Structure and activity of the acid-sensing ion channels

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Sherwood TW, Frey EN, Askwith CC. Structure and activity of the acid-sensing ion channels. Am J Physiol Cell Physiol 303: C699–C710, 2012. First published July 25, 2012; doi:10.1152/ajpcell.00188.2012.—The acid-sensing ion channels (ASICs) are a family of proton-sensing channels expressed throughout the nervous system. Their activity is linked to a variety of complex behaviors including fear, anxiety, pain, depression, learning, and memory. ASICs have also been implicated in neuronal degeneration accompanying ischemia and multiple sclerosis. As a whole, ASICs represent novel therapeutic targets for several clinically important disorders. An understanding of the correlation between ASIC structure and function will help to elucidate their mechanism of action and identify potential therapeutics that specifically target these ion channels. Despite the seemingly simple nature of proton binding, multiple studies have shown that proton-dependent gating of ASICs is quite complex, leading to activation and desensitization through distinct structural components. This review will focus on the structural aspects of ASIC gating in response to both protons and the newly discovered activators GMQ and MitTx. ASIC modulatory compounds and their action on proton-dependent gating will also be discussed. This review is dedicated to the memory of Dale Benos, who made a substantial contribution to our understanding of ASIC activity.

Keywords: acid; ASIC; acid-sensing ion channel; channel gating; fluctuation; glia; ischemia; sodium; spermine; spermidine; proton sensing; neuropeptide; pH; agmatine; spermine; psalmotoxin; channel gating

IN THE CENTRAL NERVOUS SYSTEM (CNS), fluctuations in pH occur as a result of high energy consumption and metabolic demand, elevated neuronal activity, inflammation, hypercapnia, and hypoxia (31, 76, 116, 117). Accordingly, cells utilize specific molecules to measure and respond to these pH changes to maintain proper physiology and react to pathologic insults (31, 116, 117). The acid-sensing ion channels (ASICs) are thought to represent molecular acid-sensors (78, 127). These cation channels are activated by acidic pH changes and are expressed in neurons throughout the body (127). Through work with rodent models, ASICs are known to play critical roles in various processes associated with changes in pH. For example, ASIC1 is required for fear-related behaviors in response to excess CO₂ inhalation, a condition which results in acidic pH in the brain (144). ASIC1 also facilitates seizure termination by responding to extracellular acidic pH induced by abnormally high neuronal activity (145). ASICs are important for other processes such as anxiety, depression, pain, sensory transduction, retinal function, as well as learning and memory within the hippocampus, cerebellum, and amygdala (20, 25, 40, 41, 49, 54–56, 94, 118, 130, 131). These findings suggest that acidic pH transients and ASICs play an important signaling role throughout the nervous system. Further, ASIC currents have been observed in glia, smooth muscle cells, and osteoclasts, indicating that ASICs likely play a role in the physiology of nonneuronal cells as well (36, 66, 70, 91).

ASICs also contribute to neuronal death during pathological insults. In particular, inhibiting ASIC1a attenuates neurologic damage in mouse models of stroke and multiple sclerosis, two conditions which cause long-lasting extracellular acidosis within the central nervous system (57, 101, 124, 136). These results indicate that ASICs represent novel targets for potential therapeutics (48, 51, 65, 119, 132, 135). Significant progress has been made in our understanding of ASICs. This review highlights recent advances in the structure/function of ASICs with a special emphasis on the mechanisms of ASIC activation and modulation.

