Psalmotoxin-1 and nonproton ligand interactions with acid-sensing ion channels

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PSALMOTOXIN-1 AND NONPROTON LIGAND INTERACTIONS WITH ACID-SENSING ION CHANNELS

DISSERTATION

Presented to the Graduate Council of the Graduate School of Biomedical Sciences University of North Texas Health Science Center

In Partial Fulfillment of the Requirements For the Degree of

DOCTOR OF PHILOSOPHY

By

Rachel N. Smith, M.S.

Fort Worth, TX

April 9, 2015
PSALMOTOXIN-1 AND NONPROTON LIGAND INTERACTIONS WITH ACID-SENSING ION CHANNELS

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LIST OF PUBLICATIONS

Smith RN, Gonzales EB. Protons and Psalmotoxin-1 reveal nonproton ligand stimulatory sites in chicken acid-sensing ion channel: Implication for simultaneous modulation in ASICs. Channels 2014; 8(1):49-61; PMID: 24262969; http://dx.doi.org/10.4161/chan.26978


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Smith RN, Gonzales EB. Direct activation of acid-sensing ion channels by 2-guanidine-4-methylquinazoline is mediated by the transmembrane domains. Manuscript in preparation.
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LIST OF ABBREVIATIONS

Å  angstrom
APETx2  sea anemone (*Anthopleura elegantissima*) toxin
ASIC  acid-sensing ion channel
Ca\(^{2+}\); CaCl\(_2\)  calcium; calcium chloride
CHO-K1  Chinese hamster ovarian cell line
Cl\(^-\)  chloride
CNS  central nervous system
Deg  degenerin
ENaC  epithelial sodium channel
EPSC  excitatory postsynaptic current
ETC  extracellular
FaNaC  FMRF-amide gated sodium channel
FMRF-amide  Phe-Met-Arg-Phe-NH\(_2\) peptide
GMQ  2-guanidine-4-methylquinazoline
HEK  human embryonic kidney cell line
ITC  intracellular
Mambalgins  Black Mamba (*Dendroaspis polyepsis*) toxin
MitTx  Texas Coral Snake (*Micrurus tener tener*) toxin
NPL  nonproton ligand
NPLSD  nonproton ligand sensor domain
PcTx1  Psalmotoxin-1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>ITC</td>
<td>intracellular</td>
</tr>
<tr>
<td>Na⁺</td>
<td>sodium</td>
</tr>
<tr>
<td>K⁺</td>
<td>potassium</td>
</tr>
<tr>
<td>TMD</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>τ</td>
<td>time constant</td>
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I. INTRODUCTION

NEUROTRANSMISSION, ION CHANNELS & ELECTROPHYSIOLOGY

In the mid-1800s, Santiago Ramón y Cajal postulated that structurally independent units called neurons make the cell theory relevant to the nervous system (Cajal, 1909). Following what is now known as the *neuron doctrine*, remarkable additions were made to understanding the nervous system including defining that these basic units are connected by contact points known as synapses (Sherrington, 1906). The idea of neural circuitry, where a network of neurons and synapses interact, was finally conceptualized with Cajal’s theory of *functional polarity* (Cajal, 1909). Functional polarity perceptively predicted the flow of information from the dendrite and cell body, to the axon, to another neuron (Cajal, 1909; **Figure 1**). This concept provided the framework for the organization of the mammalian reflex, and has since been the foundation for modern neuroscience research (Sherrington, 1906).

The basic synapse involves a (1) presynaptic neuron, (2) synaptic cleft, and (3) postsynaptic neuron that participate in the exchange of information from one neuron to another. This exchange of information occurs following the release of chemicals, later termed neurotransmitters, from the presynaptic nerve terminal via a synaptic vesicle (Fatt and Katz, 1950; Fatt et al., 1952; De Robertis and Bennett, 1954). Edward George Gray noted a difference in the symmetry of the pre- and postsynaptic thickening within the synapse, along with the vesicles associated with that particular synapse (Gray, 1959). In general, if the neuronal thickening is symmetrical between the pre- and postsynaptic element (*Gray’s type 1*) the contents of the variably shaped vesicles are inhibitory, and if the thickening is asymmetrical (*Gray’s type 2*) the clear, round vesicles associated with the synapse are excitatory (Gray, 1959). This synaptic vesicle release occurs as the result of an arriving action potential to the presynaptic
nerve terminal (Bernstein, 1912; du Bois-Reymond, 1849; Katz, 1969). Initially termed “action current”, Emil Du Bois-Reymond discovered a measurable electrical impulse wave that occurred following the stimulation of frog nerves (du Bois-Reymond, 1849). Using a galvanometer that he built, Du Bois-Reymond described the resting electrical potential as having an inherent negative variation only after stimulating the nerve. With his elegant findings, the field of electrophysiology was born.

The electrical stimulation of a neuron begins when an action potential is generated near the cell body by a change in the resting membrane potential (Bernstein, 1912; Figure 1). The polarized, resting potential of the neuron is due to the concentration of intracellular and extracellular ions and has a value of -70 millivolts. According to the membrane hypothesis coined by Julius Bernstein, electrically excitable cells rely on the movement of electrically charged particles, ions, in and out of the neuron depolarizes the membrane, making it more positive (Bernstein, 1912). Upon reaching threshold, the electrical impulse wave observed by Du Bois-Reymond is generated and this signal propagates down the axon toward the axon terminal. Proteins imbedded in the cell membrane are critical for the electrical stimulation of the neuron, propagation of the signal, and postsynaptic response to vesicular release. These proteins are called ion channels.

Ion channels are pore-forming proteins that allow a means for charged ions like sodium (Na$^+$), potassium (K$^+$), calcium (Ca$^{2+}$), and chloride (Cl$^-$) to pass into or out of the cell (Hille, 2001). In order to understand the electrical properties of the lipid bilayer and ion channels, we can liken the scenario to a circuit containing a capacitor (the membrane) and resistor (the ion channel) (Sherman-Gold, 1993). Just like any other resistor-capacitor (RC) circuit, Ohm’s Law becomes a guiding concept for studying ion channel properties. However, in addition to the
guiding properties of physics, other mathematical principles are key for defining the features of ion channels.

In the early 1950’s, Alan Hodgkin and Andrew Huxley proposed the first quantitative model studying the propagation of an action potential in the squid giant axon (Hodgkin & Huxley, 1952a, b, c, d, e; Keener & Sneyd, 1998). Hodgkin and Huxley mathematically defined the membrane potential as the voltage across the membrane and the selective potential for ionic currents of Na\(^+\) and K\(^+\). Other ionic currents, such as those from Ca\(^{2+}\) and Cl\(^-\), were then combined and defined as leak current. The driving force for ionic movement depends on two gradients: electrical and chemical (i.e. the electrochemical gradient), which takes into account the concentration and charge of the ion (Sherman-Gold, 1993). Mathematically, we can calculate the potential for ionic conductance with the Nernst equation and the direction of the current (into or out of the cell) by Ohm’s law. Typical membrane potentials in animal cells are between 30 and 90 mV, and action potentials generated when the membrane potential reaches a threshold value can be measured in the laboratory using electrophysiology. With electrophysiology, experimentally this measurement can occur in the form of changing voltage or passing electrical charge (i.e. current) (Sherman-Gold, 1993). By applying a known current, one could measure voltage changes across the membrane (current-clamp), or by controlling the voltage one could measure the resulting current (voltage-clamp) (Sherman-Gold, 1993). These two electrophysiological methods, current-clamp and voltage-clamp, are especially useful to study the two major classifications of ion channels.

Essentially, two major classifications of ion channels exist: ligand-gated ion channels (LGIC) and voltage-gated ion channels (VGIC). Their distinction stems from the novelty in their activation mechanisms. Fundamentally, the binding of a “ligand”, or chemical, activates LGICs,
whereas VGICs are stimulated by a change in membrane voltage. One major family of ligand-gated ion channels are heavily involved in neurotransmission throughout the central and peripheral nervous systems. These channels are known as acid-sensing ion channels.

**ACID-SENSING ION CHANNELS (ASICS)**

Among the many LGICs, acid-sensing ion channels (ASICs) are members of the epithelial sodium channel/degenerin (ENaC/deg) family, and are homologous to the molluscan sodium channels gated by FMRF-amide (FaNaC) (Garcia-Anoveros and Corey, 1997; Schild et al. 1997; Green et al., 1994). Overall, these homologous cation channels are responsible for governing various functions in mammalian (ENaC/ASICs), molluscan (deg), and nematode (FaNaC) physiology (Kellenberger and Schild, 2002; Abboud 2010; **Figure 2**). In particular, acid-sensing ion channels (ASICs) act as sensors of extracellular acidic pH changes throughout the mammalian central and peripheral nervous systems (CNS/PNS) (Krishtal, 2003). The ASIC sodium currents generated by extracellular protons was first described in sensory neurons, and have since been found in non-neuronal cells as well (Chung et al., 2010; Huang et al., 2010; Jahr et al., 2005; Lin et al., 2010). Their participation is critical in responding to fluctuations of extracellular pH that can occur during anxiety, depression, increased neuronal activity, inflammation, ischemia, learning and memory, and pain (Ziemann et al., 2008; Coryell 2009; Chesler and Kaila, 1992; Page et al., 2005; Siejo, 1988; Siejo et al., 1996; Katsura et al., 1994; Coryell et al., 2007; Wemmie et al., 2003; Wemmie et al., 2004; Ziemann et al., 2009; Bohlen 2011; Chen et al., 2002; Deval 2011; Sluka 2003). Also, advent of protons as neurotransmitters suggests that ASICs have more significance than simply being a “reactionary” mediator of pathology, and they may act to help maintain homeostatic conditions (Du et al., 2014).
CHANNEL STRUCTURE AND DOMAIN FUNCTIONS

For mammalian ASICs, multiple subtypes (ASIC1a, 1b, 2a, 2b, 3, and 4) are available to form functional channels (Bassilana et al., 1997; Bässl er et al., 2001; Chen et al., 1998; Waldmann et al. 1997a; Waldmann et al. 1997b; Carnally et al., 2008; Gonzales et al., 2009; Jasti et al., 2007; Hesselager et al., 2004). Each ASIC subunit consists of an extracellular domain (ETC) with roughly two-thirds of the ~550 amino acid subunit length, two transmembrane domains (TMDs), and intracellular (ITC) amino- and carboxyl- termini (Saugstad et al., 2004; Figure 3A). The publication of the three-dimensional crystal structure using a truncated chicken ASIC1 (cASIC1), revealed that three individual subunits form a functional channel consisting of 12 β-sheets and 7 α-helices in the ETC domain as well as lining the pore (Jasti et al., 2007; Figure 3B). Primarily the ETC domain is critical for ligand binding and upon channel activation ASICs select for are sodium-selective with modest permeabilities to K⁺ and even Ca²⁺ during excitotoxicity (Nedergaard et al., 1991). Following multiple crystal structures, the likeness of cASIC1 was compared to a hand holding a ball and was given divisions of: β-ball, finger, forearm, knuckle, palm, thumb and wrist (Gonzales et al., 2009; Jasti et al., 2007; Figure 3C). The interface of each subunit contains an acidic pocket whereby sensing pH changes are this regions primary purpose (Gonzales et al., 2009; Jasti et al., 2007). Importantly, following proton activation, Na⁺ is ushered through the channel based on residues with “trigonal antiprism geometry” creating equilateral triangles that coordinate Na⁺ dehydration (Gonzales et al., 2009). This creates the ideal coordination for Na⁺ through the channel based on the number of subunits, symmetry, and the radii of a hydrated Na⁺ ion (Nightingale, 1959). Additionally, this suggested residue geometry revealed the first glimpse at how these equilateral triangles could extend
through the pore of the channel (Gonzales et al., 2009). Given the understanding that Na$^+$ is the ideal permeant ion, mechanistically, the channel undergoes movement of the thumb/wrist regions that result in the opening the channel (Gonzales et al., 2009).

Rotation of the transmembrane domains yields an expanded pore of the channel that is weakly selective for Na$^+$ over K$^+$ ($P_{Na}:P_{K} = 3$ to 30:1) (Bässler et al., 2001; Hoagland et al., 2010; Sutherland et al., 2001; Waldmann et al., 1997a; Yang and Palmer, 2014; Baconguis and Gouaux, 2012). It is the expansion of the pore that depolarizes the local membrane potential in order to continue or promote neuronal excitation (Grunder and Pusch, 2015). Some studies have reported that the pore of ASICs is considered to be significantly wider than their relatives, the ENaCs, since Ca$^{2+}$, NH$_4^+$, and guanidinium can permeate some ASIC subtypes (Yang and Palmer, 2014). There are similarities of ASIC/ENaC pore lining residues, yet TM1-residues appear to have a larger impact on the lining of the pore in ASICs than ENaCs (Kellenberger et al., 1999a; Kellenberger et al., 1999b; Schild et al., 1997; Sheng, 2000; Sheng et al., 2001; Snyder et al., 1999; Bässler et al., 2001; Coscoy et al., 1999; Carattino and Della Vecchia, 2012; Li et al., 2011). The extracellular most portion of ASIC-TM2 creates the entrance of the pore with a single, negatively charged aspartate residue (Paukert et al., 2004). It has been suggested that this site, known as the outer/extracellular vestibule, acts as the location where Na$^+$ ions concentrate prior to channel activation, and that mutations of this aspartate residue leads to reductions in Na$^+$ selectivity and conductance (Yang and Palmer, 2014; Grunder and Pusch, 2015). Furthermore, Na$^+$ ion accumulation within the outer vestibule appears to be aided by cation-π interactions mediated by a conserved tyrosine residue slightly more extracellular than the aspartate residue (Baconguis et al., 2014).
The earliest of crystal structures shows the pore of ASICs extending in a direct path through the lipid bilayer (Jasti et al., 2007; Gonzales et al., 2009). While these early structures were well-analyzed three-dimensional pictures of ASICs, the pore, particularly the carboxyl- half of TM2 (TM2b), has recently been adjusted to adopt a perpendicular conformation to the amino-end of TM2 (TM2a) (Baconguis et al., 2014). This oversight may have been because the desensitized pore (Gonzales et al., 2009) is more rigid in comparison to the open channel conformation (Baconguis et al., 2014). This would cause the overlapping symmetry of the TM2b with the neighboring subunit to obscure this perpendicular orientation. Perhaps most importantly, TM2 houses the narrowest portion of the channel where the selectivity filter is located (Baconguis et al., 2014). For ASICs, the selectivity filter is comprised of a conserved three-residue motif: Glycine-Alanine-Serine (GAS). The 6.2 Å distance between the atoms of the backbone oxygen associated with the glycine residue of each subunit creates an approximate 3.6 Å pore radius; perfect for coordinating a fully hydrated Na\(^+\) ion (Nightingale, 1959; Baconguis et al., 2014). Although this scenario for Na\(^+\) selectivity appears ideal, other factors such as large pore size and overall rigidity are important to note, and ultimately lead to lesser Na\(^+\) selectivity in ASICs than ENaCs (Dudev and Lim, 2015).

Following full proton activation, the continuous presence of protons will result in ASIC desensitization (Sutherland et al., 2001; Babini et al., 2002; Chen et al., 2005; Yagi et al., 2006; Sherwood et al., 2011). Some residues involved in proton affinity are also involved in ASIC desensitization (Babini et al., 2002; Liechti et al., 2010; Paukert et al., 2008). Prolonged stimulation with protons causes the channels to remain in a constant desensitized state, while the desensitized state does not necessarily require sustained agonist activity to be energetically favorable (Jones and Westbrook, 1996; Gründer & Pusch, 2015). The open-desensitized shift
appears to occur in the ETC and TMDs following analysis of toxin-bound crystal structures (Baconguis et al., 2014; Baconguis & Gouaux, 2012; Gründer & Augustinowski, 2012). It also is apparent that important interactions in β-sheet linkers (β1-β2, β11-β12) and residues in the TMDs stabilize the desensitized state (Frey et al., 2013; Li et al., 2010a; Roy et al., 2013; Springauf et al., 2011; Gonzales et al., 2009; Jasti et al., 2007). Desensitization occurs slowly in ASICs, although the true speed of desensitization is thought to be faster at physiological temperature, or 37°C (Bassilana et al., 1997; Bässler et al., 2001; Hesselager et al., 2004; Sherwood et al., 2011; Sutherland et al., 2001; Askwith et al., 2001). The true rationale behind the slow desensitization of ASICs is not known, however, it has been speculated that this feature may be important in protecting neurons during prolonged acidotic conditions (Friese et al., 2007; Xiong et al., 2004). Although mechanisms of proton activation and desensitization as a family are similar, the biophysical properties differ based on the ASIC subtype in question.

SUBUNIT DISTRIBUTION & PROPERTIES

Multiple ASIC subtypes can assemble to form functioning homo- or heterotrimeric channels, however ASIC2b and ASIC4 are only functional when in the heteromeric conformation (Bassilana et al., 1997; Bässler et al., 2001; Chen et al., 1998; Waldmann et al. 1997a; Waldmann et al. 1997b; Carnally et al., 2008; Gonzales et al., 2009; Jasti et al., 2007; Hesselager et al., 2004). Their extensive distribution throughout mammalian physiology, make targeting ASIC subtypes viable for pharmacological agents (Table 1). Subunit composition is still not entirely understood, however the central nervous system consists of mainly homomeric ASIC1a and ASIC1a/2a heteromers (Askwith et al., 2004; Baron et al., 2002; Vukicevic et al., 2004; Wu et al., 2004; Sherwood et al., 2011). Within the peripheral nervous system, ASIC3 is
critical for sensing pain (Benson et al., 2002; Deval et al., 2008; Hattori et al., 2009; Lin et al., 2008; Linguela et al., 1997; Sutherland et al., 2001; Waldmann et al., 1997a). However, ASIC3 appears to have a larger role within the mammalian CNS than initially thought, and ASIC stoichiometry is flexible (Delaunay et al., 2012; Bartoi et al., 2014). In a recent publication, this flexibility may be manipulated by the neuron in order to preferentially respond to extracellular stimuli (Bartoi et al., 2014).

Extensive electrophysiological studies have shown that inherent differences exist in the biophysical properties, such as activation and desensitization, of the ASIC subtypes (Babini et al., 2002; Sutherland et al., 2001; Waldmann et al., 1997a, Waldmann et al., 1997b; Wemmie et al., 2006; Figure 4). Using a variety of (e.g. COS7, HEK, CHO-K1) or heterologous expression systems like Xenopus oocytes, several groups have determined that ASIC1 and ASIC3 are the most sensitive to extracellular proton concentrations with pH50’s of 6.5 and 6.6, respectively (Babini et al., 2002; Sutherland et al., 2001; Waldmann et al., 1997a, Waldmann et al., 1997b; Wemmie et al., 2006; Sherwood et al., 2009; Bartoi et al., 2014; Table 2). When pH values fall slightly below physiological pH of 7.35, ASICs activate on the order of milliseconds as noted by the activation time constant ($\tau_{\text{act}}$) (Bässler et al., 2001; Sutherland et al., 2001; Li et al., 2010b; Table 2). Channels containing the ASIC2a subtype are much less sensitive to changes in pH, and have a half-maximal pH (pH50) of around 5.0 (Basiliana et al., 1997; Hattori et al., 2009; Bartoi et al., 2014; Table 2).

Desensitization occurs relatively slowly, compared to activation ($\tau_{\text{desens.}}$; Table 2). Although ASIC3 desensitizes the most rapidly of the ASIC subtypes, it does not fully desensitize (Springauf and Grunder, 2010; Waldmann et al., 1997a; Yagi et al., 2006). The current that remains from incomplete desensitization is not Na+-selective like the transient current would be
The pH range in which the concentration-response profiles for activation and desensitization overlap, known as the window current, is a range in which sustained/non-desensitizing ASIC currents occur (Yagi et al., 2006). This phenomenon occurs because some channels are activated, while desensitization is still occurring in others (Grunder and Pusch, 2015). Homomeric ASIC3 and heteromeric ASIC3/2b channels possess a window current that matches cardiac ischemia pH values (pH 7.3-6.7), while others such as rat ASIC1a do not (Yagi et al., 2006). As new compounds emerge that interact with ASICs, we are reminded that it is critical to be mindful of changes in activation and desensitization kinetics, as well as the shift in window currents.

At pH values between physiological pH (7.35-7.4) and pH 6.9 transfers ASICs into steady-state desensitization without reaching the open conformation. With respect to channel activation, the proton affinity is different for steady-state desensitization (~ pH 7.25 for ASIC1, ~ pH 7.1 for ASIC3) suggesting the two processes are not linked (Grunder and Chen, 2010; Babini et al., 2002; Chen et al. 2005; Liechti et al, 2010; Sutherlan et al., 2006). However, studies have shown that mutating residues involved in channel activation for ASIC1a and ASIC3 are tightly coupled to those involved in steady-state desensitization (SSD) (Babini et al., 2007; Cushman et al., 2007; Liechti et al., 2010; Paukert et al., 2008). The Hill coefficients for SSD are large, thus suggesting many protons bind during activation (Babini et al., 2002). When plotted together, the activation and SSD curve can overlap and reveal a range of pH values at which channels are only partially activated and can spontaneously desensitize (Alijevic and Kellenberger, 2012). This window current leads to a sustained current, and shifts in either the SSD or activation curves can change the effective range of this window current (Alijevic and Kellenberger, 2012).
PROTONS & OTHER ASIC LIGANDS

Protons

Through electrophysiological and crystallographic studies, ASICs are the leading contender to interact with a novel neurotransmitter, protons (Du et al., 2014). Protons are generally not found in concentrations higher than 40 nM, which corresponds to physiological pH of 7.4 (Babini et al., 2002), but in situations of inflammation and ischemia, pH values can fall to 6.7 (Reeh and Steen, 1996; Yagi et al., 2006). It is not unreasonable that the release of synaptic vesicles can generate a pool of protons at around the same pH, and the identification of ASICs on the postsynaptic neuron creates a scenario where activation of ASICs is likely (Zha et al., 2009, Zha et al., 2006; Du et al., 2014). Another source of protons in the synaptic cleft results from the \( \text{H}^+ \)-ATPase that will insert into the presynaptic membrane upon vesicle release (Gluck et al., 1982). Using a pH-sensitive green fluorescent protein (GFP), termed pHluorin, it was confirmed that presynaptic stimulation could produce significant acidification to activate ASICs (Du et al., 2014). This activation of excitatory-postsynaptic currents (EPSCs) was sensitive to the common ASIC antagonist, amiloride, further proving that ASICs are indeed a proton-activated channel (Du et al., 2014). These new findings indicate that ASICs are much more than just sensors for extreme fluctuations in pH, and that they likely participate in the overall homeostatic balance between the extracellular and intracellular environments. Finally, other constituents of the central nervous system may contribute to increased acidity of the brain and influence ASIC activity.

