Protons and Psalmotoxin-1 reveal nonproton ligand stimulatory sites in chicken acid-sensing ion channel
Implication for simultaneous modulation in ASICs

Rachel N Smith¹ and Eric B Gonzales¹,²,³,*

¹Department of Pharmacology & Neuroscience; UNT Health Science Center; Fort Worth, TX USA; ²Institute for Aging & Alzheimer's Disease Research; UNT Health Science Center; Fort Worth, TX USA; ³Cardiovascular Research Institute; UNT Health Science Center; Fort Worth, TX USA

Keywords: 2-guanidine-4-methylquinazoline, Psalmotoxin-1, acid-sensing ion channel, gating, ion channel, neuroprotection, nonproton ligand, pain, venom peptides

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.¹,² ASICs consist of 3 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 homeric or heteromeric channels, except for ASIC2b and ASIC4, which can only exert their effects in a heteromeric channel configuration.³ 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, 2 transmembrane domains, and a large extracellular domain.

The ASIC 3-dimensional structures were determined using truncated constructs of chicken ASIC1 (cASIC1), which was cloned by Coric and colleagues,⁴ and revealed the low pH structure and the desensitized channel state.⁵ 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.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.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 cambridgei*, was shown to antagonize the ASIC1a subtype with nanomolar affinity and increases the channels sensitivity to protons. 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. While studying the PcTx1 effects on ASIC mediated calcium permeability, it was observed that PcTx1 activates chicken ASIC1 in a similar manner. 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$_{50}$ of 20 nM. The list of venom peptides is growing, as additional natural toxins that activate (coral snake toxin) and inhibit (black mamba toxin) 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. Mutating the amiloride site within the channel pore reveals a stimulatory component of amiloride activity. This provided the first glimpse of a second site that may influence ASIC activity in a stimulatory manner. Additional compounds were identified that stimulate 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. GMQ exhibits the most profound effect, as it robustly activates ASIC3 and has been shown to influence nociception via an ASIC3 mechanism. Agmatine and arcaine stimulation of ASIC3 has reduced efficacy compared with GMQ stimulation, but may be combined with other inflammatory mediators to enhance ASIC3 activity. 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 µM). 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.

The crystal structures of the PcTx1-ASIC complex were obtained using truncated cASIC1 constructs. Baconguis and Gouaux described 2 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 2 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.\(^{11,12,22}\) 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 with the 5 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 min 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 s. What remained 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 toxinASIC 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 with 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.\(^{10}\) In our studies, we supplemented the toxin test solutions with 1% BSA and ensured that this BSA concentration was influence CASIC1 activity (Fig. S1). 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 (Fig. S1A). A 1% BSA test solution had no effect on cells expressing cASIC1 (Fig. S1B and C). We observed a slight reduction in peak current following the BSA exposure that was not statistically significant (\(P = 0.4305\)) (Fig. S1C). After ensuring that BSA addition to our test solutions was not affecting our toxin-mediated responses, we attempted to remove the toxin from the channel following an initial PcTx1 (30 nM) application. Attempts to remove the toxin from the cASIC1 expressing cells failed, thus preventing our pursuit of

![Figure 2](image-url)
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 (Fig. S2). 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 2 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 2 channels, rASIC3 and cASIC1, mediate the low calcium steady-state current and the PcTx1 persistent current, respectively, as a response to 2 distinct stimuli. Following our initial attempts to interrupt the toxin-cASIC1 interaction, we compared the cASIC1 persistent and ASIC3 stead-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 s before making a complete return to the baseline. In comparison, we applied the spider toxin (30 nM) to patched cells expressing cASIC1 for 5 s and observed the cASIC1 PcTx1 persistent current for 5 min (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 2 channels generate similar current, different stimuli (nominal calcium vs. 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 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 (Fig. S2).

