel and also in a vestibule at the pore of the channel. This suggested that amiloride would compete with sodium or cations for a binding site and this held true experimentally; when the extracellular sodium concentration was reduced from 120 mM to 10 mM, the inhibition by amiloride increased by ~200%. Boosted by this experimental verification, amiloride docking poses within 10 Å of the Cs⁺ binding sites were examined. Interestingly, this captured almost 50% of the docked poses.

Extracting the lowest energy poses within these clusters and defining channel residues within 5 Å of the poses led to the delineation of multiple residues in the interaction with amiloride. Unfortunately, none of them were themselves implicated in functional mutagenesis studies of the ENaC/amiloride interactions (1,135-137). This was partially expected, as the 6-aa WYRFHY tract in the extracellular loop of ENaCs, which had been shown to play role in the amiloride interaction, is not conserved in the ASICs. The residues in the pore region though are relatively well conserved between the ENaCs and ASICs, and thus the lack of conservation of interaction residues was worrisome. The binding residues implicated are generally consistent, within a few residues of those implicated by experiments with ENaC. Closer examination offers some explanations, as the ASICs appear to have a conserved desensitization gate that is not apparent in the ENaCs. This gate is closed in the available structures. This appears to shift the accessibility of some residues. For example, the experimentally determined permeability filter in the ENaC/ASIC pore, a G/SxS tract, is actually in what appears to be the cytoplasmic vestibule of the channel (1). It will be interesting to see whether mutating these conserved ASIC residues to their counterparts in ENaC, GDI to SNL, would alter the interaction of the channel with amiloride, as would be expected by the functional analysis.
The important contribution, however, is the ability to define the domains where the Cs$^+$ cations appear to bind as amiloride binding pockets. This greatly reduces the space required to search for docking positions, and future computational work can exploit this to more rapidly and accurately search for inhibitors. The speed will come from the reduction in the search space, while accuracy can be afforded by allowing for local flexibility of the channel residues lining the pocket. This should lead to an increase in the number of drugs that can be screened and a simplification of the analysis protocol to primarily examination of the binding energies rather than the clustering steps taken to remove outliers.

To show that a virtual screening experiment is possible, we screened 29 analogs that were structurally related to amiloride. The analogs were tested against models based on either the initial higher resolution structure of the nonfunctional cASIC-1 channel or the second lower resolution structure of the functional cASIC-1 construct. We did find some differences between the docking results of ligands to the two hASIC-1 models, but the differences averaged less than 0.3 kcal/mol and appeared to correlate well with the molecular weight and complexity of the ligand. This suggests that perhaps with the size of the search space and flexibility of the ligands, we may not have sampled enough to get convergence between the two models. It could be that there are significant differences between the two structures and the docking differences are indicative of this, but the majority of the differences in the two structures appear limited to parts of the transmembrane domains, primarily a shift due to a slight bend in the wrist area attaching the helices to the extracellular loop and a larger change in residues below the desensitization gate of the
channel. At this point, it seems more probable that the docking differences are due to incomplete sampling of the docking space with the larger and more complex ligands.

However, following the outlier removal algorithm we used with amiloride, the ligands could be stratified based on whether they scored better or worse than amiloride. Four of the analogs were chosen for functional testing. Benzamil, 5-(N,N-hexamethylene) amiloride, and 3',4'-dichlorobenzamil appeared to dock better than amiloride, while benzamidine returned a binding energy that was worse than that computed for amiloride. The analogs all had known biological functions, but only benzamil had been previously been shown to inhibit acid-induced currents. Using patch clamp, the inhibitory effect of the analogs were found to correlate with the computational data, with the three drugs that scored better than amiloride inhibiting the acid induced current of hASIC-1b, while benzamidine, the drug that scored worse than amiloride, showed no inhibition of the hASIC-1b current. To reduce the chances of channel inhibition through one of the other targets of the drugs, they were screened in another cell expression system lacking endogenous NHE, which is known to be inhibited by 5-(N,N-hexamethylene) amiloride. These data describe two more amiloride analogs that inhibit ASIC-1 currents and also validate that the computational screening is capable of finding inhibitors of hASIC-1b.