Calcium

In addition to protons, there are now additional ligands that interact with ASICs (Table 3). These ligands include divalent cations. Physiologically, along with acidosis, reduced
extracellular calcium concentrations are an indicator for inflammation (Issberner et al., 1996; Deval et al., 2008; Liu et al., 2012; Talmor-Barkan et al., 2009; Gluck et al., 1982). Previous work indicates that when calcium levels are depleted, a potential “calcium-block” is removed from ASIC3, corresponding to the aforementioned aspartate residue (Immke and McCleskey, 2003). These studies have also reported that reduced calcium concentrations activate ASIC3 with a non-desensitizing current (Immke and McCleskey 2001b, 2003; Li et al., 2010). While ASIC1 is void of this effect, the proton-affinity for ASIC1 is reduced by elevated extracellular calcium concentrations (de Weille and Bassilana, 2001). It is increasingly important that extracellular calcium concentrations influence ASIC activity, albeit directly or indirectly.

**Nonproton ligands**

Aside from protons and calcium, a new group of ligands termed the *nonproton ligands* interact with ASICs by binding to the central vestibule, or palm domain, via a common guanidinium moiety in their chemical structure (Yu et al., 2010; Li et al., 2010). The prototypical nonproton ligand is the synthetic compound 2-guanidine-4-methylquinazoline (GMQ) which exhibits the greatest effect on ASICs (Yu et al., 2010; Li et al., 2010). Guanidinium-containing compounds possess many important actions biologically and can be found in many naturally occurring molecules, compounds and common pharmacological agents (Mori et al., 1987; Greenhill et al., 1993; Hiramatsu et al., 2003; Taes et al., 2008; Berlinck et al., 2008; De Deyn et al., 2009). The semi-essential amino acid, arginine, contains a positively charged guanidinium group that can interact with the negatively charged carboxylate within the active site of many ion channels and receptors (Morris et al., 2004; Masic et al., 2006; Closs et al., 2004; Sun et al., 2011). Many current guanidinium-containing pharmacological agents exploit this unique
property in order to enhance the affinity of the drug to the target. Some of these agents include, antihypertensives, antihistamines, cardiovascular drugs, anti-inflammatory drugs, antifungals, antibacterial drugs, and influenza inhibitors (Tatemoto et al., 1982; Bettio et al., 2001; Sundler et al., 1993; Heilig et al., 1990; Bottcher et al., 1993; Larsson et al., 1975; Sundler et al., 1984; Turton et al., 1997; Harfstrand et al., 1987; Pernow et al., 1987; Maturi et al., 1989; Ahlborg et al., 1992).

Recent reports indicated that guanidinium-containing compounds directly activate one ASIC subtype, ASIC3. These compounds, termed ‘nonproton ligands’, include GMQ, the arginine metabolite agmatine, and the synthetic compound arcaine (Li et al., 2010; Yu et al., 2011). In addition to ASIC3 homomeric channels, GMQ can directly activate ASIC3/1b heteromers, while other channel subtypes appear unaffected by GMQ applications (Li et al., 2010). This direct activation by GMQ occurs at a binding site distinct from proton-binding sites (Yu et al., 2010; Li et al., 2010; Alijevic & Kellenberger, 2012; Yu et al., 2011). Aside from direct activation of ASICs by binding to the nonproton ligand sensor domain (NPLSD), or central vestibule, GMQ can also induce a shift in the pH-dependent activation curve of ASIC1a, 1b, and 3 (Alijevic and Kellenberger, 2012). Interestingly, direct activation by GMQ remains an independent result of ASIC3-containing subtypes despite the residues for GMQ binding to the NPLSD being present.

Natural venom toxins

Natural venom toxins have emerged as a new area for developing novel pharmaceuticals that target and influence ASIC activity. These compounds have been isolated from snakes, sea anemones, and spiders, and include toxins like MitTx, Mambalgins, APETx2, and others (Table
3). The first toxin-ASIC interactions were identified and described for ASIC1 and ASIC3. Psalmotoxin-1 (PcTx1), isolated from the venom of the South American tarantula, *Psalmopoeus cabridgei*, was shown to antagonize the ASIC1a subtype with nanomolar affinity and increases the channels sensitivity to protons (Escoubas et al., 2000; Chen et al., 2005). In contrast, PcTx1 does not antagonize the ASIC1b subtype but acts as an agonist by mediating a large inward sodium current that does not return to the original baseline, resulting in the presence of a toxin-induced persistent current (Chen et al. 2006). While studying the PcTx1 effects on ASIC mediated calcium permeability, it was observed that PcTx1 activates chicken ASIC1 in a similar manner (Samways et al., 2009). Despite determining that ASICs clearly can interact with more than just protons, it remains unclear how multiple stimuli interact with the channel resulting in a combinatory response from the channel.

SPECIFIC AIMS

One important question remains: *Why does the prototypical nonproton ligand, GMQ, appear ASIC3 specific?* Previous studies showed that agmatine, a guanidinium-containing polyamine, can activate ASIC3 homomers, but can also activate ASIC3/ASIC1b heteromeric channels (Li et al., 2010). Complete understanding of the stoichiometry of ASIC subtypes is still not known, and recent reports suggest that ASIC1a/2a/3 are the principle heteromeric channels in skeletal muscle afferents (Gautam and Benson, 2013). If heteromeric ASIC channels are a common modality for the body to sense painful stimuli, along with other pathophysiological conditions, then guanidinium-containing compounds likely interact with more than the ASIC3 subtype. There is, however, no observable activation of other ASIC subtypes despite the residues involved in GMQ binding being present in other non-ASIC3 subtypes, specifically ASIC1.
Additionally, studies have confirmed that residues involved in ASIC3 calcium-block are not present in ASIC1 (Yu et al., 2011). Much like other ENaCs within the same superfamily, ASIC3 seems to prefer an open conformation in the absence of extracellular calcium, which may be critical to the channel’s interaction with ligands following calcium removal (Immke and McCleskey, 2003). Furthermore, GMQ, protons, and calcium interact with ASICs to regulate channel activation and their interplay is critical for understanding how ASICs are coincidence detectors (Naves and McCleskey, 2005; Xiao et al., 2013). Taken together, we speculate that the partial activation of ASIC3 when calcium is removed from the calcium-block site might have a role in the GMQ sensitivity of the channel. In order to understand how a partially activated ASIC1 could interact with GMQ, we identified a candidate compound to induce this desired effect.

Prior to the publication of the crystal structures of chicken ASIC1 (cASIC1) and Psalmotoxin-1 (PcTx1), studies indicated that PcTx1 antagonized ASIC1a (Escoubas et al., 2000; Chen et al., 2005; Baconguis et al., 2012). In contrast, PcTx1 does not antagonize the ASIC1b subtype, but acts as an agonist by mediating a large inward sodium current that does not return to the original baseline (Chen et al., 2006). During calcium permeability studies and the subsequent crystallographic study, it was observed that PcTx1 activates cASIC1 in a similar manner (Samways et al., 2009; Baconguis et al., 2012). We rationalized that PcTx1 would be an appropriate compound to partially activate cASIC1. Therefore, we hypothesized that the ability for compounds like GMQ to interact with cASIC1, is state dependent, and requires the channel to remain in a partially activated state in order to exert its effects. We sought to test this hypothesis with the following two specific aims: (1) Determine how combinations of protons,
nonproton ligands, and Psalmotoxin-1 can simultaneously modulate ASIC1 and (2) Identify if GMQ sensitivity of ASIC3 can be introduced to a nonproton ligand insensitive ASIC1 subtype.

**SIGNIFICANCE**

Several crystallographic structures using the chicken ASIC1 (cASIC1) subtype have confirmed that a prevailing theme for each of these ASIC structures is that these ion channels’ extracellular domains may sense more than simply protons (Jasti et al., 2007; Gonzales et al., 2009; Baconguis et al., 2012). The ASIC1 subtype has been, until recently, thought to be the primary proton receptor in the brain and is important in ischemic-mediated cell death (Chu et al., 2012). In addition, ASIC3 has been thought to have a substantial role in pain perception in the periphery (Deval et al., 2008; Deval et al., 2011; Sluka et al., 2003; Holzer et al., 2009; Izumi et al., 2012; Walder et al., 2010). Despite distinct roles in pathophysiological conditions, ASIC1 and ASIC3 are the most sensitive to extracellular protons and have overlapping distributions in sensory neurons in homomeric and heteromeric combinations (Deval et al., Deval et al., 2003, Deval et al., 2010, Deval et al., 2011; Mamet et al., 2002; Poirot et al., 2006; Wang et al., 2013). Furthermore, several studies on ASIC3 indicate that ASICs act as coincidence detectors to sense multiple stimuli at a given time, and that these channels can even form complexes with neighboring channels to respond to inflammatory stimuli (Birdsong et al. 2010; Deval et al., 2008; Immke and McCleskey, 2001b; Li et al., 2010; Smith et al., 2007). Despite strong evidence that ASICs are important for regulating normal and pathophysiological conditions, there remains a lack of subtype-specific ASIC-targeting compounds, specifically antagonists. Current medicinal compounds like amiloride, non-steroidal anti-inflammatory drugs (NSAIDs), 4-aminopyridine (4-AP), vegetal toxins, animal toxins, and even traditional Chinese herbs target
ASICs (Baron and Lingueglia, 2015). However, targeting ASICs as a class is much less challenging than developing specificity toward each ASIC subtype. Understanding interactions of multiple ligands, like protons, GMQ, calcium, and natural venom toxins with ASICs will progress the field toward developing ASIC subtype-specific, ligands and therapeutics.
**Fig. 1. Schematic of neuronal transmission.** Classic neurotransmission occurs with electrical stimulation at the axon hillock of a neuron. A robust impulse depolarizes the local cell membrane to threshold allowing for the generation of an action potential. The action potential is propagated down the axon terminal to synapse with a neighboring neuron. At the synaptic cleft, voltage-gated ion channels (VGIC) open allowing the flow of calcium into the presynaptic terminal. This calcium promotes the fusing of the loaded vesicle to the presynaptic membrane to release neurotransmitters (NT) along with protons (H⁺). The vesicular contents (e.g. NT and H⁺) are then able to act on the postsynaptic neuron via ligand-gated ion channels (LGIC) and promote the propagation of the signal.
Fig. 1. Schematic of neuronal transmission.
Fig. 2. Phylogenetic relationship of ENaC/DEG/ASICs. The conservation of the ENaC/DEG/ASIC family members is shown along with corresponding species. ENaC: epithelial sodium channel; DEG: degenerin; ASIC: acid-sensing ion channel; FaNaCh: FMRF amide-gated sodium channel; BLINaC: brain-liver-intestine sodium channel (sometimes referred to as ASIC5). (Figure from Abboud, 2010).
Fig. 2. Phylogenetic relationship of ASIC channels. *(Figure from Abboud, 2010)*
Figure 3. Schematic of a single ASIC subunit, including trimer and subunit domains. A, Single ASIC subunit is shown imbedded in the lipid bilayer with intracellular (in) amino- and carboxyl- termini, two transmembrane domains (TM1, TM2), and an intricate extracellular domain (out), which is overly simplified in this schematic. N- and C-termini are intracellular and lead into or extend from the first and second transmembrane domain, respectively. B, Trimeric representation of chicken ASIC1 with extracellular, transmembrane, and intracellular domains labeled. Each subunit is depicted by a different color (yellow, red, and blue). C, Single subunit of chicken ASIC1 with the finger, thumb, wrist, knuckle, β-ball, and palm domains labeled. For B and C, PDB code 4NYK was used.
Fig. 3. Schematic of a single ASIC subunit including trimer and subunit domains.
Fig. 4. Conservation of ASIC residues and activation of functional homomers. A, Topology of single ASIC subunit highlighting conserved sequences and binding sites. B, Activation of homomeric ASIC subtypes by protons in COS-7 cells. C, Proton-evoked currents recorded from (i) wild-type dorsal root ganglion neurons (WT-DRG), (ii) heteromeric ASIC subtypes in COS-7 cells, (iii) ASIC3+/- cultures mouse DRG neurons, and (iv) heteromeric ASIC1a/2a in COS-7 cells. D, ASIC1a KO mice eliminate nearly all proton-evoked currents recorded. (Figure from Wemmie et. al 2006).
Fig. 4. Conservation of ASIC residues and activation of functional homomers. (Figure from Wemmie et al. 2006).
Fig. 5. pNGFP-EU expression vector map and positive expression via green fluorescent protein. A, Wild type chicken ASIC1 (cASIC1) and rat ASIC3 (rASIC3) were inserted into a PNGFP-EU mammalian expression vector with an amino-terminal enhanced green fluorescent protein (EGFP) (Jasti et al., 2007). B, Successful transfection into Chinese hamster ovary cells (CHO-K1) was confirmed by fluorescent microscopy.
Fig. 5. pNGFP-EU expression vector map and positive expression via green fluorescent protein.
Table 1. Tissue distribution of ASIC subtype expression. *Adapted from Lin et al 2015.*

<table>
<thead>
<tr>
<th>Tissue</th>
<th>ASIC1</th>
<th>ASIC2</th>
<th>ASIC3</th>
<th>ASIC4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Central nervous system</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>amygdala</td>
<td>Y</td>
<td>Y</td>
<td></td>
<td>Y</td>
</tr>
<tr>
<td>brain</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>brain stem</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>cerebellum</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>cortex</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>hippocampus</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>muscle-motor nerve</td>
<td>Y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>olfactory bulb</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>oligodendrocyte</td>
<td>Y</td>
<td></td>
<td></td>
<td>Y</td>
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<tr>
<td>pituitary gland</td>
<td>Y</td>
<td></td>
<td></td>
<td>Y</td>
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<tr>
<td>spinal cord</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>striatum</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
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<tr>
<td><strong>Peripheral nervous system</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TG/DRG</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
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<tr>
<td>eye/retina</td>
<td></td>
<td></td>
<td></td>
<td>Y</td>
</tr>
<tr>
<td>nodose</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>cochlear</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
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<tr>
<td><strong>Skin</strong></td>
<td></td>
<td></td>
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<tr>
<td>Pacinian corpuscles</td>
<td></td>
<td></td>
<td></td>
<td>Y</td>
</tr>
<tr>
<td>Ruffini endings</td>
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<tr>
<td>Hair follicles</td>
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<tr>
<td>Meissner corpuscle</td>
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<tr>
<td>epidermal free nerve</td>
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<td>Y</td>
</tr>
<tr>
<td><strong>Vascular</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>cerebral artery</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>pulmonary artery</td>
<td></td>
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<td>Y</td>
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<tr>
<td><strong>Internal organ</strong></td>
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<td>bone marrow stromal cell</td>
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<tr>
<td>bone marrow dendritic cell</td>
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<td>Y</td>
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<tr>
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<tr>
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<td>adipose cell</td>
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<td></td>
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</tr>
<tr>
<td>bladder</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>joint</td>
<td></td>
<td></td>
<td></td>
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</tr>
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</table>
Table 2. Known functional properties of ASIC subtypes. *Adapted from Grunder and Pusch, 2015.*

<table>
<thead>
<tr>
<th>ASIC subtype</th>
<th>ASIC1a</th>
<th>ASIC1a/2a</th>
<th>ASIC1a/2b</th>
<th>ASIC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH&lt;sub&gt;50&lt;/sub&gt; activation</td>
<td>6.4-6.6</td>
<td>4.8-5.4</td>
<td>6.4</td>
<td>6.5-6.7</td>
</tr>
<tr>
<td>pH&lt;sub&gt;50&lt;/sub&gt; desensitization</td>
<td>7.2-7.3</td>
<td>--</td>
<td>7.3</td>
<td>7.1</td>
</tr>
<tr>
<td>τ&lt;sub&gt;act.&lt;/sub&gt; (ms); pH 6.0</td>
<td>6.0-13</td>
<td>--</td>
<td>--</td>
<td>&lt;5</td>
</tr>
<tr>
<td>τ&lt;sub&gt;desens.&lt;/sub&gt; (s)</td>
<td>1.2-3.5</td>
<td>0.6-0.9</td>
<td>2.6</td>
<td>0.3</td>
</tr>
<tr>
<td>τ&lt;sub&gt;recovery&lt;/sub&gt; (s); pH 7.4</td>
<td>5.0-13</td>
<td>0.6</td>
<td>--</td>
<td>0.4-0.6</td>
</tr>
</tbody>
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Table 3. Synthetic, endogenous and natural modulators of ASICs. Adapted from Baron & Lingueglia, 2015.

<table>
<thead>
<tr>
<th>Chemical structure</th>
<th>Compound Name</th>
<th>Pharmacological class</th>
<th>Effective concentration</th>
<th>ASIC subtypes involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthetic modulators</td>
<td>2-guanidine-4-methylquinazoline (GMQ)</td>
<td>Amiloride-derived</td>
<td>EC\textsubscript{50} ~ 1 mM</td>
<td>ASIC3</td>
</tr>
<tr>
<td></td>
<td>4-aminopyridine (4AP)</td>
<td>Kv channel blocker</td>
<td>IC\textsubscript{50} ~ 760 µM</td>
<td>All but ASIC3</td>
</tr>
<tr>
<td></td>
<td>A-317567</td>
<td>Amidine</td>
<td>IC\textsubscript{50} ~ 2-30 µM</td>
<td>ASIC3</td>
</tr>
<tr>
<td></td>
<td>Amiloride</td>
<td>Diuretic</td>
<td>EC\textsubscript{50} ~ 560 µM IC\textsubscript{50} ~ 5-100 µM</td>
<td>ASIC3, ASIC3 + 1b All</td>
</tr>
<tr>
<td></td>
<td>Benzothiophene methyl ammine</td>
<td></td>
<td>IC\textsubscript{50} ~ 0.22 µM</td>
<td>ASIC1a, ASIC3</td>
</tr>
<tr>
<td></td>
<td>CHF5074</td>
<td>NSAID-derived</td>
<td>IC\textsubscript{50} ~ 50 nM</td>
<td>Hippocampal ASIC channels</td>
</tr>
<tr>
<td></td>
<td>Chloroquine</td>
<td>Anti-malaria</td>
<td>IC\textsubscript{50} ~ 600 µM</td>
<td>ASIC1a</td>
</tr>
<tr>
<td></td>
<td>Diarylamidines</td>
<td>Anti-parasitic</td>
<td>IC\textsubscript{50} ~ 0.3-38 µM</td>
<td>All</td>
</tr>
<tr>
<td></td>
<td>Flurbiprofen, ibuprofen</td>
<td>NSAID</td>
<td>IC\textsubscript{50} ~ 350 µM</td>
<td>ASIC1a</td>
</tr>
<tr>
<td>Chemical Structure</td>
<td>Substance</td>
<td>Category</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Targets</td>
</tr>
<tr>
<td>--------------------</td>
<td>--------------------</td>
<td>------------------------</td>
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</tr>
<tr>
<td><img src="image1" alt="Lidocaine" /></td>
<td>Lidocaine</td>
<td>Local anesthetic</td>
<td>~ 12 mM</td>
<td>ASIC1a</td>
</tr>
<tr>
<td><img src="image2" alt="Nafamostat mesilate" /></td>
<td>Nafamostat mesilate</td>
<td>Anticoagulant</td>
<td>~ 2-70 µM</td>
<td>All</td>
</tr>
<tr>
<td><img src="image3" alt="Propofol" /></td>
<td>Propofol</td>
<td>Anesthetic</td>
<td>~ 30-300 µM</td>
<td>ASIC1a, ASIC3</td>
</tr>
<tr>
<td><img src="image4" alt="Salicylic acid, aspirin, diclofenac" /></td>
<td>Salicylic acid, aspirin, diclofenac</td>
<td>NSAID</td>
<td>~ 90-260 µM</td>
<td>ASIC2a, ASIC3</td>
</tr>
<tr>
<td><img src="image5" alt="Streptomycin, neomycin" /></td>
<td>Streptomycin, neomycin</td>
<td>Antibiotics, aminoglycosides</td>
<td>~ 32-44 µM</td>
<td>Sensory neuronal ASIC channels</td>
</tr>
<tr>
<td><img src="image6" alt="Tetracaine" /></td>
<td>Tetracaine</td>
<td>Local anesthetic</td>
<td>~ 10 mM</td>
<td>ASIC1b, ASIC3</td>
</tr>
<tr>
<td><img src="image7" alt="Tetraethylammonium (TEA)" /></td>
<td>Tetraethylammonium (TEA)</td>
<td>Cation, Kv channel inhibitor</td>
<td>~ mM range</td>
<td>ASIC1a+2a/2b</td>
</tr>
<tr>
<td><img src="image8" alt="Agmatine, arcaine" /></td>
<td>Agmatine, arcaine</td>
<td>Polyamines (GMQ-related)</td>
<td>~ 1-10 mM</td>
<td>ASIC3+1b, ASIC3</td>
</tr>
<tr>
<td><img src="image9" alt="Arachidonic acid (AA), anandamide" /></td>
<td>Arachidonic acid (AA), anandamide</td>
<td>Inflammatory mediator, cannabinoid</td>
<td>~ 1-10 µM</td>
<td>All</td>
</tr>
<tr>
<td><img src="image10" alt="Ca&lt;sup&gt;2+&lt;/sup&gt;, Mg&lt;sup&gt;2+&lt;/sup&gt;" /></td>
<td>Calcium, Magnesium</td>
<td>Cations</td>
<td>~ 2-10 mM range</td>
<td>All</td>
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<tr>
<td><img src="image11" alt="Cd&lt;sup&gt;2+&lt;/sup&gt;, Cu&lt;sup&gt;2+&lt;/sup&gt;, Gd&lt;sup&gt;3+&lt;/sup&gt;, Ni&lt;sup&gt;2+&lt;/sup&gt;, Pb&lt;sup&gt;2+&lt;/sup&gt;" /></td>
<td>Cadmium, Copper, Gadolinium, Nickel, Lead</td>
<td>Cations</td>
<td>~ µM to mM range</td>
<td>All</td>
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<tr>
<td><img src="image12" alt="Dynorphins" /></td>
<td>Dynorphins</td>
<td>Opioid neuropeptides</td>
<td>~ 26-33 nM</td>
<td>ASIC1a, ASIC1b</td>
</tr>
<tr>
<td></td>
<td>Neuropeptides</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Subtype(s)</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>---------------------------</td>
<td>----------------</td>
<td>--------------------------</td>
<td></td>
</tr>
<tr>
<td>FMRFamide and Rfamide-related peptides</td>
<td>Neuropeptides</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~ 10-50 µM</td>
<td>ASIC1a, ASIC1b, ASIC3</td>
<td></td>
</tr>
<tr>
<td>NH&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Cations</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~ 1-10 mM</td>
<td>ASIC1a</td>
<td></td>
</tr>
<tr>
<td>Nitric oxide (NO donors)</td>
<td>Inflammatory/ischemia mediator</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~ 10-100 µM</td>
<td>All</td>
<td></td>
</tr>
<tr>
<td>Serotonin</td>
<td>Inflammatory mediator</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~ 41 µM</td>
<td>ASIC3</td>
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<tr>
<td>Spermine</td>
<td>Polyamines</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~ 495 µM</td>
<td>ASIC1a, ASIC1b, ASIC1a+2a</td>
<td></td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Cations</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; ~ 120 µM; IC&lt;sub&gt;50&lt;/sub&gt; ~ 26-61 µM; ~ 10 nM</td>
<td>ASIC1a, ASIC1b, ASIC1a+2a</td>
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### Natural modulators