Overview of ASIC Gating and Structure

There are four ASIC genes in mammals (ASIC1–4) which encode at least six distinct ASIC subunits termed ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, and ASIC4 (4, 11, 16, 24, 46, 58, 62, 68, 92, 103, 128). Alternate isoforms, “a” and “b”, of ASIC1 and ASIC2 differ in the NH₂-terminal third of the protein, and the location of the alternate splice site is very similar between ASIC1 and ASIC2 (16, 92). Three individual ASIC subunits associate to form active channels which can be homomeric (composed of uniform subunits) or heteromeric (composed of varied subunits) (71). The current kinetics and

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ASICs can open in the absence of pH changes by the addition of an activator or by the acidic pH changes (27, 127). Some ASICs (ASIC1a homomeric channels, ASIC1a/2b heteromeric channels, and human ASIC1b channels) are also permeable to divalent cations such as calcium, suggesting that they could play a particularly important role in intracellular signaling as well as membrane excitability (64, 115, 127).

ASICs are activated by a rapid increase in the concentration of extracellular protons (acidic pH) (127). ASICs typically desensitize/inactivate rapidly after proton-induced activation and produce characteristic transient currents, although specific channels can show some level of a sustained acid-dependent component to their current (78, 126, 127). Exposure to mildly acidic pH (not low enough to induce substantial activation) or slow acidification results in steady-state desensitization (9). When steady-state desensitization is induced, ASICs fail to respond to additional decreases in extracellular pH. Multiple studies suggest that induction of steady-state desensitization prevents acidosis-mediated death of neurons and results in behavior and neuronal death in rodents (27, 127). Exposure to mildly acidic pH (not low enough to induce substantial activation) or slow acidification results in steady-state desensitization (9).

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Like all degenerin (DEG)/epithelial sodium (ENaC) channels, individual ASIC subunits have characteristic topology with intracellular NH$_2$ and COOH termini and two transmembrane domains separated by a large extracellular cysteine-rich region. The publication of two chicken ASIC1 (cASIC1) crystal structures (Protein Data Bank 2QTS and 3HGC) has lent invaluable insight into our understanding of ASIC structure and function, and it has spurred a flurry of investigations into the domains responsible for ASIC gating (59, 71). Both structures are from channels crystallized at low pH and are, presumably, of the desensitized channel. Only the second structure published (3HGC) was solved using a cASIC1 protein which could produce functional acid-gated channels (59). Overall, the structures are similar, but there are significant differences, particularly within the transmembrane domain (59).

ASICs display a chalice-like shape with the large extracellular domain projecting substantially above the membrane (Fig. 1A) (59, 71). Individual subunits associate in a threefold axis arrangement within the trimeric channel. Within the extracellular domain of the trimeric channel, there are numerous intersubunit contact sites and crevices. The most notable of these represents the acidic pocket, a region of intrasubunit contacts which play a role in pH-dependent gating and binding of some modulators (Fig. 1A) (59, 71). Four main solvent

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**Fig. 1.** Overview of the acid-sensing ion channel 1 (ASIC1) crystal structure. A: surface view of trimeric chicken ASIC1 (cASIC1a) created with the UCSF Chimera package from Protein Data Bank (PDB) ID: 3HGC (98). The coloration indicates domains identified by Jasti et al. (59, 71): transmembrane domains 1 and 2 (TM1 and TM2, red); wrist (red); palm (blue); knuckle (cyan); finger (purple); thumb (green); and β-ball (orange). Notice that ASICs display a chalice shape with a large extracellular domain. The acidic pocket is located at the interface between 2 subunits and is indicated by the black box (71). B: one subunit of cASIC1 in ribbon format. Coloration indicates the specific domains identified by Jasti et al. (71): The extracellular domain also has 12 β-sheets (β1–12), 7 α-helices (α1–7), and 7 disulfide bonds (illustrated in pink). Note that one of the disulfide bonds is obscured from view by the upper region of the palm domain. The extracellular domain also contains a Cl$^-$ (red sphere) binding site in the thumb domain. Images for all figures were rendered using the crystal structure of the functional cASIC1 (PDB ID: 3HGC) and the UCSF Chimera package (98). Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, funded by grants from the National Institutes of Health, National Center for Research Resources (2P41RR001081) and National Institute of General Medical Sciences (9P41GM103311). [Adapted by permission from Macmillan Publishers Ltd: Nature (Ref. 59, copyright 2009, and Ref. 71, copyright 2007).]
vestibules are arranged vertically within the central core of the protein (upper, central, extracellular, intracellular) (59). The extracellular domain of each subunit resembles a clenched hand, and subregions in the extracellular domain include the wrist, palm, finger, knuckle, thumb, and β-ball domain (Fig. 1B) (59, 71). The palm domain is the central element of each subunit, being composed of four large β-strands that span most of the large extracellular domain (Fig. 1B). The “β-ball” region exists between the palm, thumb, and tip of the finger (59, 71). A chloride ion is bound to each subunit within the extracellular domain at the interface of the thumb, finger, and palm (Fig. 1B) (59, 71). The “wrist” domain connects the “hand” area (palm, finger, knuckle, thumb, and β-ball) to the transmembrane domain through two well-ordered loops. The transmembrane region is composed of six individual continuous α-helices with each subunit contributing two helices (corresponding to transmembrane domain 1 and 2) (59, 71). ASICs also have intracellular NH₂ and COOH termini which are not represented in the crystal structures. These domains play an important role in channel gating, ion permeation, intracellular protein-protein contacts, and modulation by intracellular molecules (34).