Table 1. Summary of pH activation and steady-state desensitization for cASIC1 in the absence and presence of GMQ

|          | pH_{50} | pH_{50} 95% CI | nH      | nH, 95% CI |
|----------|---------|---------------|---------|------------|
| **Activation** |         |               |         |            |
| Control  | 6.65 ± 0.01 | 6.62 – 6.67    | 4.55 ± 0.54 | 5.93 – 3.16 |
| GMQ      | 6.98 ± 0.03** | 6.90 – 7.06    | 2.21 ± 0.38** | 3.19 – 1.23 |
| SSD      |         |               |         |            |
| Control  | 7.53 ± 0.00 | 7.52 – 7.53    | 5.82 ± 0.08 | 5.57 – 6.08 |
| GMQ      | 7.48 ± 0.00** | 7.47 – 7.50    | 5.75 ± 0.19 | 5.16 – 6.34 |

** indicates different from control, p < 0.01. Control, WT cASIC1; GMQ, represents cASIC1 in the presence of 0.3 mM GMQ; CI, Confidence Interval; Mean ± SEM, n ≥ 6.
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).18,19,21,25 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 2 sites that elicit different responses: 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 were applied to cells at pH 7.35 and 8.0, respectively (Fig. 5A and B). 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 pH50 of 6.65 ± 0.01 and nH 4.55 ± 0.54; GMQ pH50 of 6.98 ± 0.01 and nH of 2.21 ± 0.38) (Fig. 6B; 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 s), 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 min 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 with the pH 6.0 control (Fig. 7B). Each of these GMQ mediated currents exhibited slower activation and deactivation kinetics than what was observed with proton-mediated ASIC activity. The GMQ mediated activation rise times (10–90%) were fit to a monoexponential equation and yielded time constants (τ) 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.

![Figure 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.](image-url)
Figure 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 are 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 µM) 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_{max}) as a function of pH.

Figure 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 are presented as mean ± SEM of at least 4 individual cells and significance was determined using unpaired Student t test (*, P < 0.05 compared with control).
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 s 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 and the architecture of the channel pore. 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. 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. 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 s) 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 min). 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. 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 overshoot toward 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. 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 and the protein’s use as a crystallographic construct along with the first ASIC protein structure 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 PcTx1-cASIC1 complex. 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.

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. 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

---

**Figure 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. 32).
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. 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 3, GMQ is the most potent, with maximal efficacy, and stimulates ASIC3 at concentrations as low as 1 µM when applied under mild acidity (pH 7.0). 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 pH50 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. 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.

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
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 2 guanidinium groups), antagonized current in all homomeric ASIC subtypes tested, with the most robust and potent inhibition observed in ASIC1b. Through in-silico modeling using the desensitized state ASIC structures, 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 2 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-α and MitTx-β) activated ASIC1a, 1b, and 3 in a concentration-dependent manner and increased the ASIC2a proton potency, despite sharing no homology to PcTx1.4 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.6 Both the human and rat ASIC1a subtypes are antagonized by PcTx1 under normal conditions. Chen and Gruber have shown that PcTx1 activation can be 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 2 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).32 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.23 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 2 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.19 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 seek 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 ASIC3.33 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 enhances 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

©2014 Landes Bioscience. Do not distribute.
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

Cell culture and transfection
Wild type cASIC1 (cASIC1) and rat ASIC3 with an N-terminal enhanced green fluorescent protein (EGFP) encoded in a pNGFP-EU 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. Subculturing 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 pNGFP-cASIC1 or pNGFP-ASIC3 cDNA and a 4 ml Lipofectamine LTX (Life Technologies) according to manufacturer's instructions. Transfected cells were used for patch clamp electrophysiology 24–48 h 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.) 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 while low pH test solutions were adjusted to pH 7.35 using N-methyl-D-glucamine (NMDG) 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 application. businessman.

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 Clamp 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 s), recordings were filtered at 2 kHz and sampled at 5 kHz.