<table>
<thead>
<tr>
<th>Peptide</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Subtype(s)</th>
</tr>
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<tbody>
<tr>
<td>APETx2</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; ~ 37 nM to 2 µM</td>
<td>ASIC3+1a/1b, ASIC3/2b, ASIC3</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; ~ 0.2 µM</td>
<td>Sensory neuronal ASIC channels</td>
</tr>
<tr>
<td>Gastrodin</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; ~ 0.2 µM</td>
<td>Sensory neuronal ASIC channels</td>
</tr>
<tr>
<td>Peptide</td>
<td>Mambalgins</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; ~ 11-250 nM</td>
</tr>
<tr>
<td>Peptide</td>
<td>MitTx</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; ~ 9-830 nM</td>
</tr>
<tr>
<td>Peptide</td>
<td>Compound</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td>---------</td>
<td>----------</td>
<td>----------------</td>
</tr>
<tr>
<td>Paeoniflorin</td>
<td>Vegetal compound</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; ~ 5 µM</td>
</tr>
<tr>
<td>Pctx1</td>
<td>Spider peptide toxin (<em>Psalmopoeus cambridgei</em>)</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; ~ 0.4-4 nM 100-190 nM</td>
</tr>
<tr>
<td>PhcrTx1</td>
<td>Sea anemone peptide toxin (<em>Phymanthus crucifer</em>)</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; ~ 100 nM</td>
</tr>
<tr>
<td>Puerarin</td>
<td>Vegetal flavinoid</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; ~ 9-38 µM</td>
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<tr>
<td>Sevanol</td>
<td>Lignan from thyme</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; ~ 0.3-1.8 mM</td>
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<tr>
<td>Thallassiolin B</td>
<td>Vegetal flavinoid</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; ~ 27 µM</td>
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55, 281 – 290.


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II. PROTONS AND PSALMOTOXIN-1 REVEAL NONPROTON LIGAND STIMULATORY SITES IN CHICKEN ACID-SENSING ION CHANNEL: IMPLICATION FOR SIMULTANEOUS MODULATION IN ASICS

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ABSTRACT

Acid-sensing ion channels (ASICs) are proton-sensitive, sodium-selective channels expressed in the nervous system that sense changes in extracellular pH. These ion channels are sensitive to an increasing number of nonproton ligands that include natural venom peptides and guanidine compounds. In the case of chicken ASIC1, the spider toxin Psalmotoxin-1 (PcTx1) activates the channel, resulting in an inward current. Furthermore, a growing class of ligands containing a guanidine group has been identified that stimulate peripheral ASICs (ASIC3), but exert subtle influence on other ASIC subtypes. The effects of the guanidine compounds on cASIC1 have not been the focus of previous study. Here, we investigated the interaction of the guanidine compound 2-guanidine-4-methylquinazoline (GMQ) on cASIC1 proton activation and PcTx1 stimulation. Exposure of expressed cASIC1 to PcTx1 resulted in biphasic currents consisting of a transient peak followed by an irreversible cASIC1 PcTx1 persistent current. This cASIC1 PcTx1 persistent current may be the result of locking the cASIC1 protein into a desensitized transition
state. The guanidine compound GMQ increased the apparent affinity of protons on cASIC1 and decreased the half-maximal constant of the cASIC1 steady-state desensitization profile. Furthermore, GMQ stimulated the cASIC1 PcTx1 persistent current in a concentration-dependent manner, which resulted in a non-desensitizing inward current. Our data suggests that GMQ may have multiple sites within cASIC1 and may act as a “molecular wedge” that forces the PcTx1-desensitized ASIC into an open state. Our findings indicate that guanidine compounds, such as GMQ, may alter acid-sensing ion channel activity in combination with other stimuli, and that additional ASIC subtypes (along with ASIC3) may serve to sense and mediate signals from multiple stimuli.

INTRODUCTION

The acid-sensing ion channel (ASIC) is emerging as a robust sensor for extracellular pH in a variety of pathologies, such as stroke, pain, and mental health diseases. ASICs consist of three protein subunits arranged around a central pore that is primarily sodium selective, and are activated by protons (H+). A variety of ASIC subtypes (ASIC1a, 2a, 2b, 3, and 4) are available to form either homomeric or heteromeric channels, except for ASIC2b and ASIC4, which can only exert their effects in a heteromeric channel configuration. These proton sensitive channels are members of a larger family of trimeric ion channels, the epithelial sodium channel/Degenerin (ENaC/DEG) family. Members of the ENaC/DEG family are presumed to share similar subunit topology, consisting of intracellular amino- and carboxyl-termini, two transmembrane domains, and a large extracellular domain.

The ASIC three-dimensional structures were determined using truncated constructs of chicken ASIC1 (cASIC1), which was cloned by Coric and colleagues, and revealed the low pH
structure\textsuperscript{5} and the desensitized channel state.\textsuperscript{6} These structures showed the intricacy of the ASIC extracellular domain, the location of potential proton-binding sites involved in ASIC channel activation, the ASIC desensitization gate, and potential cation binding sites at the mouth of the channel pore.\textsuperscript{6} These channel structures highlighted how similar ASICs/ENaCs are to a once thought to be unrelated cation channel, the ATP sensitive purinergic-gated ion channel (P2X receptor), despite both channels are activated by different agonists.\textsuperscript{7, 8} The prevailing theme for each of these ASIC structures is that these ion channels’ extracellular domains are intricate and may sense more than simply protons.

Natural venom toxins have emerged as a new area for developing novel pharmaceuticals that target ion channels and influence ASIC activity. Each of the functional ASICs has demonstrated sensitivity to these venom toxins. The first toxin-ASIC interactions were identified and described for ASIC1 and ASIC3. Psalmotoxin-1 (PcTx1), isolated from the venom of the tarantula *Psalmopoeus cabridgei*, was shown to antagonize the ASIC1a subtype\textsuperscript{9} with nanomolar affinity and increases the channels sensitivity to protons.\textsuperscript{10} In contrast, PcTx1 does not antagonize the ASIC1b subtype but acts as an agonist by mediating a large inward sodium current that does not return to the original baseline, resulting in the presence of a toxin-induced persistent current.\textsuperscript{11} While studying the PcTx1 effects on ASIC mediated calcium permeability, it was observed that PcTx1 activates chicken ASIC1 in a similar manner.\textsuperscript{12} Other toxins influence ASIC activity. A toxin obtained from the venom of *Anthopleura elegantissima* (Pacific coast sea urchin), APETx2, inhibits ASIC3 with a measured IC\textsubscript{50} of 20 nM.\textsuperscript{13} The list of venom peptides is growing, as additional natural toxins that activate (coral snake toxin)\textsuperscript{14} and inhibit (black mamba toxin)\textsuperscript{15} ASICs have been reported.
Natural peptide toxins are examples of exogenous peptides that influence ASIC activity through direct interactions. However, the intricacy of the ASIC extracellular domain suggested that there could be other molecules that could influence the channel’s activity. Within ASIC3, the commonly used ASIC antagonist amiloride can stimulate and inhibit channel activity in a pH-dependent manner. At modest pH changes (pH 7.4 to 7.0), amiloride enhances ASIC3 mediated current but inhibits the transient current of low pH activation.\textsuperscript{16} Mutating the amiloride site within the channel pore reveals a stimulatory component of amiloride activity.\textsuperscript{17} This provided the first glimpse of a second site that may influence ASIC activity in a stimulatory manner. Additional compounds were identified that stimulated the activity of ASIC3. These compounds, termed ‘nonproton ligands’, contain a characteristic guanidine group and include 2-guanidine-4-methylquinazoline (GMQ), the arginine metabolite agmatine, and the synthetic guanidinium compound arcaine.\textsuperscript{18} GMQ exhibits the most profound effect, as it robustly activates ASIC3 and has been shown to influence nociception via an ASIC3 mechanism.\textsuperscript{18} Agmatine and arcaine stimulation of ASIC3 has reduced efficacy compared to GMQ stimulation, but may be combined with other inflammatory mediators to enhance ASIC3 activity.\textsuperscript{19} Furthermore, GMQ reduced the proton potency of ASIC1a to more acidic conditions and altered the steady-state desensitization of ASIC1a, 1b, 2a, and 3. The proton-mediated ASIC current was reduced with increasing GMQ concentrations that are greater than concentrations needed to activate ASIC3 (<300 mM).\textsuperscript{20} A conserved glutamate residue within the ASIC central vestibule, the equivalent residue to Glu-80 in cASIC1, has been identified to mediate the GMQ stimulatory effects in ASIC3.\textsuperscript{18,19,21}

The crystal structures of the PcTx1-ASIC complex were obtained using truncated cASIC1 constructs.\textsuperscript{22,23} Baconguis and Gouaux described two PcTx1-ASIC1 crystal structures
using a construct (termed “D13”) where 13 and 63 amino acids were removed from the channel’s respective amino and carboxyl termini. These two channel states represent a high pH toxin-bound state (pH 7.25) and the low pH toxin bound state (pH 5.5). If PcTx1 is administered at either pH, there is an observed cASIC1 PcTx1-mediated current that does not return to pre-PcTx1 baseline values, with subsequent low pH applications resulting in a diminished inward current. The PcTx1 activation of cASIC1 is in contrast to the pharmacological and experimental data observed in the studies of the spider toxin and ASIC1a.

In the present report, we sought to assess the influence of PcTx1 and the nonproton ligand GMQ on transiently expressed chicken ASIC1 using patch-clamp electrophysiology. We observed, similar to previous reports, that toxin induced a cASIC1 PcTx1 persistent current that was irreversible. Furthermore, we observed that subsequent application of low pH, in the absence of additional PcTx1, test solution induced a reduction of PcTx1 steady state current, reminiscent of H+-induced desensitization observed in ASIC1b, but not in ASIC1a. The cASIC1 PcTx1 persistent current was enhanced by the prototypical ASIC nonproton ligand 2-guanidine-4-methylquinazoline in a concentration-dependent manner with unique slow activation and deactivation kinetics. Additionally, GMQ increased the cASIC1 proton sensitivity and decreased the steady-state desensitization of cASIC1. However, GMQ failed to directly activate cASIC1, which is in contrast to the direct activation associated with this guanidine ligand at ASIC3. Based on our data, we propose that there are two stimulatory nonproton ligand sites present in the chicken ASIC1 subtype, with one of these sites sharing properties with the ASIC nonproton ligand sensor domain. Our data provide evidence to suggest that the nonproton ligand sensor domain is present in other ASICs, but is only accessible in a state-dependent manner.
RESULTS

*PcTx1 induces an irreversible and persistent cASIC1 current.*

We sought to characterize the interaction of the Psalmotoxin-1 (PcTx1) in wild type cASIC1. Previous reports described the toxin’s agonist activity in the chicken ASIC construct and the resulting toxin persistent current. To confirm this response, we transiently transfected CHO cells with a fluorescently labeled cASIC1, which provided visual confirmation of protein expression using fluorescent microscopy. We exposed patch-clamped cells to 3, 10, and 30 nM PcTx1 (Fig. 1A). Since the toxin binds irreversibly, each toxin exposure was performed on toxin-naïve cells. Psalmotoxin-1 at 30 nM concentration showed maximal peak current amplitude as compared to the 3 and 10 nM PcTx1 responses (Fig. 1B). We utilized the saturating concentration of 30 nM PcTx1 in our subsequent studies to observe the toxin’s maximal effects on cASIC1. Working with the saturating concentration of toxin, we first applied a pH 6.0 solution while holding the cell at a constant -70 mV and measured the resulting peak current amplitude (Fig. 2A). The pH 6.0 solution application resulted in a robust ASIC transient inward current followed by proton-mediated desensitization, as expected. Following a 2-minute washout period, we applied PcTx1 (30 nM) to the patched cells. The spider toxin activated a cASIC1 transient peak current amplitude that was 40% of the measured amplitude of the low pH mediated current (0.40 ± 0.04, n = 7) (Fig. 2A). The toxin-mediated transient current desensitized, but did not return to the pre-toxin baseline. Both the low pH and toxin were applied to the patched cells for 5 seconds. The remainder of the toxin-mediated current persisted for the duration of the experiment. A second pH 6.0 solution application was applied to the cASIC1 PcTx1 persistent current to assess the proton sensitivity of the toxin-ASIC complex and to determine if toxin could be removed using protons for further characterization of the toxin-
channel interaction (Fig. 2A). The second low pH application resulted in a transient inward current, which exhibited a reduced peak current as compared to the pre-PcTx1 pH 6.0 control response (0.23 ± 0.03, n = 7) (Fig. 2B). Furthermore, the low pH application resulted in a return to the pre-PcTx1 persistent current levels during the duration of the test pulse. Upon cessation of the low pH solution application, the cASIC1 PcTx1-mediated persistent current returned. The PcTx1 persistent current was 22% of the control current amplitude (0.22 ± 0.03, n = 7) (Fig. 2C).

Previous reports using PcTx1 indicated that bovine serum albumin (BSA) was added in order to minimize tube absorption of the toxin. In our studies, we supplemented the toxin test solutions with 1% BSA and ensured that this BSA concentration did not influence cASIC1 activity (Supplementary Fig. 1). In our initial BSA-free PcTx1 experiments we subjected cASIC1 positively transfected CHO cells to pH 6.0 followed by PcTx1 (30 nM) and repeated this experiment with BSA containing solutions (Supplementary Fig. 1A). A 1% BSA test solution had no effect on cells expressing cASIC1 (Supplementary Fig. 1B and 1C). We observed a slight reduction in peak current following the BSA exposure that was not statistically significant (p=0.4305) (Supplementary Fig. 1C). After ensuring that BSA addition to our test solutions was not affecting our toxin-mediated responses, we attempted to remove the toxin from the cASIC1 expressing cells failed, thus preventing our pursuit of generating a complete PcTx1 concentration-response profile. Applications of toxin at pH 6.0 resulted in an increase in the cASIC1 transient peak current amplitude, suggesting that PcTx1 enhances proton activation and induces a persistent current at lower pH (Supplementary Fig. 2).

The PcTx1-mediated transient and persistent current in cASIC1 was intriguing. Previous studies have reported that a persistent current in cASIC1 is present when PcTx1 is applied to
cells expressing the protein. However, the striking aspect of this channel response to the toxin was how similar the current is to currents generated by the ASIC3 subtype. ASIC3 has been shown to generate an inward current in response to two specific stimuli: 1) the removal or chelation of the extracellular divalent cation calcium and 2) shifting the external pH to modest acidic conditions. First, simply removing calcium from the external environment, either by chelating or using nominal (approximately 0 mM) divalent cation solutions, will activate ASIC3. Second, at modest pH changes (pH 7.4 to 7.0), ASIC3 mediate current followed by a steady state current that is observed for the duration of the exposure. We considered that these two channels, rASIC3 and cASIC1, mediate the low calcium steady-state current and the PcTx1 persistent current, respectively, as a response to two distinct stimuli. Following our initial attempts to interrupt the toxin-cASIC1 interaction, we compared the cASIC1 persistent and ASIC3 steady-state currents. Using pH 7.35 as our base solution, we subjected ASIC3 expressing cells to a nominal calcium external solution (Fig. 3A). ASIC3 generated an inward transient peak current followed by desensitization that resulted in a channel mediated steady-state current. A typical steady-state current in ASIC3 will remain until the external calcium is returned to the initial concentration. In our experiment, ASIC3 was exposed to the nominal calcium environment for 60 seconds before making a complete return to the baseline. In comparison, we applied the spider toxin (30 nM) to patched cells expressing cASIC1 for 5 seconds and observed the cASIC1 PcTx1 persistent current for 5 minutes (Fig. 3B). A single, 5 s application of PcTx1 leaves the channel in a current-conducting state and fails to return to a non-conducting state. Although these two channels generate similar current, different stimuli (nominal calcium versus toxin application) are required to generate these similar channel responses.
**GMQ alters the proton-sensitivity of cASIC1.**

Based on our observation, the cASIC1 PcTx1 persistent current and the low calcium environment ASIC3 currents are similar. If these currents are similar, we considered that identified ASIC3 modulators might influence this cASIC1 PcTx1 persistent current. These compounds, termed nonproton ligands, contain a guanidinium group that carries a single positive charge when applied within the working range of pH values for ASIC activity. These nonproton ligands include the endogenous arginine metabolite agmatine, the synthetic compound arcaine, and 2-guanidine-4-methylquinazoline (GMQ), which robustly activates ASIC3 (Fig. 4A)\(^\text{18, 19, 21}\). Furthermore, these compounds share similarity to the prototypical ASIC antagonist amiloride, which also contains a guanidinium group (Fig. 4B). However, GMQ and amiloride exert different effects on ASIC activity. The guanidinium group, which serves as a monovalent cation, may interact with ASIC at two sites that elicit different responses: stimulation through the nonproton ligand sensor domain (within the central vestibule at the base of the extracellular domain) and antagonism through the pore-blocking site (amiloride site). We sought to determine if GMQ influences either cASIC1 activity under standard conditions or the cASIC1 PcTx1 persistent current. The cASIC1 expressing cells were exposed to GMQ (0.3 mM) in the absence of PcTx1 and was applied to cells at pH 7.35 and 8.0, respectively (Fig. 5A, 5B). Despite being exposed to different conditioning pH, GMQ failed to elicit a response in cASIC1.

Although GMQ alone did not affect cASIC1, we explored the possibility that GMQ may influence the channel in combination with proton-mediated activation (Fig. 6). We began by determining the activation profile for WT cASIC1 using a conditioning pH of 8.0 and measuring the peak current amplitude for decreasing pH (increasing proton concentrations). Each response was normalized to pH 6.0 (Fig. 6A). Additionally, we determined the steady-state desensitization
(SSD) curve associated with the WT cASIC1 channel by bathing cells in each conditioning pH and measuring the peak current amplitude at pH 6.0 (Fig. 6A). We reproduced the activation and SSD profiles in the presence of GMQ (0.3 mM) and noted a leftward shift in the activation profile (WT pH<sub>50</sub> of 6.65 ± 0.01 and nH 4.55 ± 0.54; GMQ pH<sub>50</sub> of 6.98 ± 0.01 and nH of 2.21 ± 0.38) (Fig. 6B and Table 1). The presence of GMQ in the conditioning solution resulted in a statistically significant decrease in SSD proton sensitivity (WT 7.53 ± 0.00; GMQ 7.48 ± 0.00).

Since acid-sensing ion channels are keenly sensitive to pH values near the activation and SSD profile interface, we investigated if nominal calcium could result in channel activity at the activation-SSD interface (approximately pH 7.2). Using a 10 pA response as our cutoff, we observed no measurable response when cASIC1 was exposed to nominal calcium at the cASIC1 activation-SSD profile interface (data not shown).