The Pore Region

Transmembrane domain 1 (TM1) and transmembrane domain 2 (TM2) form the pore of ASICs (Fig. 2). The α-helices are arranged at an approximately 50° angle to the membrane plane in the crystal structure (59). Transmembrane domain 2 predominantly lines the lumen of the pore. Transmembrane domain 1 contacts the lipid bilayer and makes numerous intrasubunit contacts with TM2 as well as intersubunit contacts with TM1 and TM2 of the adjacent subunit. Only the COOH-terminal portion of TM1 appears to line the pore (86). Within the crystal structure, the extracellular vestibule (approximately 12 Å high by 8 Å wide) is separated from an intracellular vestibule (approximately 10 Å high and 15 Å wide at its base) by the desensitization gate (59). Approximately half of the extracellular vestibule is within the plane of the lipid bilayer. The other half is encased in the extracellular domain of the channel. Ions likely flow into the extracellular vestibule through three oval-shaped fenestrations (approximately 4 × 10 Å) located between subunits at the boundary of the extracellular region and lipid bilayer (59). These three ion pathways are located in the wrist domain which also plays a critical role in channel gating (see below). Although there is no continuous opening through the central axis of the desensitized channel’s extracellular domain, conformational changes could create a central pathway which also allows ion passage.

The desensitization gate. The second transmembrane domain of each subunit crosses about one third of the way through the bilayer region to form the desensitization gate (59). Within the crystal structure, the desensitization gate constriction is formed from residues D433-G436 with the side chain of D433 forming the extracellular boundary of the desensitization gate (Fig. 2, A and B) (59). Transmembrane domain 2 is highly conserved between ASICs and among DEG/ENaC channels in general (77). It has been known for many years that in other DEG/ENaC channels the residue immediately adjacent to the desensitization gate (D432 in cASIC1) plays a critical role in channel gating (Fig. 2, A and B). This amino acid position is often referred to as the degenerin or “Deg” residue, and mutation of residues in this location can produce channels whose gating is dramatically perturbed. This was first discovered when mutation of this residue in the Caenorhabditis elegans channel DEG-1 produced constitutively open channels which caused neurodegeneration (hence the “Deg” designation) (22). For some ASIC subunits, mutations in the corresponding residue produce channels with whole cell currents showing constitutive activity, delayed desensitization, or alterations in the pH dependence of channel activation (1, 23, 128). Interestingly, such mutations at the Deg residue often do not dramatically affect ion selectivity in ASICs, which is surprising given their location near the tight constriction within the transmembrane pore (23, 59, 83). Mutations of the residue which forms the boundary of the desensitization gate, D433 in cASIC1, also affect channel gating and the side chain at position 433 may be important for stabilizing (or destabilizing) the gate (83). Mutation of D433 also does not profoundly alter ion selectivity, suggesting that the selectivity filter and desensitization gate are separate in ASICs (83, 86).