Patched cells were allowed to equilibrate in external wash solution for a minimum of 5 min prior to experimentation. Test solutions were applied for 5 s and patched cells were washed with external wash solutions for a minimum of 2 min between test solution applications. A typical patch clamp experiment lasted more than 30 min. 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 s followed by exposure to pH 6.0 test solution (without GMQ) for 5 s, 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 8.1 (OriginLab). Nonproton ligand (GMQ) activation time constants (τₐ) 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 Student t test on a minimum of three individual cells. Data are presented as the mean ± SEM.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
We thank Mandy McBroom-Zahn for technical assistance. We thank the members of the Gonzalez laboratory for critical reading of the manuscript. We thank Eric Gouaux (Vollum Institute) for generously providing the enhanced green fluorescent protein amino terminally tagged chicken ASIC1 and rat ASIC3 constructs. We thank Nathalie Sumien for helpful discussion regarding the manuscript.

Author Contributions
Smith RN and Gonzales EB conceived the project, developed recording protocols, analyzed data, and wrote the manuscript. Smith RN collected the data and made figures. Gonzales EB supervised the overall project.
Funding Disclosure
This work was supported by the UNT Health Science Center Intramural Seed Grant and an American Heart Association Beginning Grant-in-Aid (12BGI1882001) (to Gonzales EB). Smith RN is supported by a fellowship on an NIH National Institute on Aging T32 AG020494.