**GMQ enhances the PcTx1 induced persistent current.**

Despite having little effect on cASIC1 under normal conditions, we continued to investigate the influence of GMQ by applying the nonproton ligand to the channels when the cASIC1 PcTx1 persistent current had been established. Our recording protocol for the GMQ mediated effects on the cASIC1 PcTx1 persistent current included the initial pH 6.0 test solution, followed by the induction of the PcTx1 persistent current (application of 30 nM PcTx1 for 5 seconds), and followed by another application of the pH 6.0 test solution for confirmation of the establishment of the cASIC1 PcTx1 persistent current (Fig. 7A). Each test pulse was separated by at least 2 minutes of washout with the external solution. The cASIC1 PcTx1 persistent current was consistent throughout the recording, which is shown with a dashed line (Fig. 7A). The nonproton ligand GMQ stimulated the persistent current in a concentration-dependent manner at
GMQ concentrations of 0.1 (0.02 ± 0.01), 0.3 (0.19 ± 0.05), and 1 mM (0.50 ± 0.11) as compared to the pH 6.0 control (Fig. 7B). Each of these GMQ mediated currents exhibited slower activation and deactivation kinetics than what is observed with proton-mediated ASIC activity. The GMQ mediated activation rise times (10-90%) were fit to a monoexponential equation and yielded time constants (tact) for 0.3 and 1 mM GMQ concentrations of 1.03 ± 0.22 s and 0.96 ± 0.14 s, respectively. At the highest concentration of GMQ tested (1 mM), we observed a small rebound current at the end of the GMQ application. This rebound current suggests that GMQ may antagonize the GMQ stimulated cASIC1 PcTx1 persistent current via a channel blocking mechanism.

A rebound current occurs following amiloride blockade of the PcTx1 persistent current.

The cASIC1 PcTx1 persistent current was observed in the functional characterization of the truncated construct used to solve the PcTx1-ASIC1 crystal structure. Baconguis and Gouaux exposed the toxin-mediated current to amiloride in combination with additional PcTx1. Although amiloride failed to reduce the PcTx1 persistent current, amiloride did antagonize the PcTx1 mediated transient current. Since GMQ, the prototypical ligand for the nonproton ligand sensor domain, stimulated the cASIC1 PcTx1 persistent current, we sought to determine if amiloride had an effect on the cASIC1 PcTx1 persistent current in the absence of additional toxin. After establishing the PcTx1 persistent current, we subjected the cASIC1 PcTx1 persistent current to increasing concentrations of antagonist amiloride (0.01, 0.1, and 0.5 mM) (Fig. 8). The cASIC1 PcTx1 persistent current response to amiloride was apparent at the lowest concentration used (0.01 mM). Initially, amiloride reduces the cASIC1 PcTx1 persistent current (as observed by the immediate reduction in the PcTx1 persistent current baseline), followed by a steady return
to the toxin persistent current baseline (Fig. 8A). This was observed for amiloride at concentrations of 0.1 and 0.5 mM. At the completion of the 5-second amiloride exposure, a rapid rebound current was observed that increased in amplitude with increasing amiloride concentration (Fig. 8A). The amiloride rebound current amplitude at 0.01 and 0.1 mM were 10 and 50% of the pH 6.0 control current (0.10 ± 0.04 and 0.51 ± 0.20, respectively) (Fig. 8B). Furthermore, the 0.5 mM amiloride rebound current approached the same current amplitude of the pH 6.0 test solution (1.14 ± 0.4). The mean rebound peak current amplitude at the end of the amiloride application was observed to be either greater than or less than the initial steady-state current at 0.01 mM concentrations of the antagonist (1.37 ± 0.13) (Fig. 8C). Furthermore, the amiloride rebound currents generated after cessation of the application on the cASIC1 PcTx1 persistent current for 0.1 and 0.5 mM of the antagonist were 2.98 ± 1.02 and 5.49 ± 2.03, respectively (Fig. 8C). Our data suggest that amiloride influences the cASIC1 PcTx1 persistent current when further stimulation of additional toxin is absent and reflect the direct effect of amiloride on the established cASIC1 PcTx1 persistent current and not the transient toxin-mediated activation of cASIC1.

DISCUSSION

Psalmotoxin-1 elicits a persistent current in wild type chicken ASIC1.

The spider toxin, Psalmotoxin-1 (PcTx1), has been used to selectively target and inhibit neuronal ASIC1a to characterize the role of these proton-sensitive channels in stroke and acidosis-induced cell death. Psalmotoxin-1 is neuroprotective in these studies, suggesting that targeting ASIC1a may be a suitable target for novel neuroprotective interventions. Recently revealed ASIC protein crystal structures provide views into how the channel’s architecture
relates to function, revealing the trimeric arrangement of protein subunits \(^5\) and the architecture of the channel pore. \(^6\) However, the PcTx1-bound ASIC structure, and accompanying functional assessment, provided evidence to suggest that cASIC1 acts differently than the ASIC1a subtypes targeted in the neuroprotection studies. \(^22\) The PcTx1-bound ASIC structure revealed an open channel conformation and included an observed amiloride-insensitive persistent current. These structural studies utilized a modified cASIC1 construct (named “D13”), where 13 residues from the amino terminus and 63 residues from the carboxyl terminus were removed to improve crystallographic resolution. The D13 construct had a determined pH\(_{50}\) of 6.41 ± 0.02 and a Hill coefficient of 3.1. Wild type cASIC1, in our studies, had an apparent pH\(_{50}\) of 6.67 ± 0.01 and Hill coefficient of 4.06. The differences observed in proton affinity and Hill coefficients between WT cASIC1 and D13 could be attributed to the difference in protein terminal residues. The PcTx1 activation profiles were different, as 30 nM PcTx1 in our hands was the most efficacious. In the D13 construct, the PcTx1 EC\(_{50}\) was 188 nM. \(^22\) The difference in apparent affinities could be due to the truncation of the D13 construct’s termini. Our current report focused on the functional significance of this PcTx1 interaction with wild type cASIC1 and its interaction with guanidine compounds. Consistent with previous reports, we observed a cASIC1 PcTx1 persistent current after a brief application (5 seconds) of the spider toxin. Furthermore, this cASIC1 PcTx1 persistent current appeared irreversible, as it remained throughout our experiments and persisted during a long recording protocol (20 minutes). These cASIC1 PcTx1 persistent currents precluded the determination of a concentration-response profile due to the toxin’s irreversible activity. These cASIC1 PcTx1 persistent currents have been observed previously in cells that express both heterologous and endogenous cASIC1. \(^12\) We observed proton-mediated stimulation of the persistent current at pH 6.0, but the proton-mediated activation underwent proton-induced
desensitization similar to the pre-PcTx1 pH 6.0 channel activity. The current returned to the pre-PcTx1 baseline during the low pH application, but upon its end, the cASIC1 PcTx1 persistent current was re-established. This transient current, followed by over-shoot towards the pre-PcTx1 baseline, differs from what has been observed previously. This overshoot current was not observed when the PcTx1 persistent current was exposed to low pH.\textsuperscript{22} In this case, low pH and PcTx1 were applied simultaneously and may influence the cASIC1 PcTx1 persistent current. One possibility for this discrepancy is that the addition of toxin along with low pH test solution prevented the return to pre-toxin baseline current. In our study, we exposed the cASIC1 PcTx1 persistent current to a range of pH values and did not reverse the effect. Moreover, we observed proton-dependent changes in current. At all the pH values tested, the cASIC1 PcTx1 persistent current was reduced while exposed to increased protons. This suggests that the PcTx1 persistent current is a toxin-bound desensitized current that can transition to the open state with lower pH values (< pH 6), but still undergoes proton-dependent desensitization during the low pH exposure. Furthermore, low pH is unable to remove the toxin from the PcTx1-cASIC1 complex.

One question that has lingered with the identification of chicken ASIC1\textsuperscript{4} and the protein’s use as a crystallographic construct along with the first ASIC protein structure\textsuperscript{5} has persisted: which ASIC subtype does cASIC1 resemble? Also, are the described structures of ASIC representative of all acid sensing ion channels? For example, the prototypic ASIC antagonist, amiloride, failed to antagonize the PcTx1 persistent current of the truncated ASIC construct used for structure determination.\textsuperscript{22} Furthermore, amiloride also failed to inhibit the window current mediated by modest pH changes in ASIC3\textsuperscript{16}, suggesting that the toxin-bound cASIC1 crystal structure may represent the structure of ASIC3 while mediating this window current.\textsuperscript{22} Located predominantly in the periphery, ASIC3 is sensitive to changes in extracellular
calcium and subjecting ASIC3 expressing cells to low calcium conditions results in a non-desensitizing inward current.\textsuperscript{28} Exposure to more alkaline conditions (higher pH) results in an increase in this window current; exposure to acidic conditions (low pH) results in a robust inward current that is followed by ASIC3 desensitization.\textsuperscript{24} These ASIC3 currents are similar to our observed cASIC1 PcTx1 persistent currents and suggests that these currents, despite being generated by different ASIC subtypes, are similar.\textsuperscript{22} Our data provide an example in both the ASIC3 and cASIC1 activity to show that these two channels, although under normal conditions function distinctly with unique channel activity, can generate similar response. The resulting response is dependent on the presence of unique ligands, such as PcTx1, or the removal of stabilizing cations, such as calcium. Taken together, the ASIC architecture described by the available protein crystal structures may represent channel states in other ASIC subtypes and may be exploited to describe specific ASIC states of these other subtypes. For cASIC1, PcTx1 is necessary to mimic the ASIC3 mediated current in nominal calcium conditions. In other conditions, cASIC1 fails to mediate a current in nominal calcium conditions.

\textit{GMQ increases the cASIC1 apparent proton affinity.}

Nonproton ligands have become the focus of study within the ASIC family of ion channels as they act as direct activators of ASIC3 and modulate the channel in the absence of proton activation.\textsuperscript{18} These first ligands included 2-guanidine-4-methylquinazoline (GMQ), the arginine metabolite agmatine, and the synthetic arcaine. Each of these nonproton ligands contains a guanidinium group that is ionized within the working pH range of ASIC. Of these three, GMQ is the most potent, with maximal efficacy, and stimulates ASIC3 at concentrations as low as 1 mM when applied under mild acidity (pH 7.0).\textsuperscript{18} Recently, residues within the ASIC
central vestibule have garnered attention as the site of action for these guanidine nonproton ligands. The stimulatory effects of nonproton ligands have been attributed to a site within the ASIC central vestibule, which sits within the channel’s β-sheet core. The residues that have been identified as crucial for the nonproton ligand stimulation in ASIC3 are found in other ASIC subtypes including the central vestibule of cASIC1 (Leu 78, Glu 80, Gln 277, Gln 279, Arg 370, and Glu 417). We anticipated that GMQ would demonstrate some influence on cASIC1. However, we failed to observe GMQ mediated stimulation when applied alone to patched cells expressing the cASIC1 construct at either pH 8.0 or 7.35. This lack of GMQ stimulation under normal conditions is consistent with the lack of stimulation observed in other ASIC subtypes when GMQ is present.

However, guanidine compounds such as GMQ may have effects on the proton activation and steady-state desensitization profiles of cASIC1. We characterized the influence of GMQ on cASIC1 by probing the nonproton ligand’s effects on wild-type cASIC1 in the absence of toxin. Both cASIC1 activation curves and steady-state desensitization (SSD) curves were generated, and to our knowledge, this is the first investigation to characterize these parameters in the chicken ASIC1 construct. Based on our observations, the cASIC1 construct shares characteristics with other ASIC1 subtypes. With respect to the GMQ influence on the proton SSD profile, the guanidine compound shifted the profile to the right, increasing the cASIC1 SSD pH50 from 7.53 ± 0.00 to 7.48 ± 0.00 (Fig. 6). This change in SSD pH50 is consistent with the GMQ influence on the SSD profiles of ASIC1a, ASIC1b, ASIC2 and ASIC3. The SSD pH50s decrease for each of these acid-sensing ion channels to more acidic pH. The most profound effect of GMQ on cASIC1 is observed in the proton activation profile.
When GMQ is present, the proton activation profile is similar to the WT cASIC1 activation curve, with an apparent leftward shift to pH 6.98 ± 0.03 from pH 6.65 ± 0.01 in WT cASIC1. In other ASIC subtypes, like ASIC1a, 1b and 2a, GMQ is associated with a reduction of proton potency, which is observed as a rightward shift in the proton activation curve to more acidic pH. However, in ASIC3, the proton activation curve is shifted to the left, suggesting there is an apparent increase in proton potency in the presence of GMQ. Our findings indicate that the GMQ effects on cASIC1 are similar to ASIC3 in this respect. Furthermore, the ASIC3 window current is increased in the presence of GMQ. The wild type window current, found at the interface of the proton activation and steady-state desensitization curves, accounts for a small fraction of the ASIC3 current (less than 10% of peak current). In the presence of GMQ, the ASIC3 window current increases to greater than 60% of the ASIC3 peak current. The resulting expansion and shift of the ASIC3 window current by GMQ explains the profound effects of the guanidine compound on ASIC3 activity. In cASIC1, the apparent window current intersection of the SSD and activation curves occurs at pH 7.18 and accounts for less than 1% of the wild type peak current while GMQ shifts the window current to slightly more alkaline pH (7.28) but only increases the window current to no more than 15% of peak current. Furthermore, exposing cASIC1 to nominal calcium at pH 7.2 does not induce a window current (data not shown). In contrast, similar conditions in the presence of ASIC3 would result in a significant window current. Although the window current is not significantly altered in cASIC1, one cannot rule out that GMQ has similar actions on both cASIC1 and ASIC3. In the case of cASIC1, GMQ increases the apparent proton potency, but does so without generating the characteristic GMQ steady-state current (a non-desensitizing current) as observed by many previous studies. These studies have attributed the GMQ effect on this non-desensitizing current as the interaction
with the identified nonproton ligand sensor domain, which limits the desensitization of the channel. In two recently published findings, the residues associated with the central vestibule, and by extension the nonproton ligand sensor domain, move during ASIC desensitization to a collapsed conformation.\textsuperscript{29,30} One explanation for the apparent GMQ stimulation could be that the nonproton ligand, and other guanidine compounds, may bind at a second site within the ASIC protein architecture that is stimulatory and that remains stimulatory at modest pH ranges (pH 7.2 to 6.5). In our studies, we did not observe this. This provides the strongest evidence for a novel GMQ site, a site that lacks the GMQ-mediated steady-state current within this modest pH range. If GMQ accessed and mediated its effects via a nonproton ligand sensor domain, there would be an appreciable GMQ steady-state current. We observed no such current within this range in cASIC1.

\textit{The cASIC1 Pctx1 persistent current is sensitive to GMQ.}

The shift in the proton activation curve in the presence of GMQ suggests that the guanidine compound interacts with cASIC1, but does so in a manner inconsistent with interaction with the nonproton ligand sensor domain. However, the lack of GMQ direct stimulation does not rule out that the nonproton ligand sensor domain is functional within cASIC1. Perhaps, access to the nonproton ligand sensor domain and subsequent activity could be dependent on the ASIC state. For example, amiloride paradoxically enhances the ASIC3 window current during mild acidosis, but antagonizes the ASIC3 transient proton-mediated current, suggesting the state of the channel dictates activity.\textsuperscript{16} Amiloride may both inhibit the established cASIC1 Pctx1 persistent current and hinder cASIC1 desensitization. The result would appear as an amiloride-induced rebound current, as seen in this report. This amiloride stimulation has been
attributed to a site distinct from the amiloride antagonist site (located within the ASIC pore)\textsuperscript{17} and, most recently, attributed to the nonproton ligand sensor domain.\textsuperscript{25} Nonproton ligands stimulate ASIC3 in a concentration-dependent manner, often enhancing the proton-mediated current and reducing the observed channel desensitization.\textsuperscript{18} We observed that GMQ stimulated the cASIC1 PcTx1 persistent current in a concentration dependent manner and lacked desensitization, which is reminiscent of the ASIC3 response to activation with protons and GMQ.\textsuperscript{18} The GMQ cASIC1 persistent current stimulation differed from proton-mediated activation, as the measured GMQ activation time constants are slower than proton activation. When GMQ activates ASIC3, the nonproton ligand induces an inward current with steady-state current.\textsuperscript{18} The nonproton ligand GMQ acts to eliminate desensitization. The GMQ stimulation in the presence of the cASIC1 PcTx1 persistent current provides strong evidence that the nonproton ligand sensor domain is present in cASIC1, but only accessible when another ligand modifies the channel’s architecture providing a path to the stimulatory protein domain. The observed difference in GMQ activation and desensitization rates suggests that GMQ interaction with the PcTx1-bound cASIC1 protein is either: 1) due to limited access to the stimulus nonproton ligand sensor domain, or 2) the subsequent GMQ-induced conformational change to a conducting channel state is slow to occur.

\textit{GMQ activity on the cASIC1 PcTx1 persistent current suggests that there is a single site for activity.}

The nonproton ligand GMQ increased the cASIC1 PcTx1 persistent current in a concentration dependent manner. Furthermore, we observed that the increase in current had an estimated Hill coefficient of 1, which is similar to experimentally determined Hill values for
GMQ in ASIC3 stimulation. Yu and colleagues determined that in the presence of either increasing calcium or increasing protons, the GMQ Hill coefficient was near 1, which would support the contention that GMQ acts at a single site.\textsuperscript{18} Additionally, GMQ stimulates the cASIC1 PcTx1 persistent current with an observed slower activation than activation mediated by simply protons. We determined that both 300 mM and 1 mM GMQ stimulated the cASIC1 PcTx1 persistent current with statistically similar activation time constants. Our data provide compelling evidence to suggest that GMQ utilizes a single site to stimulate the cASIC1 PcTx1 persistent current. The likeliest candidate for the site of action for GMQ stimulation is the central vestibule site within the cASIC1 extracellular domain. However, we cannot rule out other potential sites of action for GMQ stimulation of the cASIC1 PcTx1 persistent current. Other nonproton ligands that contain at least one guanidinium group influence ASIC activity. The diarylamidines, a class of guanidinium compounds that include diminazene and the nuclear stain DAPI (both contain two guanidinium groups), antagonized current in all homomeric ASIC subtypes tested, with the most robust and potent inhibition observed in ASIC1b.\textsuperscript{31} Through in-silico modeling using the desensitized state ASIC structures\textsuperscript{5, 6}, several diarylamidines were shown to interact with sites in the exterior of the extracellular domain, away from the proposed proton, nonproton ligand sensor domain, and amiloride sites. This external ASIC nonproton ligand site has yet to be characterized further, but remains a likely candidate for mediating nonproton ligand activity in the acid-sensing ion channel. Furthermore, there is a possibility that the PcTx1-cASIC1 complex has exposed additional binding sites, which could expand the array of potential nonproton ligand sites and library of additional ASIC modulators. Furthermore, the enhancement of proton affinity by GMQ suggests that another site exists to explain these effects.
Amiloride exhibits dual activity on the cASIC1 PcTx1 persistent current.

Amiloride has been used as an antagonist for acid-sensing ion channels, despite the ligand’s dual role in ASIC3 activity. Here, amiloride both antagonizes the cASIC1 PcTx1 persistent current and results in a rebound current that is observed at the end of the amiloride exposure. Baconguis and Gouaux showed that when the persistent current was observed, amiloride reduced the PcTx1 mediated transient current and failed to modulate the persistent current. One clear difference with our study is that we chose to focus on the cASIC1 PcTx1 persistent current in the absence of further PcTx1 stimulation. Amiloride may perform two roles when modulating the cASIC1 PcTx1 persistent current. The normally antagonistic amiloride may block the cASIC1 PcTx1 persistent current through interaction with the amiloride inhibitory site within the channel pore. However, the return to the cASIC1 PcTx1 persistent current baseline during amiloride application suggests that the guanidine compound is binding to another site. This return is similar to the slow activation kinetics observed in GMQ stimulation of the same persistent current. We speculate that the interaction with the nonproton ligand sensor domain mediates the return to the cASIC1 PcTx1 persistent current baseline. Furthermore, amiloride may relieve the channel from desensitization that results in a rebound current at the end of the amiloride exposure. Thus, amiloride has dual roles in the modulation the cASIC1 PcTx1 persistent current. Amiloride has shown dual actions in other ASIC subtypes, more specifically in ASIC3, where the guanidine compound can antagonize the channel when the channel is activated by protons, as evidenced by a reduction in the proton-mediated transient current but enhances the ASIC3 window current. Both of these effects are observed for the PcTx1 interaction with cASIC1. The PcTx1 cASIC1 transient current is inhibited by amiloride; however, amiloride has no effect on the persistent current in the continued presence of toxin. Both the
inhibitory and stimulatory activity of amiloride on the cASIC1 PcTx1 persistent current provides evidence to suggest that the cASIC1 protein architecture has sites similar to ASIC3 and are nonproton ligand sensing sites.

cASIC1 stimulation by toxin is similar to ASIC1b.