Ion selectivity. The TM2 domain predominantly lines the pore and, thus, is assumed to mediate ion permeation and form the selectivity filter. In an elegant series of experiments using lamprey ASIC1, the role of TM2 was examined using cysteine scanning studies and mutational analysis (86). These studies suggest that the location of the narrowest part of the pore is different in the closed versus the open state of the channel. In
the closed state, data were consistent with a constriction around D433, similar to that observed in the desensitized crystal structure (59, 86). In the open state, however, the smallest constriction was located mid-way through the pore around G443-A444-S445 in cASIC1 (Fig. 2, A and B) (86). Mutation of G443 profoundly affected ion permeability, supporting the theory that this region is important for ion selectivity (86). Mutation of A445 in human ASIC1a (hASIC1a), which corresponds to A444 in cASIC1a, as well as L441 in hASIC1a also affects ion permeability, further supporting this hypothesis (138). This is in stark contrast to mutations of G432 or D433, which do not profoundly affect ion permeability and are located at the narrowest region in the desensitized channel (83). These results suggest that the selectivity filter in ASICs exists at the 443–445 location within TM2.

The TM2 domains of the different ASIC subunits are highly conserved with few amino acid substitutions. Yet, individual subunits confer distinct ion selectivity to ASICs. Studies capitalizing on the difference between subunits have indicated that the ion selectivity of ASICs is affected by residues in the region intracellular to transmembrane domain 1 (pre-TM1) (16, 42). Specifically, substitutions within this region can alter selectivity for monovalent ions (Na/K) as well as the divalent (16, 42). Since these residues do not appear to line the pore region, it is thought that they affect ion selectivity indirectly. The NH2-terminal pre-TM1 region is also home to the “HG” motif (His-Gly), which plays an important, but not completely understood, role in the activity of DEG/ENaCs (17, 61). Mutation of residues in the pre-TM1 region has been shown to affect channel gating, suggesting it plays a defined and important role in ASIC gating as well as selectivity (14, 16, 42, 99, 107). This region is not illustrated in the current crystal structures and future studies are necessary to determine how the pre-TM1 region contributes to ion selectivity and channel gating.

Pore block. Multiple compounds are thought to inhibit ASICs via blocking the transmembrane pore. Tetraethylammo-
nium (TEA), a nonspecific inhibitor of potassium channels, inhibits ASIC1a/2a and ASIC1a/2b heteromeric channels at concentrations commonly used to inhibit potassium channels (115). Based on voltage dependence and TEA’s action on other channels, it is thought that TEA inhibits heteromeric ASICs via pore blocking. The diuretic amiloride and related compounds also inhibit ASICs (127, 128). It is thought that amiloride and related compounds may inhibit ASICs through a pore-blocking mechanism, and modeling studies have suggested that amiloride interacts with residues near the desensitization gate (105, 110). However, amiloride binding to additional sites within the extracellular domain can also have consequences on channel gating (3, 105, 110).

In addition to pharmacological pore blockers, ASICs are sensitive to calcium-mediated pore block. Calcium has a vari-
ety of effects on ASICs (19, 122, 127). Early studies suggested that removal of calcium was sufficient for ASIC3 opening, and a model was proposed in which calcium displacement by proton binding was solely responsible for a transition from the closed state of the channel to the open state (67). However, experiments with ASIC1a suggest that calcium modulation of ASICs is more complex, and that calcium allosterically affects channel opening (96, 143). Yet, ASICs clearly display low-affinity calcium-dependent block that, based on limited volt-
age-dependence, is likely due to calcium ions binding to an area outside the deepest part of the membrane spanning region (96). Interestingly, mutation of a residue that lines one of the fenestrations thought to be critical for ion permeation (corre-
sponding to E426 in cASIC1) in conjunction with mutation of the “gate” residue at the base of the extracellular vestibule (D433 in cASIC1) has been shown to eliminate calcium block in rat ASIC1a (rASIC1a) (96). Studies of residues around E426 indicate that this region may also undergo conformational changes during gating, suggesting that this region is involved in the translation of signals from the extracellular domain to changes in the conductive state of the pore (95). Whether E426 or D433 is involved directly in coordinating calcium or whether they allosterically affect calcium-mediated block of ASIC1a has yet to be determined.