References
1. Gründer S, Chen X. Structure, function, and pharmacology of acid-sensing ion channels (ASICs): focus on ASIC1a. Int J Physiol Pathophysiolog Pharmacol 2010; 2:73-94; PMID:21383888
2. Sherwood TW, Frey EN, Askwith CC. Structure and activity of the acid-sensing ion channels. Am J Physiol Cell Physiol 2012; 303:C699-710; PMID:22843794; http://dx.doi.org/10.1152/ajpcell.00188.2012
3. Hesselager M, Timmermann DB, Ahring PK. pH Dependency and desensitization kinetics of heterologously expressed combinations of acid-sensing ion channel subunits. J Bio Chem 2004; 279:11006-15; PMID:14703823; http://dx.doi.org/10.1074/jbc.M305370200
4. Coric T, Zheng D, Gerstein M, Canessa CM. Proton sensitivity of ASIC1 appeared with the rise of fishes and changes of residues in the region that follows TM1 in the ectodomain of the channel. J Physiol 2005; 568:725-35; PMID:16002453; http://dx.doi.org/10.1113/jphysiol.2005.077734
5. Jasti J, Furukawa H, Gonzales EB, Gouaux E. Structure of acid-sensing ion channel 1 at 1.9 Å resolution and low pH. Nature 2007; 449:316-23; PMID:17882215; http://dx.doi.org/10.1038/nature06163
6. Gonzales EB, Kawate T, Gouaux E. Pore architecture and ion sites in acid-sensing ion channels and P2X receptors. Nature 2009; 460:599-604; PMID:19645889; http://dx.doi.org/10.1038/nature08218
7. Kawate T, Michel JC, Birdsong WT, Gouaux E. Crystal structure of the ATP-gated P2X4(0) ion channel in the closed state. Nature 2009; 460:592-8; PMID:19645888; http://dx.doi.org/10.1038/nature08198
8. Hatori M, Gouaux E. Molecular mechanism of ATP binding and ion channel activation in P2X receptors. Nature 2012; 485:207-12; PMID:22355247; http://dx.doi.org/10.1038/nature10101
9. Escoubas P, De Weille JR, Lecoq A, Diochot S, Medrzhadzsky KF, Zhou S, King D, Sánchez EE, Burlingame AL, Baumba AI, Julius D. A heteromorphic Texas coral snake toxin targets acid-sensing ion channels to produce pain. Nature 2011; 479:410-4; PMID:22094702; http://dx.doi.org/10.1038/nature10607
10. Diochot S, Baron A, Salinas M, Kalbacher H, Gründer S. The tarantula complex with the gating modifier Psalmotoxin 1. Nat Neurosci 2008.12.002
11. Jasti J, Furukawa H, Gonzales EB, Gouaux E. Structure, function, and pharmacology of acid-sensing ion channels (ASICs): focus on ASIC1a. Int J Physiol Pathophysiolog Pharmacol 2010; 2:73-94; PMID:21383888
12. Sherwood TW, Frey EN, Askwith CC. Structure and activity of the acid-sensing ion channels. Am J Physiol Cell Physiol 2012; 303:C699-710; PMID:22843794; http://dx.doi.org/10.1152/ajpcell.00188.2012
13. Bohnen CJ, Cheshire AT, Sharif-Naeini R, Hesselager M, Timmermann DB, Ahring PK. pH Dependency and desensitization kinetics of heterologously expressed combinations of acid-sensing ion channel subunits. J Bio Chem 2004; 279:11006-15; PMID:14703823; http://dx.doi.org/10.1074/jbc.M305370200
14. Coric T, Zheng D, Gerstein M, Canessa CM. Proton sensitivity of ASIC1 appeared with the rise of fishes and changes of residues in the region that follows TM1 in the ectodomain of the channel. J Physiol 2005; 568:725-35; PMID:16002453; http://dx.doi.org/10.1113/jphysiol.2005.077734
15. Jasti J, Furukawa H, Gonzales EB, Gouaux E. Structure of acid-sensing ion channel 1 at 1.9 Å resolution and low pH. Nature 2007; 449:316-23; PMID:17882215; http://dx.doi.org/10.1038/nature06163
16. Gonzales EB, Kawate T, Gouaux E. Pore architecture and ion sites in acid-sensing ion channels and P2X receptors. Nature 2009; 460:599-604; PMID:19645889; http://dx.doi.org/10.1038/nature08218
17. Adams CM, Snyder PM, Welsh MJ. Paradigmatic stimulation of a DEG/ENaC channel by amiloride. J Bio Chem 1999; 274:15500-4; PMID:10386442; http://dx.doi.org/10.1074/jbc.274.22.15500
18. Yu Y, Chen Z, Li WG, Cao H, Feng EG, Yu F, Liu H, Jiang H, Xu TL. A nonproton ligand sensor in the acid-sensing channel. Neuron 2010; 68:61-72; PMID:20292097; http://dx.doi.org/10.1016/j.neuron.2010.09.001
19. Li WG, Yu Y, Zhang ZD, Cao H, Xu TL. ASIC3 channels integrate agmatine and multiple inflammatory signals through the nonproton ligand sensing domain. J Biol Chem 2010; 285:36059-70; PMID:20729462; http://dx.doi.org/10.1074/jbc.M109.092687
20. Keller O, Kellenberger S. Molecular determinants of desensitization in an ENaC/degenerin channel. FASEB J 2013; PMID:24081065; http://dx.doi.org/10.1096/fj.13-236808
21. Chen X, Qiu L, Li M, Dinnagel S, Orser BA, Xiong ZG, MacDonald JF. Diarylaminides: high potency inhibitors of acid-sensing ion channels. Neuropharmacology 2010; 58:1045-53; PMID:20114056; http://dx.doi.org/10.1016/j.neuropharm.2010.01.011
22. Bacoongus I, Hattori M, Gouaux E. Unanticipated parallels in architecture and mechanism between ATP-gated P2X receptors and acid sensing ion channels. CurrOpinStruct Biol 2013; 23:277-84; http://dx.doi.org/10.1016/j.sbi.2013.04.005
23. Li WG, Xu TL. ASIC3 channels in multimodal sensory perception. ACS Chem Neurosci 2011; 2:26-37; PMID:22778854; http://dx.doi.org/10.1021/cn100094b

Supplemental Materials
Supplemental materials may be found here: http://www.landesbioscience.com/journals/channels/article/26978

www.landesbioscience.com Channels 61

©2014 Landes Bioscience. Do not distribute.