In this report, we confirmed that PcTx1 activates cASIC1 and subsequently focused on the resulting PcTx1 persistent current and its sensitivity to nonproton ligands, such as GMQ and amiloride. We observed that PcTx1 irreversibly alters cASIC1 activity, which is distinct from the more frequently studied PcTx1 antagonism of ASIC1a. This PcTx1 activation of cASIC1 is comparable to other identified natural venom peptides. For example, the Texas coral snake toxin, MitTx (consisting of MitTx-a and MitTx-b) activated ASIC1a, 1b, and 3 in a concentration-dependent manner and increased the ASIC2a proton potency, despite sharing no homology to PcTx1.14 Psalmotoxin-1 activates cASIC1, which is unexpected as the chicken ASIC protein shares considerable sequence homology (89%) to human and rat ASIC1a and less homology to ASIC1b.4 Both the human and rat ASIC1a subtypes are antagonized by PcTx1 under normal conditions. Chen and Grunder have shown that PcTx1 activation can by observed in ASIC1a by lowering calcium.10 Conversely, PcTx1 is an agonist for ASIC1b, where the toxin activates this ASIC subtype at modest pH.11 In these ASIC1b studies, the PcTx1 induced channel activity exhibited a persistent current similar to the one outlined in this report. Furthermore, the application of low pH and amiloride resulted in a decline of this ASIC1b PcTx1 persistent current that parallels our observations when subjecting the cASIC1 PcTx1 persistent current to low pH. This decline is reversible, as observed in both our study and the published ASIC1b PcTx1 study. Despite sharing considerable homology to ASIC1a, it may be appropriate to
consider that cASIC1 is functionally similar to ASIC1b despite having considerable amino acid similarity to ASIC1a due to the PcTx1 activation. Thus, the use of PcTx1 in areas where ASIC1b is expressed should be cautioned and may result in persistent activity of ASIC1b. Further assessment of PcTx1 and other natural peptides is warranted before proceeding to more complex studies where mixed populations of acid-sensing ion channels are involved.

2-guanidine-4-methylquinazoline (GMQ) acts as a molecular wedge to stimulate the cASIC1 PcTx1 persistent current.

Our study provides evidence to suggest that there are two stimulatory GMQ sites in cASIC1. The first stimulatory site increases the cASIC1 apparent proton affinity as seen by a reduction in the activation profile’s pH50. A similar shift in proton affinity is observed in ASIC3, but in the case of cASIC1, there was no observed GMQ-mediated steady-state current at nominal changes in pH. The second site and the ligand’s stimulation of the cASIC1 PcTx1 persistent current are similar to what is observed in wild-type ASIC3 responses. This second site may be the ASIC nonproton ligand sensor domain. How do we reconcile that GMQ can stimulate the cASIC1 PcTx1 persistent current but not have a significant effect on low pH channel activity or on the channel in the absence of protons? We propose a hypothetical model to describe this cASIC1 PcTx1 persistent current and interaction with GMQ, as an extension of the Baconguis and Gouaux model for P2X and ASIC gating (Fig. 9). The high cASIC1 PcTx1 crystal structure shows a toxin-bound ASIC in an apparent open conformation, with an expanded pore domain that lacks a channel gate, or occlusion. However, the cASIC1 PcTx1 persistent current occurs after channel desensitization. Following activation of the channel, the cASIC1 PcTx1 transient current desensitizes to the toxin-induced persistent current and is mediated by an
equilibrium between two conformations, the toxin-bound open and inactivated/desensitized states. Furthermore, this proposed PcTx1-cASIC1 persistent current state might have a collapsed central vestibule that further stabilizes the inactive channel conformation. When GMQ is applied, the guanidine compound serves as a “molecular wedge”, working to force apart the ASIC central vestibule by binding to the nonproton ligand sensor domain. This GMQ “molecular wedge” requires time to occur, either by reaching the nonproton ligand sensor domain or inducing the conformational change. In doing so, GMQ may increase the probability of re-entering a toxin-activated state and reducing the probability of returning to the inactivated state. Either scenario may account for the slow activation observed in the GMQ stimulation of cASIC1 PcTx1 persistent current. The PcTx1 peptide, once bound to cASIC1, introduces a new set of channel conformations that may be susceptible to other ligand modulators.

Currently, there is sufficient evidence to suggest that the acid sensing ion channels have evolved to sense multiple stimuli. Our current study supports that position and shows that cASIC1 may be similar to ASIC3 when PcTx1 binds to the channel. Inflammatory mediators such as arachidonic acid, lactic acid, and the endogenously available agmatine modulate ASIC3, with demonstrated cumulative efficacy. These additive effects may contribute to the mediation of pain sensation following inflammation. Although ASIC3 has been shown to sense these inflammatory mediators that enhance mild acidic ASIC activation, ASIC3 was thought to be unique in sensing a multitude of signals that influence channel activity. The stimulation of the cASIC1 PcTx1 persistent current by GMQ suggests that other ASIC subtypes may sense multiple stimuli and have unique, unreported effects on channel activity. This synergistic ASIC modulation should be considered in the design of novel therapeutics that seeks to target the nonproton ligand sensor domain. Furthermore, previously described guanidinium-containing
ligands (e.g. arginine and other naturally occurring compounds) that have no direct effect on channel activity should be revisited in other ASIC subtypes in the context of multiple stimuli modulation, as described for ASIC3.\textsuperscript{32} The growing class of ASIC modulating natural venom toxins may pave the way for novel combinatorial stimulation, or antagonism, of ASIC activity. In the present scenario, the natural venom peptides offer the tools to study these multimodal effects in additional ASIC subtypes.

In summary, we have confirmed that PcTx1 activates wild type cASIC1 in a similar manner to the truncated ASIC constructs and irreversibly induces a persistent current. The nonproton ligand GMQ enhance cASIC1 proton sensitivity and stimulates the cASIC1 PcTx1 persistent current. We propose that GMQ mediates these properties via two stimulatory sites, with one of these sites being the nonproton ligand sensor within the ASIC extracellular central vestibule. It is clear, based on our study, that there are additional sites for guanidine containing compounds within the ASIC protein architecture. Although GMQ acts as a “molecular wedge” to stimulate the cASIC1 PcTx1 persistent current, other guanidine compounds may influence the channel differently (see amiloride in this study). Finally, we have provided compelling evidence to suggest that other ASIC subtypes, besides ASIC3, may act as receptors to integrate multiple stimuli and mediate a response.

MATERIALS AND METHODS

\textit{Cell culture and transfection.} Wild type cASIC1 (cASIC1) and rat ASIC3 with an amino-terminal enhanced green fluorescent protein (EGFP) encoded in a pNEGFP mammalian expression vector were kind gifts from Eric Gouaux (Vollum Institute, Portland OR). Both
EGFP-cASIC1 and EGFP-ASIC3 were transiently expressed in Chinese hamster ovary cells (CHO-K1) and were cultured in T25 flasks in Ham’s culture media (Life Technologies), containing 10% fetal bovine serum (Phenix Research) at 37° C in a 5% CO₂ water-jacketed incubator. Subculturating and transfection were performed when the cells reached 70-80% confluency with cells plated on square (9 x 9 mm) glass coverslips. CHO-K1 cells were transfected using 1-2 mg pNEGFP-cASIC1 cDNA and a 4 ml Lipofectamine LTX (Life Technologies) according to manufacturer’s instructions. Transfected cells were used for patch clamp electrophysiology 24-48 hours post transfection and successful transfection was confirmed using fluorescent microscopy.

**Electrophysiology.** Whole cell and outside-out patch-clamp recordings were obtained using thick-walled borosilicate glass capillary tubes (Sutter Instrument Company), which were pulled to a resistance of 3-6 MΩ (Flaming/Brown, P-87/PC, Sutter Instrument Co., Novato, CA) and subsequently fire-polished. Recording pipettes were filled with internal solution consisting of (in mM): KCl (100), NaCl (5), HEPES (40), EGTA (10), MgCl₂ (5) and adjusted to pH 7.4 using N-methyl-d-glucamine. Transfected cells were perfused with an external recording solution containing (in mM): NaCl (150), KCl (5), HEPES (5), MES (5), and CaCl₂ (1). External wash solution was adjusted to pH 7.35 while low pH test solutions were adjusted using HCl. Psalmotoxin-1 (PcTx1) was commercially obtained (Peptide International) and stock solutions made on the day of the experiments. Bovine serum albumin (BSA) was added to a final concentration of 1% w/v in all test solutions that contained PcTx1 to minimize toxin absorption to tubing and plastics. Psalmotoxin-1 stock solutions were diluted in standard external solution.
with 1% BSA. The nonproton ligand 2-guanidine-4-methylquinazoline (GMQ) was dissolved in dimethylsulfoxide (DMSO).

Solution exchange was obtained through the use of an array of capillary tubes arranged perpendicularly to a similar array that delivered control solutions, through the digital control of PTFE solenoid valves using a ValveLink8.2 controller (AutoMate Scientific) with a solution exchange rate of approximately 5 ms. Whole-cell and outside-out patch clamp recordings were performed on an inverted fluorescent microscope (Nikon) using an Axopatch 200B patch amplifier (Axon Instruments), filtered at 5 kHz, and sampled at 10 kHz for offline analysis in pClamp 10.0 (Molecular Devices). Cells were voltage-clamped at -70 mV. Offline analysis was performed with Clampfit 10.0 analysis software and Origin 8.1 (OriginLab). For longer recordings (>30 sec), recordings were filtered at 2 kHz and samples at 5 kHz.

Patched cells were allowed to equilibrate in external wash solution for a minimum of 5 minutes prior to experimentation. Test solutions were applied for 5 seconds and patched cells were washed with external wash solutions for a minimum of 2 minutes between test solution applications. A typical patch clamp experiment lasted more than 30 minutes. During this time, there was no diminishment of the cASIC1 PcTx1 persistent current. After each experiment with PcTx1 application, the recording dish chamber and coverslip were replaced to prevent previous PcTx1 exposure from contaminating subsequent recordings. Activation experiments were performed in the presence and absence of GMQ in the test solutions. Steady-state desensitization experiments were performed in the presence and absence of GMQ in the conditioning solutions. For steady-state desensitization experiments, patch-clamped cells were exposed to conditioning pH (with and without GMQ) for 60 seconds followed by exposure to pH 6.0 test solution
(without GMQ) for 5 seconds, followed by a return to the conditioning pH. Responses were normalized to the peak current amplitude of the pH 6.0 recording.

Data analysis. Data were analyzed using the statistical software package Origin 8.2 (OriginLab). Nonproton ligand (GMQ) activation time constants (t_{act}) were determined by fitting the 10-90% activation rise-time to a monoexponential equation using the Levenberg-Marquardt method (Clampfit 9.0). The peak current amplitudes of PcTx1 mediated current, PcTx1 persistent current, and GMQ mediated current were normalized to their respective pH solution controls. Statistical significance was determined utilizing unpaired Student’s t-test on a minimum of three individual cells. Data is presented as the mean ± SEM.
Fig. 1. cASIC1 is maximally sensitive to 30 nM PcTx1. A, Representative whole-cell patch clamp recordings of separate cells subjected to PcTx1 (3, 10, or 30 nM) normalized to a pH 6.0 (pre-PcTx1) control pulse, for comparison, are shown (holding potential: -70 mV). Test solutions were applied for 5 seconds and returned to the normal bath solution (pH 7.35). Whole-cell recordings are scaled for comparison. Horizontal scale bar is in seconds (s). B, Summary of normalized peak current for 3, 10 and 30 nM (0.02 ± 0.01; 0.35 ± 0.05; 0.40 ± 0.04 of the pH 6.0 control, respectively) PcTx1 in pH 7.35 following pH 6.0 (pre-PcTx1) control is shown. Data is presented as mean ± SEM of at least 3 individual cells.
Fig. 1. cASIC1 is maximally sensitive to 30 nM PcTx1.
Fig. 2. cASIC1 PcTx1 persistent current is sensitive to pH. A, Whole-cell patch clamp recordings of pH 6.0 (pre-PcTx1), PcTx1 mediated, and pH 6.0 (post-PcTx1) in cASIC1 are shown (holding potential maintained at -70 mV). Test solutions were applied for 5 seconds and returned to the normal bath solution (pH 7.35). Horizontal and vertical scale bars are seconds (s) and picoAmperes (pA), respectively. B, Summary of normalized peak current of the PcTx1 transient current (0.40 ± 0.04, n=7) and the pH 6.0 following the establishment of the cASIC1 PcTx1 persistent current (0.23 ± 0.03, n=7) are shown. C, Summary of the cASIC1 PcTx1 persistent current (0.22 ± 0.03, n=7) are shown. Data is presented as mean ± SEM of at least seven individual cells and significance was determined using paired Student’s t-test (*, p<0.05 compared to control). (PcTx1-PC, PcTx1 persistent current).
Fig. 2. cASIC1 PcTx1 persistent current is sensitive to pH.
Fig. 3. The cASIC1 PcTx1 persistent current is similar to the ASIC3 low calcium environment current. A, Whole-cell recording of rASIC3 following a shift from calcium containing external (1 mM) to a nominal calcium external solution (~0 mM CaCl$_2$) at pH 7.35 are shown. Scale bars: time, in seconds (horizontal) and nA (vertical). B, Representative whole-cell recording of cASIC1 showing a five second application of PcTx1 (30 nM) at pH 7.35 is presented. The duration of the recording is 5 minutes. Scale bars: time, in seconds (horizontal) and pA (vertical).
Fig. 3. The cASIC1 PcTx1 persistent current is similar to the ASIC3 low calcium environment current.
**Fig. 4. Structures of guanidinium compounds.** The nonproton ligand chemical structure of (A) 2-guanidine-4-methylquinazoline (GMQ) and the common ASIC antagonist (B), amiloride are shown. The guanidinium group within both compounds is highlighted with a dashed box.
Fig. 4. Structures of guanidinium compounds.
Fig. 5. GMQ fails to directly activate cASIC1. A, Patch-clamp recordings of GMQ (0.3 mM) at pH 7.35 following a pH 6.0 control test pulse are shown. B, Patch-clamp currents of GMQ (0.3 mM) at pH 8.0 following a pH 6.6 control test pulse are shown. No measurable current was observed in either condition. Data are representative of n ≥ 3 cells.
Fig. 5. GMQ fails to directly activate cASIC1.
Fig. 6. GMQ shifts the proton sensitivity and steady-state desensitization of cASIC1. A, Representative outside-out patch clamp recordings for WT cASIC1 activation for selected test pulses are shown. Conditioning pH for each pulse is pH 8.0. B, Summary of pH dependence of cASIC1 activation (solid line) and steady-state desensitization, or SSD, (dashed line) profiles under control conditions for WT cASIC1 are shown. The half-maximal pH response (pH$_{50}$) and Hill slope (nH) values for the cASIC1 activation were 6.65 ± 0.01 and 4.55 ± 0.54, respectively. The mean pH$_{50}$ and Hill slope (nH) values for cASIC1 steady-state desensitization were 7.53 ± 0.00 and 5.82 ± 0.08, respectively. Data is presented with normalized current ($I/I_{max}$) as a function of pH. C, Representative outside-out patch clamp recordings for cASIC1 activation in the presence of GMQ (0.3 mM) for selected test pulses are shown. D, Summary of pH dependence of cASIC1 activation (solid line) and steady-state desensitization, or SSD, (dashed line) in the presence of GMQ (300 mM) are shown. The half-maximal pH response (pH$_{50}$) and Hill slope (nH) values in the presence of GMQ were 6.98 ± 0.03 and 2.21 ± 0.38, respectively, while the mean SSD pH$_{50}$ and SSD Hill slope (nH) values for the GMQ SSD curve are 7.48 ± 0.00 and 5.75 ± 0.19, respectively. Data are presented as the mean ± SEM of at least 6 patched cells with normalized current ($I/I_{pH \, 6.0}$) as a function of pH.
Fig. 6. GMQ shifts the proton sensitivity and steady-state desensitization of cASIC1.
**Fig. 7. GMQ stimulates the cASIC1 PcTx1 protein complex.** A, Whole cell recording of pH 6.0, PcTx1 (30 nM), and GMQ concentration (0.1, 0.3, 1 mM). Horizontal and vertical scale bars are in seconds (s, horizontal axis) and picoAmperes (pA, vertical axis), respectively. The zero current (blackened dashed lines) and cASIC1 PcTx1 persistent current (red dashed line) are indicated. B, Summary of concentration-dependent GMQ stimulation of PcTx1-cASIC1 current at 0.01 mM (0.02 ± 0.01), 0.3 mM (0.19 ± 0.05), and 1 mM (0.50 ± 0.11). The GMQ peak current amplitude was normalized to the pH 6.0 control current. Data is presented as mean ± SEM of at least four individual cells and significance was determined using unpaired Student’s t-test (*, p<0.05 compared to control).
Fig. 7. GMQ stimulates the cASIC1 PcTx1 protein complex.
Fig. 8. Amiloride influences the cASIC1 PcTx1 persistent state in two ways. A, Whole-cell recordings of the cASIC1 PcTx1 persistent current at low pH with amiloride. Amiloride (0.01, 0.1, and 0.5 mM) inhibited the cASIC1 PcTx1 persistent current. B, Summary of amiloride rebound current and low pH peak current. Observed amiloride rebound current was normalized to pH 6.0 peak current. Compared to the control response, normalized amiloride peak current at 0.01, 0.1, and 0.5 mM were 0.10 ± 0.04, 0.51 ± 0.20, and 1.14 ± 0.4, respectively. Observed amiloride rebound current was normalized to the PcTx1 persistent current. Compared to the PcTx1 persistent current, the normalized amiloride peak current at 0.01, 0.1, and 0.5 mM were 0.88 ± 0.45, 4.19 ± 1.96, and 9.44 ± 4.06, respectively. Data is presented as mean ± SEM of at least three individual cells.
Fig. 8. Amiloride influences the cASIC1 PcTx1 persistent state in two ways.
**Fig. 9. Proposed model of PcTx1 mediated gating of cASIC1.** Hypothetical toxin-mediated cASIC1 gating pathway is shown. PcTx1 activates ASIC1 inducing an expanded transmembrane (TM) domain region (**left**). After the initial PcTx1 application, the toxin-cASIC1 complex moves to a non-conducting conformation (**middle**). Furthermore, the central vestibule may collapse to mediate the non-conduction of current. The transmembrane domain occludes the pore (possibly similar to the desensitized channel conformation). Nonproton ligands, such as GMQ, may act to pry apart the central vestibule, like a “molecular wedge”, to open the channel complex (**right**). The ASIC gating schemes are depicted as the following: immovable protein scaffold (blue), central vestibule region (green), and TM domains (orange). The solved PcTx1-ASIC1 protein crystal structure is highlighted (dashed box). Model was generated with similar terminology and design for comparison to the previous models of channel gating (For review, see ref. 22).
Fig. 9. Proposed model of PcTx1 mediated gating of cASIC1.
Supplementary Fig. 1. Bovine serum albumin (BSA) has no effect on cASIC1. A, Representative whole-cell recording of PcTx1 (30 nM) addition in the absence and presence of BSA (1% w/v) addition are shown. Horizontal bar is in seconds. B, Representative outside-out recordings with a pre-BSA and post-BSA pH 6.0 test pulse are shown. Bovine serum albumin was applied at pH 7.35. Horizontal and vertical scale bars are seconds (s) and picoAmperes (pA), respectively. C, Normalized pH 6.0 peak currents (post-BSA) were compared to the control (pre-BSA) pH 6.0 peak currents and were not significantly different (post-BSA mean was 0.86 ± 0.15, p=0.4035). Data are presented as mean ± SEM of three individual cells.
Supplementary Fig. 1. Bovine serum albumin (BSA) has no effect on cASIC1.
Supplementary Fig. 2. PcTx1 persistent current remains despite altering pH following toxin addition. A, Representative whole-cell recordings depicting pH 6.0 alone (pre-PcTx1), pH 6.0 in combination with PcTx1 (30 nM), and a post-PcTx1 pH 6.0 test pulse from the same cell are shown. At least a two-minute washout at the conditioning pH (pH 7.35) and a return to the persistent current baseline separates each solution exchange. Whole cell recordings are scaled for comparison. Vertical and horizontal scale bar are pA and seconds, respectively. B, Representative whole-cell recordings from the same cASIC1 expressing CHO cell are shown. Depicted, in order (from left to right): pH 6.0 control, 30 nM PcTx1, pH 6.0 washout, pH 7.0, 5.0, and 4.0. (Conditioning pH = 7.35). Each recording is separated by one-minute washout between exposures, which was sufficient to return to the previous baseline. Horizontal and vertical scale bars are seconds (s) and picoAmperes (pA), respectively. Solid black bars indicate test solution application.
Supplementary Fig. 2. PcTx1 persistent current remains despite altering pH following toxin addition.
### Table 1. Summary of pH activation and steady-state desensitization for cASIC1 in the absence and presence of GMQ

<table>
<thead>
<tr>
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<th>pH$_{50}$</th>
<th>pH$_{50}$, 95% CI</th>
<th>nH</th>
<th>nH, 95% CI</th>
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<tr>
<td><strong>Activation</strong></td>
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<tr>
<td>Control</td>
<td>6.65 ± 0.01</td>
<td>6.62 – 6.67</td>
<td>4.55 ± 0.54</td>
<td>5.93 – 3.16</td>
</tr>
<tr>
<td>GMQ</td>
<td>6.98 ± 0.03**</td>
<td>6.90 – 7.06</td>
<td>2.21 ± 0.38**</td>
<td>3.19 – 1.23</td>
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<tr>
<td><strong>SSD</strong></td>
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<tr>
<td>Control</td>
<td>7.53 ± 0.00</td>
<td>7.52 – 7.53</td>
<td>5.82 ± 0.08</td>
<td>5.57 – 6.08</td>
</tr>
<tr>
<td>GMQ</td>
<td>7.48 ± 0.00**</td>
<td>7.47 – 7.50</td>
<td>5.75 ± 0.19</td>
<td>5.16 – 6.34</td>
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</table>

Control, denotes WT cASIC1; GMQ, represents cASIC1 in the presence of 0.3 mM GMQ; ** indicates different from control, p < 0.01. All, n ≥ 6.
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III. DIRECT ACTIVATION OF ACID-SENSING ION CHANNELS BY 2-GUANIDINE-4-METHYLQUINAZOLINE IS MEDIATED BY THE TRANSMEMBRANE DOMAINS

ABSTRACT

Acid-sensing ion channels (ASICs) are trimeric, sodium-selective channels activated by extracellular protons. Although ASICs are intriguing molecular targets for pharmacological agents, there remains a lack of selective compounds that differentiate ASIC subtypes. The peripherally located ASIC3 activates with the simple removal of calcium. Additionally, nonproton ligands, like 2-guanidine-4-methylquinazoline (GMQ), have been identified to selectively activate ASIC3 via the nonproton ligand sensor domain (NPLSD). A pair of glutamates in rASIC3 (E79 and E423) responsible for GMQ activation is present in ASIC1, despite having no direct modulation effect on the channel. We proposed that direct nonproton ligand activation of ASIC3 is possible due to the actions of the transmembrane domains. Chimeric receptors combining the extracellular, transmembrane, and intracellular domains of rat ASIC3 and chicken ASIC1 were generated to individually isolate the calcium and nonproton ligand effects on channel activation. Each chimeric receptor was assessed for function using whole-cell patch clamp electrophysiology. We confirmed that rASIC3 is activated and held open when extracellular calcium concentrations are reduced with minimal proton influence (pH 8.0). Low-calcium-activation of rASIC3 is further enhanced by the addition of GMQ in a concentration dependent manner. These effects are absent in cASIC1. The chimera termed cASIC1 (rASIC3-TM/ITC) is comprised of the extracellular domain of cASIC1 and the transmembrane/intracellular domains of rASIC3, and can be activated by GMQ in the absence of
calcium, although its sensitivity to GMQ is reduced. Thus, GMQ activation was introduced in cASIC1 by replacing the transmembrane domains with those of ASIC3. This data suggests that the ASIC3 TM domains dictate NPLSD influence on channel activity.