The Extracellular Region

The extracellular region of ASICs is large and encompasses more than two thirds of the protein. It is characterized by specific protein motifs and 14 disulfide bonds which are con-
served among DEG/ENaC channels (17). The extracellular domain is responsible for acid-dependent gating, induction of desensitization/inactivation, and the response to extracellular modulators (60). It plays a critical role in channel activation and is a target for therapeutics to inhibit ASIC1 activity (48, 119). There are seven α-helices (a1–7) and 12 β-sheets (B1–12) distributed throughout the knuckle, finger, β-ball, palm, and thumb regions of the extracellular domain (Fig. 1B) (71). Two conserved glycosylation sites are located at N367 within β10 of the palm domain and N394 between the α6 and α7 of the knuckle domain (Fig. 3, A and B) (71, 75, 109). Glycosylation is required for proper trafficking to the surface and affects apparent proton sensitivity (73, 75, 109). Other regions required for proper surface expression are also contained within the extracellular domain. In ASIC3, a salt bridge between D107 located within the α1 of the finger domain (D108 in cASIC1) and R153 within the α3 of the finger domain (R161 in cASIC1) is required for proper surface localization (Fig. 3B) (139). Similarly, interaction between W287 (W288 in cASIC1) within the thumb domain and Y71 (Y72 in cASIC1) within the wrist of TM1 is also important for proper surface expression of ASIC1 and plays an integral role in gating as well (see below) (74). Chloride is coordinated by residues R310, E314, and K212 from the neighboring subunit (Fig. 3A) (71). Substitution of chloride or mutation of chloride coordinating residues results in channels that are expressed on the surface and are activated by acidic pH, but desensitize more rapidly following activation and undergo less tachyphylaxis (79). These results indicate that chloride ions play a prominent role in channel desensitization by unknown mechanisms.

Proton sensing. Although protons represent the simplest ligand, the structural mechanisms underpinning proton-in-
duced activation of ASICs has not been a simple question to answer. It is known that protons bind the extracellular domain to trigger channel activation and steady-state desensitization (127). The crystal structure of desensitized ASIC shows a solvent pocket containing many acidic residues located at a junction between the finger and thumb of one subunit and the palm domain of another (71). This location has been termed the acidic pocket (Fig. 3, B and C). Within the acidic pocket there
are two pairs of carboxyl-carboxylate side chains (Asp238-Asp350 and Glu239-Asp346 in cASIC1a) and one pair of carbonyl-carboxylate side chains within the palm of an adjacent subunit (Glu220-Asp408) which are coordinated via hydrogen bonds (Fig. 3C) (71). These side chains represent excellent candidates for proton-sensing residues because of their highly negative electrostatic potential. Further, amino acids within the acidic pocket are perfectly poised to support conformation changes in the thumb domain which, in turn, could affect conformation change in the wrist and transmembrane domains (71). In support of this theory, mutations of amino acids within the pocket result in ASICs with changes in the concentration of protons required for gating or a change in the Hill coefficient for proton-induced activation (71, 84, 97, 111, 112, 138, 141). However, elimination of all three interacting pairs results in channels with reduced apparent proton affinity, but which ultimately retain the ability to open in response to acid (84). Therefore, other residues clearly play a role in proton-induced opening of ASICs. Amino acids outside the acidic pocket have also been implicated as potential proton sensors or residues critical for apparent proton affinity (13, 39, 69, 90, 97, 120). These residues are distributed throughout the extracellular domain. Yet, defining “proton-sensing” residues is complicated by the fact that many residues can indirectly affect the apparent proton affinity without necessarily affecting proton binding. Alterations that inhibit the conformational changes required for gating, destabilize the open state, or stabilize the closed state can shift the apparent proton affinity.