In an attempt to push the limits of the technique, we compared our computational docking technique to a high throughput electrophysiology technique of a pharmaceutical company (138). Kuduk et al. used a stable cell line expressing hASIC-3a to functionally screen 60 amiloride analogs. Of those 60, four were chosen for detailed dose-response analysis. Using our blind docking technique, we found a significant correlation between
the experimental binding energy results and the best computational binding energy with the 2QTS based models. Weaker correlations were found with the average and best binding energies for the 3HGC based models, but the results are promising, suggesting that it may be possible to expand this computational technique to docking of small molecules to the other ASIC models.

Further Questions to Ask

In closing, this work has made some important contributions to the field, delineating residues involved in inhibition by PcTx-1 and amiloride, as well as describing two amiloride based analogs that were not previously known to inhibit hASIC-1b. These advancements should help in the search for new inhibitors, but there are many other questions that are left unanswered. Some of the possible avenues have already been discussed in the earlier, but there are some interesting computational and experimental techniques that can be applied to understand the ASIC-1 channel and the inhibitor interactions.

One technique that has not been explicitly discussed is the use of molecular dynamics (MD) simulations, something that has been used to postulate cation binding sites (106). The power of MD studies is due to the flexibility allowed to the protein, permitting one to explore the effect of point mutations on conformational changes. It could be hypothesized that performing MD studies using the degenerin mutant, where a single glycine residue in the pre-TM2 region is mutated into a larger, bulkier amino acid, would allow one to obtain a structure of channel in open state. One could also attempt to use MD to mimic the effect of pH changes, using a ‘constant pH’ technique where the protonation state of residues is iteratively changed from what would be expected at a starting
pH over time to obtain a structure at a target pH (139). However, the MD simulation of membrane proteins is a computationally demanding procedure and requires the careful consideration of many variables (140). For this purpose, one requires computational resources capable of performing relatively long simulations (100-200 ns), but also be able to handle large systems, as considerations of the bilayer and water box for a protein of this size dramatically increases the complexity and computational load.

It is hoped that this work shows how the use of homology modeling, docking, and electrophysiology techniques to explore the structure/function of these channels allows for the elucidation of novel inhibitors of not only ASIC-1 channels, but also the other ASICs. In theory, one can design or screen for drugs that show some specificity between different ASIC channel assemblies. By leveraging both computational and experimental techniques, a rational and targeted approach can be applied, wasting less time and money than conventional methods based on either computational or experimental techniques alone.
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APPENDIX A

SUPPLEMENTAL DATA FOR PSALMOTOXIN-1 DOCKING TO HUMAN ACID-SENSING ION CHANNEL-1
**Supplemental Table 1** shows differences between the structures; MAMMOTH (MAting Molecular Models Obtained from THeory) was used to align the structures in a sequence independent manner and calculate the unit root mean square distance (URMS) of the aligned structures, a measure analogous to the RMS deviation but better for examining similarities in structures (1,2). To quantify the structural similarity, individual subunits from the models were submitted to the MAMMOTH-mult server for a sequence independent structural alignment against the template structures. This allows one to rapidly visualize differences between the models and the template. Table 1 shows the average Unit-vector Root Mean Square (URMS) distance of the model Cα atoms as compared to the template Cα atoms. Unlike a straight forward RMS distance, URMS is a relatively localized measurement which is less swayed by areas of dissimilarity which are expected when modeling a new protein(1). MODELLER introduces some noise into the URMS measurement, as evidenced by modeling the cASIC-1 sequence against the original template. However, based solely on this measurement, the models for hASIC-1b and hASIC-2b are likely valid as they are similar to that for the template sequence control. The models for hASIC-3a and hASIC-4a appear to diverge more from the template, as would be expected due to their reduced similarity to the template.
Supplemental Table 2 shows overall scores for the models and template as calculated by PROCHECK(3), WHATCHECK(4), and ERRAT(5) using the NIH Structure Analysis and VErification Server (SAVES). PROCHECK performs many examinations of the stereochemical properties of structure and checks each residue’s Φ/ψ angle, giving the commonly used Ramachandran plot for a structure (3). The models containing residues in the disallowed region likely have some errors in the backbone structure, but may be correct in the other regions. The ERRAT software allows for the identification of regions of the models which are unusual in the manner in which atoms interact as well as a general quality score for the entire model (5). The ASIC models appear to have a similar overall quality factor as compared to the template. WHATCHECK uses a very thorough algorithm to compare the structure against observed and theoretical parameters (4). A normal protein is expected to have a score between 4 and -4 on most tests, with the majority of the population falling around zero. Obviously, an ion channel structure is not normal as compared to the majority of the proteins in the databanks, but using the template as a baseline, it becomes clear which measures can be used reliably. The backbone conformation scores for all the structures are considered ‘bad’ and conversely, the bond length and bond angle Z-scores for all the structures are normal (data not shown). However, examining the packing quality, the appearance of the Ramachandran plots, and the χ₁/χ₂ rotomer normality allows us to stratify the quality of the structures.