INTRODUCTION

Acid-sensing ion channels (ASICs) are trimeric, sodium-selective channels characterized by a large extracellular domain, two transmembrane domains per subunit, and intracellular amino- and carboxyl-termini and are members of the epithelial sodium channel (ENaC)/degenerin (DEG) family [1-6]. ASICs are activated by protons and have been implicated in ischemia, neurotransmission, and pain nociception [7-11]. Four genes encode six ASIC subtypes (ASIC1a, 1b, 2a, 2b, 3, and 4) each with extensive physiological functions [7, 12-17]. In the central nervous system, ASIC1 has been implicated in stroke as the primary proton receptor in the brain, while the peripherally located ASIC3 primarily mediates the perception of pain [11, 18-20]. Recent studies have now implicated ASIC1 involvement in pain perception as well [21].

With involvement in overlapping functions, it is increasingly important that targeting ASIC subtypes is carefully selective. However, there is a dearth of selective ligands to differentiate between ASIC subtypes. Complicating this search, ASIC subtypes have mixed interactions with newly described ASIC ligands. In particular, this includes calcium and a novel class of ligands termed ‘nonproton ligands’. Previous studies have reported that simply removing calcium can activate ASIC3 at alkaline pH (pH 8.0) by relieving the calcium block atop the channel pore [22] (Fig. 1A). Additionally, the nonproton ligand, 2-guanidine-4-methylquinazoline (GMQ), selectively activates ASIC3 at a neutral pH [23]. Other ASIC subtypes, like ASIC1, appear to be void of these effects. The pair of glutamate residues important for GMQ interaction within the
central vestibule, or nonproton ligand sensor domain (NPLSD), of ASIC3 (E79, E423) is analogous to residues in ASIC1 (E80, E417) (Fig. 1B) [23-25]. Other residues that surround the amino acids differ, but do not contribute to GMQ-ASIC3 activation. Even so, the stimulatory effects of GMQ on ASIC3 are not observed when studying ASIC1.

Our previous work showed that the GMQ reached the ASIC1 NPLSD in a state-dependent manner [26]. The publication of the crystal structure and subsequent electrophysiological studies of chicken ASIC1 (cASIC1) in complex with Psalmotoxin-1 (PcTx1) illustrated that the tarantula toxin mediates a persistent, steady state current in cASIC1 [27]. This cASIC1 PcTx1 persistent current was sensitive to GMQ, suggesting that the GMQ sensitive NPLSD is present in cASIC1. Thus, additional mechanism or channel domains must contribute to GMQ direct activation of the acid-sensing ion channel.

We sought to clarify the role the ASIC extracellular domain plays in contributing to GMQ direct activation and calcium sensitivity. To achieve this end, we generated chimeric acid-sensing ion channels by using chicken ASIC1 and rat ASIC3 (Fig. 1C) and assessed function using whole-cell patch-clamp electrophysiology. In order to identify the domains responsible for nonproton ligand interaction via the NPLSD and the calcium-block site, we designed two chimeric receptors: 1 containing the extracellular domain of cASIC1 and the transmembrane and intracellular domains of rASIC3: cASIC1 (rASIC3-TM/ITC), and 2 containing the extracellular domain of rASIC3 and the transmembrane and intracellular domains of cASIC1: rASIC3 (cASIC1-TM/ITC). By combining the presumably insensitive NPLSD of cASIC1 and the calcium-block site of rASIC3, we propose that GMQ will directly activate the chimeric cASIC1 (rASIC3-TM/ITC) following the simple removal of calcium. In the present study, we demonstrate that the one of the studied chimeric ASICs, chimeric cASIC1 (rASIC3-TM/ITC) is
sensitive to extracellular protons and the removal of calcium at alkaline pH. Furthermore, this chimeric channel was sensitive to GMQ in a concentration-dependent manner, specifically in the absence of extracellular calcium. Our data suggest that the transmembrane domains of ASIC are critical for GMQ activation via the nonproton ligand sensor domain.

RESULTS

Simple removal of calcium activates chimeric cASIC1/rASIC3.

Previous reports indicate that rASIC3 channels open when calcium is excluded or removed from extracellular recording solutions [22, 28, 29]. Additionally, rASIC1a channels respond similarly with calcium exclusion, but to a lesser degree [28]. We sought to determine if the simple removal of calcium would also activate chicken ASIC1. Utilizing whole-cell patch-clamp electrophysiology, we observed that cASIC1 is unable to open at a basic pH (pH 8.0) at nominal calcium concentrations (~ 0 mM) (Fig. 2A). However, both rat ASIC3 and the chimeric receptor cASIC1 (rASIC3-TM/ITC) open with minimal proton influence (pH 8.0) and nominal calcium (~ 0 mM) (Fig. 2B and C, respectively). Furthermore, cASIC1 (rASIC3-TM/ITC) possesses a reduced response to calcium exclusion, compared to rASIC3.

Calcium increases proton sensitivity in ASIC.

Although removing calcium did not directly activate cASIC1, we were interested in the contribution of calcium to the overall pH response of the channel. Previous studies have shown that both protons and calcium are important for gating ASICs [22, 28, 29]. Our interest was to determine the contribution of calcium on pH-mediated ASIC activation (Fig. 3A, B, and C, respectively). Representative traces and summarized activation curves for each subtype are
shown in the presence and absence of calcium by normalizing proton-mediated activation to pH 6.0 (I/I_{pH_{max}}). Interestingly, we observed cASIC1 channels exhibited a significant increase in proton sensitivity in nominal calcium concentrations; most notably activation increased with pH 7.2 and 7.4 when calcium is absent (Fig. 3A, +/- calcium, traces 4 and 5). We observed that the exclusion of calcium produces a significant increase in proton sensitivity (pH_{50} = 6.90 \pm 0.03, nH = 4.07 \pm 0.75, open squares) compared to 1 mM CaCl_2 (pH_{50} = 7.01 \pm 0.03, nH = 2.05 \pm 0.23, closed squares) (Fig. 3A). In addition to enhanced peak current, we noted an increased sustained current in nominal calcium conditions in rASIC3 (Fig. 3B). Additionally, rASIC3 undergoes a significant shift in proton sensitivity (pH_{50} = 7.2 \pm 0.04, nH = 1.30 \pm 0.14, open circles) compared to the control profile with 1 mM CaCl_2 (pH_{50} = 6.72 \pm 0.01, nH = 2.33 \pm 0.19, closed circles) (Fig. 3B). This was similar to previous reports [22]. The chimeric cASIC1 (rASIC3-TM/ITC), retains some calcium sensitivity, but appears the least affected by calcium in the extracellular recording solution (control calcium pH_{50} = 6.76 \pm 0.03, nH = 2.29 \pm 0.35, closed triangles; nominal calcium pH_{50} = 6.91 \pm 0.03, nH = 2.52 \pm 0.34, open triangles) (Fig. 3C). This data is summarized in Table 1.

*Nominal calcium concentrations are critical for cASIC1 (rASIC3-TM/ITC) activation.*

Since rASIC3 and cASIC1 (rASIC3-TM/ITC) can be activated by GMQ or removing calcium, we were interested in further understanding how both variables can affect channel activation. We confirmed that rASIC3 activates in a concentration-dependent manner when GMQ (0.3, 1, 3 mM) and in the presence of calcium (1 mM) (Fig. 4A). This concentration-dependent activation was similar in conditions with nominal calcium (~ 0 mM), however at GMQ concentrations greater than 1 mM, rASIC3 peak current amplitude began to plateau in
response (Fig. 4B). Furthermore, the ASIC3 response to GMQ in a nominal calcium environment was as robust as compared to equivalent GMQ concentrations at 1 mM CaCl$_2$. We then summarized the observed concentration-dependent GMQ activation of rASIC3 in both the presence (1 mM; closed circles) and absence (~ 0 mM; open circles) of calcium (Fig. 4C). In the presence of calcium (1 mM), GMQ increased activation of rASIC3 that begins to plateau at the maximum GMQ concentration tested (10 mM) (Fig. 4C, closed circles). When calcium is excluded from the extracellular solution (~ 0 mM), rASIC3 activation increases until the GMQ concentration is 1 mM (Fig. 4C, open circles). Higher GMQ concentrations (3 and 10 mM) tested showed a decline in rASIC3 peak current amplitude compared to the control GMQ concentration (0.3 mM). Additionally, we observed a rebound current at the end of the 3 mM GMQ exposure in the nominal calcium environment.

Unlike rASIC3, we observed that chimeric cASIC1 (rASIC3-TM/ITC) remains unable to be activated by increasing concentrations of GMQ with calcium present (1 mM) (Fig. 4D). However, within a nominal calcium environment, the chimera activated in response to increasing GMQ concentrations (0.3, 1, 3 mM) (Fig. 4E). Summarized concentration-response profiles for cASIC1 (rASIC3-TM/ITC) subjected with increasing concentrations of GMQ were normalized in comparison to 0.3 mM GMQ (Fig. 4F). The sensitivity to increasing concentrations of GMQ appeared lower in the chimera, as seen by the scale of the summarized concentration-response profiles (Fig. 4C, F). Activation of cASIC1 (rASIC3-TM/ITC) by GMQ increased without a noticeable plateau at the maximum concentration tested (10 mM) in a nominal calcium environment (Fig. 4F, open triangles). The inclusion of calcium with increasing GMQ concentrations did not yield any activation of the chimeric ASIC, making it distinct from rASIC3 (Fig. 4F, closed triangles). We did not examine higher concentrations of GMQ due to challenges
in maintaining the compound in solution.

*GMQ can rescue proton induced desensitized rASIC3 and cASIC1(rASIC3-TM/ITC).*

We know that GMQ can directly activate ASIC channels, given that the palm domain is accessible to allow GMQ to bind. Since the publication of the crystal structure of MitTx bound to cASIC1, we cannot rule out that guanidinium compounds can bind in proton-binding sites in the ETC domain, as seen by another guanidinium-containing compound, amiloride [30]. Furthermore, GMQ rescued the desensitized PcTx1 cASIC1 persistent current to an open state similar to rASIC3 [26]. We were interested to understand GMQ could act as a “molecular wedge” and rescue proton-bound desensitized ASICs back to an open state. To test this, we designed a simple recording that would expose desensitized channels to GMQ (Fig. 5). Following pH 6.0 activation, we subjected desensitized channels to GMQ (0.3 mM) while desensitized. The addition of GMQ following pH 6.0 did not revert cASIC1 desensitized channels to open channels (Fig. 5A, top trace). For rASIC3, GMQ rescued the channel from desensitization. However, this rescues current returned to the desensitized baseline prior to GMQ exposure (Fig. 5A, middle trace). Interestingly, we observed GMQ rescuing the chimeric to cASIC1 (rASIC3-TM/ITC) desensitized channels to open but did not return to a proton-induced desensitized state (Fig. 5A, bottom trace). Although there was no measureable rescue of current by GMQ in cASIC1, for rASIC3 and the chimeric cASIC1 (rASIC3-TM/ITC) currents were rescued 14 and 3 percent, respectively (Fig. 5B). This percent rescue was significant compared to the zero percent rescue recorded for cASIC1, and suggests that the ASIC transmembrane domains of ASIC3 allows for the rescue of desensitized channels to an open state.
DISCUSSION

Here, we demonstrate that the ASIC transmembrane domains contribute to the channel’s direct activation by nonproton ligands that interact with the extracellular nonproton ligand sensor domain (NPLSD). Based on our studies, and previous work, we propose that ASIC3 has evolved to accommodate NPLSD-mediated activation, via the transmembrane domains. Despite housing the NPLSD, cASIC1 and other ASICs fail to be activated by the nonproton ligands under physiological pH conditions. Furthermore, this activity is combined with the unique ASIC3 sensitivity to the divalent cation, calcium. Several studies have indicated that the proton sensitivity of ASICs is tightly regulated by extracellular calcium concentrations [7, 22, 29, 31-33]. In particular, an inverse relationship with extracellular calcium and proton sensitivity exists in ASIC1a and ASIC3 [22, 29, 31, 32, 34]. Furthermore, ASIC1a is blocked by high calcium concentrations, while physiological calcium concentrations stabilize the ASIC3 closed state [22]. In mammals, the ability for nominal calcium to activate ASIC3 to a nondesensitizing, sustained current remains a distinct gating feature of the ASIC3 subtype.

In addition to calcium-dependent activation, guanidine-containing compounds, such as GMQ (termed nonproton ligands), directly activate ASIC3 [23-25]. These ligands have a guanidinium group within their chemical structure that confers binding at the nonproton ligand sensor domain (NPLSD), or central vestibule, of ASICs [23-25]. The first chimera, cASIC1 (rASIC3-TM/ITC), was designed similarly to the Salinas chimera termed “ASIC3-loop1a” [35]. The latter chimera was not functional (data not shown), unlike the comparative Salinas construct “ASIC1a-loop3”, and we presume this may be due to the species difference between our study and that of the Salinas group [35]. Although conserved residues for binding nonproton ligands in this region are present in all ASIC subtypes (Fig. 1), only ASIC3 is directly activated by the
prototypical nonproton ligand, GMQ and other guanidine compounds. Amiloride, which possess a guanidine group, can activate ASIC3 in a manner similar to GMQ, presumably at the nonproton ligand sensor domain [23-25, 36]. The subtype-specificity of direct GMQ activation in ASICs is based on differences in ASIC3 gating mechanisms [7, 22, 37, 38]. Although cASIC1 possesses many of the features for an intact NPLSD, other ASIC protein domains appear to influence nonproton ligand activation in this channel.

Our previous work indicated that the NPLSD is intact in chicken ASIC1, but only after a persistent current is induced by the addition of Psalmotoxin-1 (PcTx1) [26]. The chimera studied here could be rescued from the desensitized state, unlike the cASIC1 wild type. This suggests that GMQ modulation is state-dependent in cASIC1. This confirms previous reports that that ASICs, especially ASIC3, are influenced by multiple stimuli and serve as coincidence detectors of multiple inflammatory signals [23]. The simple removal of calcium does not lead to open cASIC1. However, cASIC1 pH sensitivity is increased, as observed in the movement of the profile to more alkaline values in the absence of calcium. This is the first report that chicken ASIC1 activation is sensitive to calcium in this manner. This calcium sensitivity of cASIC1 may indicate similarities to human ASIC1a [33].

We have shown that the reduction of calcium to nominal levels does not result in opening cASIC1 while the pH sensitivity of cASIC1 increases when calcium concentrations are low. Two previously proposed mechanisms of channel activation in ASICs suggested that calcium (1) competes for binding with protons or (2) acts at an allosteric site for ASIC1a. These studies suggest that multiple sites are likely important for calcium-ASIC interaction [7, 22, 28, 29]. Additionally, we observed GMQ-mediated activation of rASIC3 in the presence or absence of extracellular calcium. This suggests that, at least for rASIC3, GMQ binding occurs regardless of
the calcium concentration. At nominal calcium concentrations, GMQ activation of rASIC3 is significantly reduced. One explanation for reduced efficacy in nominal calcium conditions could be that the NPLSD and central vestibule are already expanded, which leads to open channels. Exposure to GMQ may not be enhance these channels as they are unresponsive to the GMQ “molecular wedge” effect. Finally, the chimeric cASIC1 (rASIC3-TM/ITC) resembles rASIC3 activation only in conditions of nominal calcium. GMQ failed to activate cASIC1 (rASIC3-TM/ITC) in the presence of calcium. This observation supports previous hypotheses that calcium binding stabilizes the closed channel state [7, 22, 29]. In this case, GMQ is not able to open the channel, possibly due to inability to reduce calcium binding [22, 28, 29]. When GMQ-mediated activation of cASIC1 (rASIC3-TM/ITC) does occur, as in the nominal calcium environment, we observed a rebound current at higher GMQ concentrations. This can be explained by two possibilities: (1) GMQ binds at a secondary site, possibly at the proton-binding sites as observed for amiloride in the MitTx-cASIC1 crystal structure or (2) GMQ blocks ASICs at a pore-blocking site, the amiloride pore-blocking site [30]. This rebound current was observed at GMQ concentrations greater than 1 mM, and is observed in recordings of rASIC3 in low calcium to nominal calcium conditions. If the GMQ block of ASIC occurred at a pore-lining site, we expected to observe a voltage-dependence of the effect. However, the observed GMQ-induced rebound current was present at both positive and negative holding potentials, suggesting that GMQ does not block the pore to elicit a rebound current (data not shown). The voltage-independence of the GMQ rebound current suggests this site is away from the electric field of the ASIC pore. It remains unclear where these actions of GMQ occur within the ASIC protein and future work should focus on elucidating this site.
Our results confirm that the nonproton ligand sensor domain is intact in cASIC1 and that GMQ can access this site. Furthermore, we demonstrated that the ASIC transmembrane domains contribute to GMQ-induced ASIC activation and that the channel state influences GMQ activity. Moreover, if the channel is activated in a manner that mediates a sustained current, nonproton ligands could interact with the channel via the NPLSD (Fig. 6). Based on our work here, we hypothesize that all ASIC subtypes that have acidic residues at equivalent sites to rASIC3 E79 and E423 have the capacity to bind GMQ within the nonproton ligand sensor domain. Other factors, such as the ASIC transmembrane domain and the channel’s sensitivity to calcium dictate the channel’s response to GMQ. Due to the ASIC state-dependence of GMQ activity, we offer a word of caution and suggest that novel ligands (e.g. other nonproton ligands, venom toxins) that modulate acid-sensing ion channels be screened for state-dependent activity and in these proton-sensitive channels. This will provide a clearer understanding of how novel ASIC ligands interact with a complex and ubiquitously expressed protein.
METHODS

**Cell culture and ASIC expression.** Full-length chicken ASIC1 and rat ASIC3 as well the generated chimeric receptors were subcloned into a vector that attached an enhanced green fluorescent protein to the amino terminus (26). Each ASIC construct was transiently transfected into Chinese hamster ovarian (CHO) cells for electrophysiological study as previously reported (26). Only cells that exhibited fluorescence were chosen for electrophysiological study.

**Whole-cell patch clamp electrophysiology.** Patch pipettes were pulled from borosilicate glass and fire-polished with a final resistance 3-10MΩ (Flaming/Brown, P-87/PC, Sutter Instrument CO., Novato, CA). Whole-cell patch-clamp electrophysiology was performed on an inverted fluorescent microscope fitted with a FITC fluorescence filter (Nikon) that was equipped with an Axopatch 200B patch-clamp amplifier (Molecular Devices) using pClamp10 data acquisition software. Artificial external solutions were applied at room temperature and mimicked the extracellular and intracellular cellular compartment. Extracellular bath and test solutions consisted of (in mM): NaCl (150), KCl (5), MES (5), HEPES (5), with pH adjusted using HCl or N-methyl-d-glucamine (NMDG). Additionally, intracellular solutions contained (in mM): KCl (100), MgCl₂ (5), EGTA (10), HEPES (40), and NaCl (5) at pH 7.35. The extracellular base solution was used for all other solutions and modified by varying the pH, altering calcium concentrations, or adding ligands. Calcium was added at a concentration of 1mM CaCl₂ in calcium-rich solutions and omitted from those mimicking calcium-depleted solutions (nominal calcium). Solution exchange was obtained using PTFE solenoid valves with a ValveLink8.2
controller (AutoMate Scientific) with an exchange rate of approximately 5 ms across an open pipette tip.

**Data analysis.** Peak current amplitudes of test solutions were normalized against a control response in each experiment. Statistical significance was determined using unpaired Student’s $t$-test, where appropriate. Concentration-response profiles were generated for both wild type and chimeric receptors in the presence and absence of calcium to determine the pH that elicits half of the maximal response, or pH$_{50}$. The concentration response profiles were fit using a logistic equation (OriginLab 8.0).
Fig. 1. Single ASIC subunits and the residues responsible for calcium and GMQ interaction.