For example, mutation of M85L (Xenopus ASIC1, L86 in cASIC1) within the β1-β2 linker shifts the apparent proton sensitivity of ASIC1 by a mechanism that likely involves stabilizing the closed state (Fig. 3B and D) (85). More rigorous analysis is necessary to determine the connection between amino acid mutation and the proton sensitivity of the channel. Even with these important caveats, the general consensus is that there is no one proton-sensing residue for ASICs and that

Fig. 3. The location of amino acids in the extracellular domain known to be involved in ASIC gating. On the left, subunits within the multimeric channel have domains color coded as in Fig. 1. Note that one subunit within the trimeric channel has been removed to better highlight important regions. Boxes on the right indicate higher magnification of the highlighted regions showing the locations of specific amino acid residues. A: the Cl\(^{-}\) binding site and residues of interest around the thumb domain are shown in the far right box. Note that one of the residues involved in coordinating Cl\(^{-}\) (K212) is located on the adjacent subunit which is not shown. The lower box shows Y72 and W288, critical residues in the wrist domain. The loop shown in this inset is also involved in propagating the signal from extracellular modulators [i.e., RFamides (peptide terminating in the sequence arginine-phenylalanine-amide)] to channel gating (114). Residues corresponding to those involved in GMQ/agmatine binding of ASIC3 are shown in the dashed box. These residues are located within the palm regions of the trimeric channel. Note that residue E80 from one subunit (white label) interacts with E417 from the adjacent subunit (yellow label). B: residues of interest located in the finger and knuckle domains. Note that the arrow indicates that the image in A has been rotated to the right to highlight the important region. In the crystal structure (3HGC), side chains for residues K134 and R146 were unable to be visualized. The side chains shown here were inserted using the swapaa command in Chimera software. The acidic pocket is illustrated with a dashed box. The linker region is outlined by the solid box. C: higher-magnification image of the acidic pocket which is composed of the thumb and finger of one subunit and the upper region of the palm domain from the adjacent subunit. The crystal structure in this image was rotated in relation to the trimer shown in B to show the two carboxyl-carboxylate and one carbonyl-carboxylate pairs (D238-D350, E239-D346, and E220-D408). D: higher magnification of the linker region of one cASIC1 subunit. The crystal structure in this image was rotated in relation to the ribbon subunit shown in B to highlight the β1-β2 and β11-β12 linkers (pink) and residues of interest. Images were formulated using the UCSF Chimera package and PDB ID: 3HGC (59, 71, 98). [Adapted by permission from Macmillan Publishers Ltd: Nature (Ref. 59, copyright 2009, and Ref. 71, copyright 2007).]
protonation of multiple residues, possibly at multiple regions throughout extracellular domain, initiates acid-dependent activation.