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Supplemental Table 2: Validation of models was performed using the NIH SAVES metaserver. PROCHECK examines the stereochemical properties, commonly shown in Ramachandran plots. ERRAT examines pairwise atom interactions and gives an overall structure score which corresponds to the percent of the protein of good quality. WHATCHECK examines the packing of residues, the Ramachandran angles, the rotomer distribution, and many as other properties, returning general Z-scores as well as a detailed report for each residue. From this table, it is clear that the hASIC-1b and hASIC-2b structures score similarly to the template structures while the hASIC-3a and hASIC-4a structures are more divergent.
<table>
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<th>Salinas Domain</th>
<th>Jasti Domains</th>
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<th>hASIC-2b</th>
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**Supplemental Table 3** shows residues at the toxin/channel interface which have atoms within 6Å of each other as calculated by VMD 1.8.6. The corresponding domains as defined by Salinas et al and Jasti et al are also shown, as well as the corresponding residues in hASIC-2b (6,7).

**REFERENCES**

APPENDIX B

SUPPLEMENTAL DATA FOR AMILORIDE DOCKING TO HUMAN ACID-SENSING ION CHANNEL-1
Table 1: Using the NIH Structural Analysis and Verification Server at http://nihserver.mbi.ucla.edu/SAVES/, the generated models and the templates were examined for validity. Although some features of the models were considered poor or bad, such as the backbone conformation, they maintained similar or better scores as the initial template structures.

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<th>Ramachandran Plot Residues</th>
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- **Benzamidine**
- **Carbachol**
- **6-Chloro-3,5-diamino-2-pyrazinecarboxamide**
- **Amiloride**
- **5,6-Dichloroamiloride**

**2D Structures:**
- **Benzamidine**
- **Carbachol**
- **6-Chloro-3,5-diamino-2-pyrazinecarboxamide**
- **Amiloride**
- **5,6-Dichloroamiloride**
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2D Structure:

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3. [Structure Image 3]
4. [Structure Image 4]
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<tbody>
<tr>
<td>5-(N,N-Hexamethylene)amiloride</td>
<td>1794</td>
<td>4755</td>
<td>308</td>
<td>3</td>
<td>7</td>
<td>1.4</td>
<td>8</td>
<td>0</td>
<td>[Image 1]</td>
</tr>
<tr>
<td>5-(Ethylpropyl)amiloride</td>
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<td>30572</td>
<td>299.76</td>
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<td>7</td>
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<td>[Image 2]</td>
</tr>
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<td>308190</td>
<td>308.19</td>
<td>299.76</td>
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<td>4755</td>
<td>308</td>
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<td>8</td>
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<td>[Image 5]</td>
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<td>HBAC</td>
<td>XLogP</td>
<td>TC</td>
<td>TFC</td>
<td>MF</td>
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<td>3',4'-Dichlorobenzamil</td>
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<tr>
<td>Amiloride caprate</td>
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<td>319.75</td>
<td>4</td>
<td>33</td>
<td>1.7</td>
<td>54</td>
<td>0</td>
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<td>319.75</td>
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<td>1.7</td>
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<td>Amiloride caprate</td>
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<td>319.75</td>
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<tr>
<td>Drug Name</td>
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<td>MW</td>
<td>MF</td>
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<td>XLogP</td>
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<tr>
<td>2',4'-Dichlorobenzamiline</td>
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<td>388.64</td>
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<td>C13H12Cl3N7O</td>
<td>azidosalicylamide</td>
<td>3083392</td>
<td>475.89</td>
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<tr>
<td>5-N,N-Ethyl-(2-methoxy-5-nitrobenzyl)amiloride</td>
<td>130061</td>
<td>422.83</td>
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<td>1.8</td>
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<td>azidosalicylamide</td>
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<td>475.89</td>
<td>5</td>
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<td>azidosalicylamide</td>
<td>3037897</td>
<td>475.89</td>
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<td>4</td>
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<td>2.8</td>
<td>C13H12Cl3N7O</td>
<td>azidosalicylamide</td>
<td>3083392</td>
<td>475.89</td>
</tr>
</tbody>
</table>
The table above lists the 30 drugs used for docking with the ASIC-1 models in terms of their chemical name, PubChem chemical id (CID), molecular weight (MW), hydrogen bond donor count (HBD), hydrogen bond acceptor count (HBA), XLogP value (a computational prediction of the octanol/water partition coefficient), tautomer count (TC), and the 2D chemical structure. The molecular formula (MF) and the 2D chemical structure are also provided for each drug.

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>CID</th>
<th>MW</th>
<th>HBD</th>
<th>HBA</th>
<th>XLogP</th>
<th>TC</th>
<th>MW (g)</th>
<th>MF</th>
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<tbody>
<tr>
<td>5-(N-Propyl-N-buty)2,4'-dichlorobenzamil</td>
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*Supplemental Table 2: The 30 drugs used for docking with the ASIC-1 models are described above in terms of their chemical properties.*
<table>
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<tr>
<th></th>
<th>3HGC</th>
<th>2QTS</th>
<th>Both</th>
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</thead>
<tbody>
<tr>
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<td>ARG</td>
<td>64</td>
</tr>
<tr>
<td>TYR</td>
<td>67</td>
<td>LEU</td>
<td>95</td>
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<tr>
<td>GLU</td>
<td>97</td>
<td>ARG</td>
<td>190</td>
</tr>
<tr>
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<td>173</td>
<td>TYR</td>
<td>191</td>
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<tr>
<td>PHE</td>
<td>174</td>
<td>THR</td>
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<td>THR</td>
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<td>217</td>
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<td>ASP</td>
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<tr>
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</tbody>
</table>

Supplemental Table 3: Shown above are the residues which have atoms within 5Å of the docked amiloride poses described in the text and Figure 6. They are also delineated in the protein alignment found in Supplemental Figure 1.
Supplemental Figure 1: The protein alignment for the human ASIC and ENaC proteins with cASIC-1 used for construction of the ASIC-1 and ASIC-3 models is provided. Residues with a black background are identical across the 8 proteins, while a gray background represents similarity. Residues implicated in amiloride binding by prior data have been highlighted in cyan. Residues found by our work to be involved in amiloride binding are in bold if implicated by the 2QTS based model and underlined if implicated by the 3HGC based model, both if implicated by both models.
APPENDIX C

IACUC APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: June 27, 2008
TO: Dale J. Benes, Ph.D.
MCLM 704 0005
FAX: 634-2377

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Small Molecule Inhibitors of ASIC-1 (Yawar J. Qadri)
Sponsor: Internal
Animal Project Number: 080608500

On June 25, 2008, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals.

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frogs</td>
<td>C</td>
<td>30</td>
</tr>
</tbody>
</table>

Animal use is scheduled for review one year from June 2008. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 080608500 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 634-7002.

Institutional Animal Care and Use Committee
B10 Volker Hall
1970 University Boulevard
205.934.7602
FAX 205.934.1188

Mailing Address:
VH B10
1530 3RD AVE S
BIRMINGHAM AL 35204-0019