A. Calcium ion and residues important for mediating the calcium site are shown. Residues important for calcium binding are highlighted. B. Chemical structure of the prototypical nonproton ligand, 2-guanidine-4-methylquinazoline (GMQ) and residues important for GMQ binding are shown. Residues implicated in direct GMQ binding are highlighted. Corresponding residue numbers for each subtype along with the relative location within the crystal structure are shown for both the calcium and GMQ sites (A, B, respectively). Differing residues among all subunits are gray. Analogous residues are black. C. Schematic of cASIC1, rASIC3, and chimeric ASIC subunits. Single subunits for cASIC1 (left), rASIC3 (center) and cASIC1 (rASIC3-TM/ITC) (right) are shown. Residues for generating the chimeric ASIC are shown, where the amino-terminal gray residue (rASIC3) leads into the extracellular domain black residue (cASIC1), and vice versa as you move to the carboxyl-terminus.
Fig. 2. cASIC1 (rASIC3-TM/ITC) channels are calcium sensitive. Representative whole-cell patch clamp electrophysiology recordings of (A) cASIC1, (B) rASIC3, and (C) cASIC1 (rASIC3-TM/ITC) in transfected CHO-K1 cells are shown ($V_{hold} = -70$ mV). A. Representative traces showing the control (pH 6.6) activation of cASIC1 with 1 mM CaCl$_2$ and no activation when CaCl$_2$ is excluded from the extracellular recording solutions are shown. B. Representative traces of rASIC3 activation by a control test pulse (pH 6.6, 1 mM CaCl$_2$) and moderate activation with nominal CaCl$_2$ (~ 0 mM) concentrations in basic pH (pH 8.0). C. Representative traces of the chimeric receptor cASIC1 (rASIC3-TM/ITC) activated by the control test pulse (pH 6.6, 1 mM CaCl$_2$) and modest activation with nominal CaCl$_2$ (~ 0 mM) concentrations and minimal proton influence (pH 8.0). Horizontal and vertical scale bars for each set of representative traces are in seconds (s) or picoAmperes (pA), respectively.
Fig. 3. Nominal calcium solution shifts activation profiles of cASIC1, rASIC3, and cASIC1 (rASIC3-TM/ITC). A. Activation profiles of cASIC1 by pH with either 1 mM CaCl₂ (closed squares) or nominal calcium (open squares) are shown. cASIC1 pH activation displays a significant increase in proton sensitivity compared to control calcium concentrations (pH50 = 6.90 ± 0.03) and nominal calcium (pH50 = 7.01 ± 0.03). Conditioning pH for all profiles presented is pH 8.0 with corresponding test solutions of pH 6.0, 6.4, 6.8, 7.2, and 7.4 added for five seconds then returning to the conditioning pH. B. Calcium exclusion (open circles) in recording solutions produces a significant shift from the control activation profile (closed circles) (pH50 from 7.2 ± 0.04 to 6.72 ± 0.01, respectively). Activation curves are presented with normalized current (I/I_{maximum}) as a function of maximum test pH containing either 1 mM CaCl₂ and nominal calcium concentrations. C. The chimeric ASIC tested, cASIC1 (rASIC3-TM/ITC), does retain enhanced proton sensitivity when calcium is excluded from the extracellular recording solution, additionally this shift (pH50 = 6.91 ± 0.03 to 6.76 ± 0.03, respectively) is statistically significant. Data is shown with normalized currents (I/I_{maximum}) as a function of maximum test pH. Profiles presented contain either 1 mM CaCl₂ (closed triangles) or nominal calcium (open triangles) concentrations. All data is presented as the mean ± SEM of at least 5 individual cells. Data is presented with normalized current (I/I_{maximum}) as a function of maximum test pH.
Fig. 4. GMQ activates rASIC3 and cASIC1 (rASIC3-TM/ITC) in the presence and absence of extracellular calcium. A, B. Representative whole-cell patch clamp recordings of rASIC3 in the presence of (A) extracellular calcium and (B) with nominal calcium concentrations when activated with 0.3, 1, and 3 mM GMQ. C. Summary of activation of rASIC3 by increasing concentrations of GMQ in the presence (closed circles) and absence of calcium (open circles). D, E. Representative traces of cASIC1 (rASIC3-TM/ITC) exposed to increasing concentrations of GMQ (0.3, 1, and 3 mM) in the presence (D) and absence of calcium are shown (E). F. Summary of activation of cASIC1 (rASIC3-TM/ITC) with increasing concentrations of GMQ in the presence (closed triangles) and absence (open triangles) of calcium are shown. Conditioning pH for each pulse is pH 8.0. Horizontal and vertical scale bars for traces in panels A, B, D, and E are in seconds (s) and picoAmperes (pA), respectively. In panels C and F, data is presented as the mean ± SEM of at least 4 patched cells with normalized current (I/I_{0.3 mM GMQ}) as a function of GMQ concentration (mM).
**Fig. 5. GMQ rescued some of the desensitized current in rASIC3 and the chimeric cASIC1 (rASIC3-TM/ITC).** Representative traces and summarized data are shown. A. For each representative trace the conditioning pH is pH 8.0, test pulse is pH 6.0 (4 seconds), and GMQ addition is 0.3 mM (12 seconds) while maintaining low pH (pH 6.0). Representative traces of cASIC1, rASIC3, and cASIC1 (rASIC3-TM/ITC) are shown as top, middle, and bottom traces, respectively. Horizontal and vertical scale bars are in seconds (s) and picoAmperes (pA), respectively. B. Summary of percent rescue of current following GMQ application for cASIC1, rASIC3, and cASIC1 (rASIC3-TM/ITC) are shown. From left to right, the values of percent rescue are 0.00 ± 0.00, 14 ± 0.03, and 3.0 ± 0.01, respectively. Percent rescue of rASIC3 and cASIC1 (rASIC3-TM/ITC) were significant compared to the zero rescued current of cASIC1 (p < 0.01 and 0.1, respectively). Data is presented as the mean ± SEM of at least 3 patched cells with normalized current (I/I peak) as a function of test pH (pH 6.0).
Table 1. Summary of pH activation for cASIC1, rASIC3 and cASIC1 (rASIC3-TM/ITC) in the absence and presence of calcium.

<table>
<thead>
<tr>
<th>Activation</th>
<th>pH&lt;sub&gt;50&lt;/sub&gt;</th>
<th>nH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ calcium</td>
<td>- calcium</td>
</tr>
<tr>
<td>cASIC1</td>
<td>6.90 ± 0.03</td>
<td>7.01 ± 0.03***</td>
</tr>
<tr>
<td>rASIC3</td>
<td>6.72 ± 0.01</td>
<td>7.2 ± 0.04***</td>
</tr>
<tr>
<td>cASIC1 (rASIC3-TM/ITC)</td>
<td>6.76 ± 0.03</td>
<td>6.91 ± 0.03***</td>
</tr>
</tbody>
</table>

Summary of pH activation of each channel subtype studied are shown; + calcium indicates all test solutions with added CaCl<sub>2</sub> (1 mM); - calcium indicates all test solutions had no added CaCl<sub>2</sub> (~ 0 mM); *** indicates different from + calcium, p < 0.001. All, n ≥ 5.
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IV. GENERAL DISCUSSION

With the completion of the two proposed specific aims, we identified that channel state influences nonproton ligand interaction and the transmembrane domains are critical for this interaction. Previous studies with nonproton ligands, specifically GMQ, in ASICs have focused on the direct activation of ASIC3 and neglected why other ASIC subtypes appear void of this effect despite the nonproton ligand binding site being present. These studies are a start to better understanding how this novel class of ligands interacts with ASICs and expands if they could be used for pharmacological targeting of ASIC subtypes.

Our first study using whole-cell and outside-out patch clamp electrophysiology was designed to test if ASIC1 Na\(^+\) current could be potentiated by GMQ following persistent activation of the channel by a natural venom toxin, Psalmotoxin-1 (PcTx1; 30 nM). We hypothesized that the partial activation of chicken ASIC1, induced by PcTx1, is critical for GMQ binding in the case of the ASIC1 subtype. We observed that GMQ could enhance the PcTx1-persistent current in a concentration-dependent manner, thus validating that ASIC1 contains an intact nonproton ligand sensor domain. This occurred despite multiple conditioning pHs showing that GMQ (0.3 mM) cannot directly activate ASIC1. We also show that GMQ (0.3 mM) can sensitize the channel to protons, shifting the activation pH\(_{50}\) from 6.65 ± 0.01 to 6.98 ± 0.03. This occurs based on a reduced Hill coefficient (4.55 ± 0.54 to 2.21 ± 0.38) arguing a loss of proton binding sites at the expense of GMQ filling those sites. Steady-state desensitization is less affected by the addition of extracellular GMQ (0.3 mM), although the shift in pH50 is still significant (7.53 ± 0.00 to 7.48 ± 0.00). At the conclusion of this set of experiments, we created a hypothetical model in which GMQ acts as a molecular wedge to pry apart the transmembrane domains upon binding in the central vestibule where the nonproton ligand sensor domain is
housed. In the case of ASIC1, in order for GMQ to reach the nonproton ligand sensor domain, the channel requires a persistently activated state.

Another portion of this study included the effect of amiloride on the cASIC1-PcTx1 protein complex. Traditionally, amiloride acts as a pore blocker for the ASIC family. However, we observed that once the PcTx1 persistent current ensues, amiloride paradoxically stimulates the channel after a brief attempt to block. This stimulation was observed in the form of a rebound current that increased with increasing concentrations of amiloride. We concluded from this study that amiloride likely binds to a secondary site mediating the stimulation of the channel after the pore block.

Now that we validated our initial hypothesis that the nonproton ligand sensor domain is intact in ASIC1, we were interested in understanding which portion of ASIC3 mediates this unique feature of GMQ-mediated direct activation. Another distinct feature of ASIC3 activation is its unique relationship with extracellular calcium. Reductions in extracellular calcium (< 1 mM) cause ASIC3 to open and remain open until calcium concentrations are returned to normal (1-2 mM). This trait is similar to the persistent current induced by the addition of PcTx1 in our original study. We proposed that the ability for ASIC3 to respond to extracellular calcium in this manner is critical for GMQ-mediated direct activation, and were interested in whether we could introduce this feature to ASIC1.

Using previously studied chimeric ASICs, we created a chimera that retains the calcium block site of ASIC3 and the nonproton ligand sensor domain of ASIC1, which we termed cASIC1 (rASIC3-TM/ITC). We showed that the ASIC1 is not sensitive to the removal of extracellular calcium, while ASIC3 and cASIC1 (rASIC3-TM/ITC) remained calcium sensitive. Furthermore, we showed for the first time that although the simple removal of calcium does not
activate ASIC1, calcium removal significantly sensitizes the channel to proton additions by shifting the pH\textsubscript{50} from 6.9 ± 0.03 in 1 mM calcium to 7.01 ± 0.03 in nominal calcium conditions. Similarly, ASIC3 and the chimeric ASIC underwent a similar increase in proton sensitivity with the removal of extracellular calcium (7.2 ± 0.04 to 6.72 ± 0.01 for ASIC3; 6.91 ± 0.03 to 6.76 ± 0.03 for cASIC1 (rASIC3-TM/ITC)).

To test the ability for GMQ to activate the channel, we were interested in looking at how the two ASIC subtypes directly activated by calcium removal (ASIC3 and the chimera) respond to nonproton ligand (GMQ) additions. As expected, ASIC3 activates with GMQ additions in a concentration-dependent manner regardless of the extracellular calcium concentration. Furthermore, supporting our hypothesis, the chimeric ASIC was potentiated by increasing concentrations of GMQ in the absence of extracellular calcium. This effect was not observed when extracellular calcium concentrations were at 1 mM indicating that this characteristic is critical for the GMQ-mediated activation to be observed. Taken together, our second set of experiments indicate that the transmembrane and intracellular domains of ASIC3 are critical for GMQ-mediated direct activation. Additionally, we can introduce the GMQ sensitivity to ASIC1, which was previously not activated by direct additions of GMQ. Taken with our previous experiments, we can conclude that given the precise circumstances, we cannot rule out that guanidinium compounds could activate ASIC1 and this eliminates GMQ as a candidate for selectively activating ASIC3. We constructed this final model taking into account how calcium now influences direct GMQ-mediated activation of ASIC3, along with the interaction of PcTx1 and ASIC1 (Figure 1).
Fig. 1. Proposed interplay between PcTx1, calcium and nonproton ligand-mediated activation in ASIC1 and ASIC3. Here we show how PcTx1 binding and calcium leaving its binding site at the top of the channel pore result in the partial activation of ASIC1 and ASIC3, respectively. This partial activation then results in GMQ binding the nonproton ligand sensor domain within the central vestibule. The nonproton ligand then acts as a molecular wedge which forces apart the transmembrane domains even further causing channel potentiation.
V. CONCLUSIONS AND FUTURE DIRECTIONS

The acid-sensing ion channel family remains a robust sensor for changes in extracellular pH in the central and peripheral nervous systems. Despite their ubiquitous expression, targeting ASIC subtypes with pharmacological agents in a selective manner remains difficult. Our studies have contributed to a better understanding of how the nonproton ligand sensor domain can activate other ASIC subtypes. Specifically, we showed that (1) the nonproton ligand sensor domain is intact in ASIC1, (2) a guanidinium compound (GMQ) can potentiate ASIC1 current following partial activation with a natural venom toxin (Psalmotoxin-1; PcTx1), (3) the transmembrane domains are important for narrowing down why ASIC3 is uniquely activated by GMQ addition, and (4) ligand interaction with ASICs is state-dependent. Although our studies did not yield a site-specific target for ASIC subtypes, we are confident that our additions to the ASIC literature are critical for the future development of drugs selectively targeting the ASIC subtypes.

GMQ-mediated ASIC activation likely involves another binding site.

We showed that GMQ increases the proton sensitivity of chicken ASIC1 and does not directly activate the channel on its’ own (Smith and Gonzales, 2014). Additionally, Baconguis and Gouaux solved cocrystals with a dimer of amiloride, another guanidinium compound, within the proton binding sites (Baconguis et al., 2014). Their work showed definitively that a guanidinium compound could bind within a proton binding site and this may be a viable explanation for the shift in proton sensitivity that we observe with the pH concentration-response profile of cASIC1. Although other studies are needed to confirm this hypothesis, it remains
feasible that GMQ could increase the proton-sensitivity of ASICs through binding at the proton binding site.

*Other guanidinium compounds.*

Although our studies focused primarily on GMQ as our choice guanidinium compound, many other guanidinium compounds could interact with ASICs. Endogenous compounds like the polyamines, agmatine and spermidine, may come in contact with ASICs as byproducts of arginine degradation. In order to further understand the modulatory effects that guanidinium compounds could have on ASICs, additional studies would be beneficial to understand their importance. In addition, their may be exogenous dietary supplements that contain a guanidinium moiety in their chemical structure, making ASICs a potential target for drug-supplement interactions not yet understood.

*Heteromeric v. homomeric ASICs.*

Our studies are a start in determining the true ability for guanidinium compounds to alter other homomeric ASIC1 and ASIC3 activation. In order to understand the exact affect that guanidinium compounds have on ASICs *in vivo*, a logical next step would be to consider studying heteromeric ASICs *in vitro*. It has been shown that ASIC1a/2a/3 heteromers are important for sensing painful stimuli, and this may be true for ASICs in other physiological situations as well. In general, ASIC1 and 3 are considered the principal acid sensors from the ASIC family, however ASIC2a in heteromeric conformations may alter currently accepted biophysical properties of the channel.
REFERENCES


APPENDIX A: A REVIEW OF CREATINE SUPPLEMENTATION IN AGE-RELATED DISEASES: MORE THAN A SUPPLEMENT FOR ATHLETES

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ABSTRACT

Creatine is an endogenous compound synthesized from arginine, glycine, and methionine. This dietary supplement can be acquired from food sources such as meat and fish, along with athlete supplement powders. Since the majority of creatine is stored in skeletal muscle, dietary creatine supplementation has traditionally been important for athletes and bodybuilders to increase the power, strength, and mass of the skeletal muscle. However, new uses for creatine have emerged suggesting that it may be important in preventing or delaying the onset of neurodegenerative diseases associated with aging. On average, 30% of muscle mass is lost by age 80, while muscular weakness remains a vital cause for loss of independence in the elderly population. In light of these new roles of creatine, the dietary supplement’s usage has been studied to determine its efficacy in treating congestive heart failure, gyrate atrophy, insulin insensitivity, cancer, and high cholesterol. In relation to the brain, creatine has been shown to
have antioxidant properties, reduce mental fatigue, protect the brain from neurotoxicity, and improve facets/components of neurological disorders like depression and bipolar disorder. The combination of these benefits has made creatine a leading candidate in the fight against age-related diseases, such as Parkinson’s disease, Huntington’s disease, amyotrophic lateral sclerosis, long-term memory impairments associated with the progression of Alzheimer’s disease, and stroke. In this review, we explore the normal mechanisms by which creatine is produced and its necessary physiology, while paying special attention to the importance of creatine supplementation in improving diseases and disorders associated with brain aging and outlining the clinical trials involving creatine to treat these diseases.

INTRODUCTION

The usage of dietary supplementation in the United States is a multibillion-dollar industry, where creatine (N-[aminoiminomethyl]-N-methyl glycine) accounts for over 4 million kg and $200 million annually. Creatine is an endogenous molecule found in all cells in the body and is synthesized in the kidney, liver, and pancreas using the amino acids arginine, glycine and methionine before entering the bloodstream. From the plasma, creatine is transported into the cells via the creatine transporter protein (CRT). This transporter is critical for the distribution of creatine throughout the cells as well as for traversing the blood brain barrier (BBB), giving creatine access to the central nervous system (CNS).

Nearly 95% of creatine stores reside in skeletal muscle with the remaining 5% found in the brain, liver, testes, and kidneys. Perhaps the most well understood role of creatine in physiology is its participation in energy production. More specifically, creatine maintains the intracellular levels of adenosine triphosphate (ATP) in skeletal muscle. This source of ATP is
produced via oxidative phosphorylation, which is regulated by the mitochondria\(^9\). Within just a few seconds, muscle contraction utilizes the entire ATP store (2-5 mM) found in skeletal muscle\(^10\). ATP is regenerated using the phosphocreatine system where phosphocreatine donates its phosphate group to adenosine diphosphate (ADP) to form ATP. This reaction occurs rapidly and reversibly via the enzyme creatine kinase (CK), making the ATP replenishing capacity of both phosphocreatine and creatine kinase high. Conversely, at rest, ATP donates a phosphate group to creatine in order to replenish phosphocreatine stores for future muscle contraction use.

Although primarily associated with energy production, mitochondria play an important role in the production of reactive oxygen species, dysregulation of calcium, excitotoxicity, and premature cellular death\(^{11-14}\). Likewise, creatine has important implications in antioxidant mechanisms, controlling intracellular calcium concentrations, regulating extracellular glutamate concentrations, and preventing the opening of the mitochondrial permeability transition pore (MPT)\(^{15-20}\). With evidence for creatine’s critical role in cellular bioenergetics, the phosphocreatine system in energy buffering, and the aforementioned implications in mechanisms associated with mitochondrial dysregulation, it is no surprising that creatine is the subject of investigation for improving the status of patients with neurodegenerative diseases that either result or progress by some mechanism of energy insufficiency.

**PHYSIOLOGICAL CREATINE CONCENTRATIONS AND CREATINE SUPPLEMENTATION**

There is a maximum capacity for the synthesis of endogenous creatine. To increase these levels, patients and athletes turn to creatine supplementation. These individuals that take in foods rich in creatine tend to have higher creatine levels\(^{21,22}\). The transport of creatine into cells is
limited, since the capacity of creatine transport within each muscle cell is only 160 mmol/kg\textsuperscript{23}. The possible beneficial effects of creatine are negligible if the creatine transporter is not functioning or if the maximal concentration of creatine within the cell has been reached. Evidence suggests that additives with creatine supplementation like proteins, carbohydrates, alpha lipoic acid, and D-pinitol can stimulate the movement of creatine into the cell, making creatine an ideal supplement for athletes with increased protein and carbohydrate intake\textsuperscript{24-27}.

In general, a 70 kg human has a total creatine pool of 120 grams with 2 grams per day production from both dietary and endogenous sources\textsuperscript{8,28}. Like many other supplements, supplementation reduces the normal physiological creatine production. This reduction is reversible as creatine supplementation is terminated\textsuperscript{8}. Athletes use creatine supplementation to increase creatine phosphate stores. Elevated phosphocreatine leads to the phosphorylation of ADP to ATP and aids in limiting energy depletion during rapid muscle movement. Multiple studies have indicated significant improvements in sprint performance, body mass, fat-free body mass, weightlifting volumes, oxygen uptake and overall exercise performance following creatine supplementation\textsuperscript{29-40}. Creatine loading in athletes can require 20 grams per day of supplementation, while maintenance dosing is roughly 5 grams per day. These studies dosed the subjects in a similar fashion. Serum creatine levels reached 2.17 mM and 0.8 mM at 2.5 hours following a 20 gram and 5 gram creatine bolus, respectively\textsuperscript{41,42}. Creatine is excreted in the urine as creatinine with a daily turnover of 2 grams per day. Although creatine supplementation results in reduced, but reversible natural creatine production, creatine supplementation appears to have few unwanted side effects\textsuperscript{43}. Thus, creatine is an attractive dietary supplement for athletes.
Prior to the usage of creatine as an athletic enhancer, creatine has been the focus of research to understand the dietary supplement’s role in physiology for 150 years. Creatine supplementation became popular during the Barcelona Olympic Games as it was shown to enhance athletic performance\textsuperscript{44}. Around the same time, two studies showed that creatine enhanced exercise performance via oral creatine ingestion\textsuperscript{23,45}. With a clear understanding of the creatine/phosphocreatine system and its relation to the ADP/ATP energy metabolism in the mitochondria, studies began to shift their focus to understanding creatine’s role in pathophysiological conditions.

In addition to athletic performance, creatine usage has expanded to treat pathophysiological conditions including gyrate atrophy, post-stroke depression, congestive heart failure, chronic musculoskeletal pain disorders, atherosclerotic diseases and cisplatin nephrotoxicity\textsuperscript{28,46,47}. Furthermore, a recent review proposed prophylactic creatine supplementation could reduce chances of preterm labor or hypoxic-ischemic encephalopathy\textsuperscript{48}. Extensive research has demonstrated that the availability of phosphocreatine plays a role in skeletal muscle pathology and the associated pain can be alleviated by the intake of exogenous creatine\textsuperscript{49}. New studies indicate that creatine plays a role in age-related neurological diseases and reduced brain functionality associated with Parkinson’s disease, Huntington’s disease, amyotrophic lateral sclerosis (ALS), long-term memory deficits, Alzheimer’s disease, and stroke. In the subsequent text, we will discuss creatine’s role in these neurodegenerative conditions.

BENEFITS OF CREATINE SUPPLEMENTATION IN AGE-ASSOCIATED DECLINES IN THE BRAIN

\textit{General benefits}
Aging is associated with lower levels of creatine and phosphocreatine, specifically in the skeletal muscle. Phosphocreatine regeneration rates following exercise fall approximately 8% each decade after age 30. Creatine supplementation increases both creatine and phosphocreatine from 10-40% in athletes. Furthermore, a recent review described a meta-analysis of the role creatine supplementation and resistance training plays on muscle health in an aging population. Based on the analysis of 13 published, creatine had an overall beneficial effect on aged individuals muscle mass. Since the creatine transporter can readily transport creatine from the bloodstream across the BBB, it is reasonable to suggest that exogenous supplementation of creatine would increase concentrations in the brain, where endogenous creatine levels may be diminished as a person ages. In neurodegenerative disorders (as outlined below), creatine may help slow the progression of each condition.

**Parkinson’s disease**

Parkinson’s disease (PD) is a neurodegenerative disorder resulting from the loss of dopamine neurons in the midbrain with symptoms becoming apparent when approximately 60% of these neurons are lost. Notable symptoms of PD include resting tremor, postural instability, bradykinesia, loss of muscle mass, strength, and increased ability to fatigue. The treatment measures for PD involve early detection of the disease and understanding how to slow PD progression once symptoms have been reported. Rodent models often used for the study of PD are induced by toxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), to mimic the pathogenesis and progression of the disease marked by dopaminergic neuron loss, mitochondrial dysfunction, and oxidative stress. In particular, complex I of the electron transport chain within the mitochondria is deficient in patients with Parkinson’s disease. The fact
that postural instability, loss of muscle mass, and strength all occur in the progression of PD, and are coupled with creatine’s ability to alter cellular energetics, has led to the hypothesis that the creatine dietary supplementation could minimize the associated symptoms with Parkinson’s disease.

An early study testing the benefit of creatine in MPTP-induced Parkinson’s disease mice showed significant neuroprotection with 1% creatine supplementation in diet\textsuperscript{55}. In 2006, a group of investigators at the National Institute of Neurological Disorders and Stroke (NINDS) began a phase III clinical trial for creatine in 200 patients affected by Parkinson’s disease after second phase preliminary data showed that creatine was able to slow down the progression of the disease\textsuperscript{56}. A year later, a double-blind study compared the control group (no creatine supplementation) to the test group (20 Parkinson’s disease patients), which received a creatine loading dose of 20 grams per day for 5 days and a creatine maintenance dose of 5 grams per day thereafter\textsuperscript{57}. The purpose of this study was to specifically explore if creatine could help increase muscle strength in idiopathic Parkinson’s disease patients. Both groups received resistance training during the study. A difference was observed in the creatine supplemented group with some of the strength exercises used versus the control group\textsuperscript{57}. In September 2013, the NINDS announced that the phase III clinical trial for creatine use in Parkinson’s disease was halted because the study would result in an observable significant difference\textsuperscript{58}. The test subjects tolerance for creatine or creatine associated side effects were not the cause for stopping the trial. The patients in this creatine clinical trial received 10 grams creatine daily for up to 5 years. Although this outcome is disappointing, the ability for creatine to alter energy dysfunction in addition to muscle strength may still have a combinatory effect with other Parkinson’s disease
drug treatments. The possibility remains that creatine may be beneficial for Parkinson’s disease patients, but more work needs to be done to demonstrate the dietary supplements efficacy.

**Huntington’s disease**

Huntington’s disease (HD) is a neurodegenerative disease where the onset of symptoms occurs in midlife. Once the symptoms begin, a patient can expect to live, on average, 20 more years\(^{59}\). Those that develop Huntington’s disease possess genetically inherited mutations in the number of cytosine-adenine-guanine (CAG) repeats in the *huntingtin* gene responsible for producing the huntingtin protein\(^{60-63}\). The huntingtin protein is expressed throughout the central and peripheral nervous systems. Upon the onset of HD symptoms, the patient begins to exhibit changes in mood, cognition, and motor coordination\(^{60-63}\). In addition to contributing to an abnormal gait, resting tremor and even epileptic seizures associated with Huntington’s disease, the mutant huntingtin protein product leads to impaired energy metabolism\(^{64}\). Without a cure for Huntington’s disease, the impairment of energy metabolism offers an avenue and target for new therapies. Furthermore, there is an observed reduction in the phosphocreatine and inorganic phosphate ratio in Huntington’s disease patients’ muscle tissue, which may indicate the Huntington mutation’s involvement in dysregulating the phosphocreatine/creatine ratio\(^{65}\). In a mitochondrial toxin Huntington’s disease mutant mouse model, R6/2, creatine is hypothesized to act as a means of buffering, or providing a larger phosphocreatine pool for rapid conversion of ADP to ATP, energy within the cell\(^{5,66-68}\). Furthermore, as an extension of the work done by Matthews and colleagues\(^{68}\), the Ferrante group\(^{69}\) showed that lifespan was extended by 9.4, 17.7 and 4.4% when supplemented with 1, 2, or 3% creatine in the diet, respectively\(^{69}\).
Several Huntington’s disease transgenic mice strains have been developed to study the dysfunction in energy metabolism, the electron transport enzymes in the mitochondria, and excessive excitotoxicity associated with the disease. Supplementation with exogenous creatine in one transgenic mouse model showed improved motor performance, reduced atrophy of neurons, and huntingtin protein aggregates, and an observed increased survival rate or lifespan. Following these studies, a phase II clinical trial ensued to assess creatine tolerability given at a dosage of 8 grams per day to Huntington’s disease patients. From these studies, Hersch and colleagues determined that 8-hydroxy-2’-deoxyguanosine (8OH2’dG), a marker for damaged DNA, was abnormally high in patients with HD, but was reduced after creatine treatment. In an interview regarding the phase III clinical trial of creatine usage in Huntington’s disease patients, the CREST-E (Creatine Safety, Tolerability, and Efficacy) clinical trial, Hersch reported that 8OH2’dG had returned to normal levels. Subsequently, there was an observed reduction in brain deterioration rate when patients were supplemented with creatine and that creatine kinase is a potential biomarker for HD. The potential of creatine as a viable therapy for HD remains to be seen and the results from CREST-E clinical trial will provide some indication to the dietary supplement’s utility. Thus, creatine supplementation remains a potential therapy for Huntington’s disease, however further studies are needed.

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS), or Lou Gehrig’s disease, is marked by the loss of voluntary muscle control from the progressive degeneration of motor neurons with subsequent neuronal loss resulting in paralysis. The cause and cure for ALS remain elusive. The most promising treatment is the drug riluzole, which only increases the lifespan of those
with the disease by 6 months\textsuperscript{82}. To complicate the search for effective treatments, as many as 50\% of ALS patients experience cognitive impairment that is revealed when they undergo specialized testing for neuropsychological deficits\textsuperscript{83}. In addition to motor neuron loss observed in ALS patients, cognitive impairment most often associated with the frontotemporal region of the brain is not always present\textsuperscript{83}. Furthermore, despite identifying a genetic overlap in ALS and other neurodegenerative disease mechanisms, the sporadic occurrence of cognitive impairment in ALS patients obscures understanding a clear etiology of the disease\textsuperscript{83}.

At a molecular level, ALS is characterized by altered glutamate homeostasis, oxidative damage, elevated intracellular calcium concentrations, mitochondrial swelling, and electron transport chain complex I deficiencies leading to reduced energy intake\textsuperscript{84-87}. Generally, mutations in the gene responsible for the production of the enzyme, superoxide dismutase (SOD1) are common in many cases of familial ALS\textsuperscript{80}. The loss of function associated with SOD1 mutations reported in ALS translates to the accumulation of toxic free radicals from superoxide generated by the mitochondria\textsuperscript{80,88,89}. This suggests that the build up of free radicals results in altered energy production. Thus, creatine may serve as an energy alternative that is beneficial for ALS patients.

Klivenyi and colleagues studied transgenic mice with a mutated human SOD1 gene and assessed the neuroprotective effects of creatine. This was in response to the promotion of survival and improved motor coordination they observed with long-term creatine supplementation\textsuperscript{90}. Along with the proposed creatine benefits in protecting neurons from insufficient energy production, the results indicated that creatine administration protected neurons from oxidative damage. In contrast, two completed clinical trials in 2003 and 2004 tested oral creatine supplementation and provided little notable improvements in lifespan, muscle
strength, or motor unit numbers in patients with ALS\textsuperscript{91,92}. Although there is an observed trend toward enhanced survival following creatine supplementation, these studies distinguished between large differences (30-50\% difference). Due to the high threshold for observing significant differences, there remains a possibility that creatine has a subtle effect. Currently, the long-term effects of creatine supplementation are being studied in a phase II clinical trial associated with the Northeast Amyotrophic Lateral Sclerosis Consortium (NEALS).

\textit{Long-term memory}

The ability to encode new memories (working memory), recall previous events (episodic/long-term/declarative memory) and short-term (primary/active) memory declines with age\textsuperscript{93,94}. Previous reports indicated that phosphocreatine stores are quickly depleted upon brain activation, while ATP concentrations remain constant\textsuperscript{95,96}. A reduction in creatine levels may have an effect on the immediate recall of knowledge based on the dramatic drop in phosphocreatine levels from brain activation. In 2003, Rae and colleagues questioned if creatine supplementation enhanced intelligence in healthy subjects\textsuperscript{97}. For 6 weeks, subjects were given 5 grams of creatine orally per day. Following this protocol, the subjects showed improvements in working memory and intelligence utilizing the backward digit span and Raven’s Advanced Progressive Matrices tasks, respectively. Furthermore, each of the participants were vegetarians, which supported the role that exogenous creatine has on increasing participants’ serum creatine levels. Using magnetic resonance spectroscopy to measure creatine levels, it was determined that creatine levels increased in the brain, solidifying that creatine is not solely distributed in skeletal muscle\textsuperscript{98}. Creatine supplementation has been considered for the improvement of memory in the elderly. One such study in the United Kingdom reported that subjects with an average age of 76
saw improvements in long-term memory when supplemented with 20 grams per day of creatine for 1 week. In addition to improvement in the long-term memory task, the elderly subjects improved in both forward and backward spatial recall as well as forward number recall. Based on these results, the investigators concluded that creatine could enhance cognition in elderly subjects, although follow up studies have not elucidated a mechanism by which creatine does this. Currently, there are few studies focused on the role that creatine supplementation plays on cognition and memory. In addition, how creatine improves memory in the aforementioned studies is yet to be understood at the molecular and cellular levels.

*Alzheimer’s disease*

Alzheimer’s disease (AD) is a neurodegenerative disease marked by neurofibrillary plaques and tangles in the brain. One of the challenging characteristics of AD is the inability to definitively determine if a patient has the disease while alive. Only during the postmortem exam the disease can be definitively diagnosed. However, these hallmarks can be observed in the postmortem brains of individuals that did not display dementia or deteriorated cognitive function. As the disease progresses, the symptoms include severe dementia, confusion, and the loss of long-term memory. Although the onset of AD is not entirely understood, studies have shown that high-energy metabolism precedes the onset of AD while there are increased levels of myo-inositol, an important structural component of lipids, in comparison to the relative creatine concentrations. Furthermore, this increase in the ratio of myo-inositol and creatine precedes the onset of dementia in individuals with Down’s syndrome. Creatine was shown to be protective of rat hippocampal neurons when confronted with beta-amyloid (Aβ) toxicity. One possible mechanism to intervene with the progression of Alzheimer’s disease is creatine kinase.
Creatine kinase is responsible for the conversion of ATP to ADP and vice versa\textsuperscript{105} and tends to be susceptible to high levels of oxidative damage in the brains of Alzheimer’s disease patients\textsuperscript{105}. In Alzheimer’s disease, creatine kinase activity is reduced by as much as 86\% along with a reduction of in creatine kinase protein expression of 14\%, which suggests that the Alzheimer’s disease brain has lower levels of phosphocreatine in the beginning stages of the disease\textsuperscript{101,106}. Although creatine kinase levels were reduced, studies have questioned the involvement of creatine upon finding deposits of the molecule in amyloid precursor protein (APP) in transgenic mice\textsuperscript{107}. The solubility of creatine in an aqueous solution is 100 mM, however the total creatine concentration in the brain only reaches 20 mM\textsuperscript{10}. Possible explanations for the origin of these creatine deposits in the transgenic mice models include: (1) spillage from neuronal cell death, (2) excess oligodendrocyte production of creatine, (3) limited creatine uptake by the CRT and (4) the oxidation of creatine kinase that limits the formation of phosphocreatine\textsuperscript{108-111}. Although each of these possibilities has been studied extensively, it remains unclear to the exact origin of the creatine deposits. This further reiterates that the exact role of creatine in AD is still yet to be understood and may be more complicated than previously thought.

\textit{Stroke}

Stroke is “defined as an acute neurologic dysfunction of vascular origin with sudden (within seconds) or a least rapid (within hours) occurrence of symptoms and signs corresponding to the involvement of focal areas in the brain”\textsuperscript{112}. In the past decade, stroke has been the second leading cause of death\textsuperscript{113}. Ischemic stroke, which is more common\textsuperscript{114,115}, occurs when the brain is deprived of glucose and oxygen due to insufficient blood supply. This causes acidosis, an increase in intracellular calcium, and the formation of reactive oxygen species leading to
ischemic cell death\textsuperscript{116}. Symptoms of ischemic stroke include sudden numbness on one side of the body, vision disturbances, difficulty in speaking and understanding, imbalance, and loss of coordination. The available treatments for ischemic stroke consist of the use of tissue plasminogen activator (tPA)\textsuperscript{117} and/or surgical treatments. The commonly used in vitro model for ischemic stroke involves oxygen-glucose deprivation\textsuperscript{118,119} and rodent model for stroke involves mechanical or thromboembolic occlusion of cerebral vessel to mimic cerebral ischemia\textsuperscript{120-123}.

An early study involving eight stroke patients has demonstrated that creatine and phosphocreatine content is reduced in the ischemic brain\textsuperscript{124}. In rat hippocampal slices, pre-incubation with creatine (0.03-3 mmol/L) dose-dependently reduced damage due to anoxia\textsuperscript{125}. Another in-vitro study in rat hippocampal slices showed dose dependent increase in phosphocreatine concentration and delay in anoxic depolarization after incubation with 1mM creatine\textsuperscript{126}. Creatine was shown to exert protective antioxidant effect in U937 human promonocytic cells after oxidative damage\textsuperscript{127}.

Creatine has been shown to be neuroprotective in an experimental model of anoxia in neonatal mice supplemented with creatine\textsuperscript{128-130}. Also, rats subjected to creatine pretreatment before cerebral hypoxia showed a reduction (25\%) in the volume of edematous brain tissue compared when compared to control\textsuperscript{131}. In 2004, mice supplemented with 2\% creatine in diet showed reduced neuronal damage compared to control groups following middle cerebral artery occlusion that causes ischemic stroke. The study indicated that this beneficial effect of creatine is due to the restoration of energy depletion and inhibition of caspase activation along with some other unknown mechanisms\textsuperscript{132}. These studies support the fact that creatine could be a potential compound to be used as a prophylactic, or preventative, dietary supplement in patients at high
risk for stroke. Still, more work is needed to demonstrate the efficacy of creatine to prevent stroke and to develop a creatine supplementation regimen to help patients at risk for stroke to avoid the debilitating event.

CONCLUSIONS

Creatine has the potential to elicit positive effects in muscle strength, memory, and has further influence on neurodegenerative conditions. It remains to be seen if creatine has the ability to alter age-associated, progressive neurodegenerative disorders once individuals are in intermediate or late stages of the disease. However, based on the creatine interaction with energy metabolism and subsequent neuroprotective mechanisms, the interest for studying alternative uses for creatine in physiology is enhanced. Unfortunately, the phase III clinical trial for Parkinson’s disease and creatine was halted. However, there are ongoing clinical trials for creatine. Creatine supplementation is in a phase III clinical trial for the treatment of the energetic deficiencies in Huntington’s disease. With promising effects thus far, CREST-E remains continually funded by the National Center for Complementary and Alternative Medicine (NCCAM) and the Food and Drug Administration (FDA) as the largest therapeutic trial ever for Huntington’s disease. The usefulness of this compound may prove an important deterrent at beginning stages of dementia or even for increasing muscle strength in Parkinson’s disease patients. Furthermore, creatine may serve as a preventative treatment for the long-term consequences of stroke but may play a more complicated role in Alzheimer’s disease. Despite the wide range of uses for creatine supplementation, this dietary supplement should be the focus of additional studies for the treatment of age-related diseases. Going forward, one must consider if there are other mechanisms for which creatine acts to be protective and beneficial. These
alternative mechanisms and the molecular/cellular targets for creatine remain to be determined and fully characterized.
REFERENCES


APPENDIX B: CURRICULUM VITAE

RACHEL N. SMITH, M.S.

EDUCATION AND RESEARCH

PH.D. BIOMEDICAL SCIENCES: 2011-ANTICIPATED MAY 2015
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Sigma Xi Honor Society Inductee 2014

Honorable Mention for Excellent Scientific Poster 2014
INS Neuroscience Symposium, University of Texas at Austin

Defense with Distinction, Grant Writing Qualifying Exam 2013
UNT Health Science Center, Fort Worth Texas

MANUSCRIPTS AND PUBLICATIONS

Smith RN, Gonzales EB. Protons and Psalmotoxin-1 reveal nonproton ligand stimulatory sites in chicken acid-sensing ion channel: Implication for simultaneous modulation in ASICs. Channels 2014; 8(1):49-61; PMID: 24262969; http://dx.doi.org/10.4161/chan.26978

Smith RN, Gonzales EB. Direct Activation of ASICs by 2-guanidine-4-methylquinazoline is Mediated by the Transmembrane Domains. In preparation.

PRESENTED ABSTRACTS

POSTER PRESENTATIONS

Rachel N. Smith, M.S. & Eric B. Gonzales, Ph.D.
Acid-sensing ion channel modulation by nonproton ligands: the influence of divalent cations

Biophysics: San Francisco, CA February 2014
Rachel N. Smith, M.S. & Eric B. Gonzales, Ph.D.
Modulation of chicken ASIC1 by 2-guanidine-4-methylquinazoline (GMQ) in the absence and presence of Psalmotoxin-1

The Institute for Neuroscience Annual Neuroscience Symposium (INS): UT Austin January 2014
Rachel N. Smith, M.S. & Eric B. Gonzales, Ph.D.
Modulation of chicken ASIC1 by 2-guanidine-4-methylquinazoline (GMQ) in the absence and presence of Psalmotoxin-1

NDOGS Receptions: UNT Health Science Center June 2013
National Directors of Graduate Studies
Rachel N. Johnson, M.S. & Eric B. Gonzales, Ph.D.
Nonproton ligand activation is linked to the ASIC3 calcium block site

UNH Health Science Center Research Appreciation Day April 2013
Rachel N. Johnson, M.S. & Eric B. Gonzales, Ph.D.
Nonproton ligand activation of ASIC3 is mediated by calcium depletion

Experimental Biology: Boston, MA April 2013
Rachel N. Johnson, M.S. & Eric B. Gonzales, Ph.D.
Nonproton ligand activation is linked to the ASIC3 calcium block site

UNT Health Science Center Research Appreciation Day April 2012
Rachel N. Johnson, M.S. & Eric B. Gonzales, Ph.D.
Development of a Soluble Acid-Sensing Ion Channel Extracellular Domain for Novel Drug Design
UNT Health Science Center McNair Scholars Program  
Jose L. Ontiveros, Jr., Rachel N. Johnson, Rebecca L. Cunningham, Ph.D., Eric B. Gonzales, Ph.D.  
Presence of ASIC and P2X receptors in Parkinson’s Disease Model  
July 2011

UNT Health Science Center Health Disparities Conference  
Rachel N. Johnson & Eric B. Gonzales, Ph.D.  
P2X receptor expression in male- and female-derived epithelial cancers  
June 2011

Experimental Biology: Washington, D.C.  
Gonzales EB, Johnson RN, Bilal K, and Pearce V  
Deregulated Progenitors Express Altered Acid-Sensing Ion Channels  
Note: Selected for special poster session sponsored by ASIP Breast Cancer Scientific Interest Group  
April 2011

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Rachel N. Johnson & Eric B. Gonzales, Ph.D.  
Pre-Crystallization Study of a Member of the MYM Gene Family, ZNF237, by Fluorescence-Detection-Size-Exclusion Chromatography (FSEC)  
April 2010

ORAL PRESENTATIONS

Society Advancement of Chicanos & Native Americans in Science: Los Angeles, CA  
October 2014  
Rachel N. Smith, M.S. & Eric B. Gonzales, Ph.D.  
What happens first? How calcium and guanidino compounds influence the acid-sensing ion channel

UNT Health Science Center Research Appreciation Day  
April 2011  
Rachel N. Johnson & Eric B. Gonzales, Ph.D.  
Differential expression of P2X receptors in male- and female-derived epithelial cancers