The role of calcium in proton-dependent activation. The gating behavior of many ASICs is intricately related to the concentration of extracellular calcium (19, 45, 127). As discussed above, calcium can block the pore of ASICs (67, 96). In addition, the apparent proton sensitivity of ASIC1 and ASIC3 is inversely related to extracellular Ca\(^{2+}\) concentration (67, 112, 127, 143). Multiple studies suggest that Ca\(^{2+}\) and protons compete for binding to the channel, and evidence supports a model where calcium binding favors the closed state whereas proton binding favors the open or desensitized state (67, 127, 143). Work with ASIC3 suggested that a calcium ion blocks the pore of ASIC3 and removal of the calcium ion alone (induced by proton binding to the calcium-coordinating amino acids) could open the channel (67). This model implied that no conformation change was necessary for ASIC3 to open, only calcium removal. These observations were supported by the fact that at very low calcium concentrations, ASIC3 displays a small noninactivating current at basal pH (7.4) (67). Yet, protons dramatically enhanced channel opening even in the absence of calcium, suggesting the presence of protons enhanced gating in a manner independent of calcium block (67). In further support of a more complex gating mechanism, studies with ASIC1 show that there are significant allosteric changes upon opening of the channel (143). Several recent studies on ASIC1a support a twofold effect. Calcium blocks the ion permeation pore of ASIC1a (96). In addition, Ca\(^{2+}\) decreases the apparent proton sensitivity of ASIC1a by an undetermined mechanism (96, 143). This is supported by ASIC1a mutants where calcium pore block is abolished, but the calcium-dependent shifts in apparent proton sensitivity remain intact (96). These studies suggest that there are two calcium-binding sites in ASIC1a: one that predominantly determines pore block and one that predominantly mediates calcium regulation of proton-induced gating. Further, calcium-mediated inhibition of apparent proton sensitivity can be separated from the apparent proton sensitivity of activation, suggesting that multiple regions of the protein are important for this process (112). Whether protons and calcium compete directly for binding at one site or whether the calcium binding affects proton-induced activation allosterically remains to be determined.

Acid-evoked steady-state desensitization. ASICs undergo steady-state desensitization (also termed steady-state inactivation) in which the channel enters the desensitized state without obviously transitioning through the open state (9). This process occurs with exposure to a mild increase in proton concentration over time on the order of seconds to minutes (9, 67, 122). Once steady-state desensitization has occurred, little to no current is observed with a further increase in proton concentration. In fact, the ASIC inhibitor psalmotoxin 1 (PcTx1) elicits neuroprotective effects by promoting steady-state desensitization of the channel (29, 113, 134). Steady-state desensitization, like activation, is mediated by proton binding to the extracellular domain of the channel (9). Multiple studies suggest that proton-dependent activation and steady-state desensitization are coupled (12, 90, 114). For example, the protein domains involved in steady-state desensitization overlap with those involved in proton-dependent activation (12, 90, 114). Residues in the acidic pocket play a role in the apparent proton sensitivity of steady-state desensitization and activation (141). In addition, the concentration of calcium inversely affects the apparent proton affinity of both steady-state desensitization and activation (9, 122). Finally, the venom peptide PcTx1 affects the proton dependence of both steady-state desensitization and activation of ASIC1a (29). These results suggest that there are similar mechanisms responsible for proton-dependent activation and steady-state desensitization. However, there are important differences between proton-induced steady-state desensitization and activation. The apparent proton affinity for steady-state desensitization is much higher than activation and occurs at more neutral pHs (9). The proton-dose response curve of steady-state desensitization has a greater Hill coefficient and displays significantly more cooperativity (9). These results suggest that steady-state desensitization may utilize “high-affinity” proton-binding sites which foster desensitization over time rather than activation. In support of this idea, alterations of certain titratable residues (specifically E235 and E254 in hASIC1a) can specifically affect the apparent proton sensitivity of steady-state desensitization without profoundly affecting the pH\(_{50}\) for activation (90). Channel modulators can also affect steady-state desensitization specifically, or differentially affect the apparent proton affinity of steady-state desensitization and activation (50, 113, 129). Further, some amino acids play a prominent role in defining the apparent proton sensitivity of activation or steady-state desensitization, suggesting that residues can play distinct roles in proton-dependent gating (43, 85, 90). Importantly, mutation of N415 (cASIC1) within the β11-β12 linker preferentially affects the apparent proton sensitivity of steady-state desensitization (Fig. 3B and D) (82). Another consideration is that the channel conformation evoked by steady-state desensitization is similar to the desensitized state that occurs after proton-mediated activation (67), and no studies have shown that they are distinct conformational states. Thus, protein regions involved in desensitization following activation might also be expected to affect steady-state desensitization. The fact that induction of steady-state desensitization likely requires a large conformational change suggests that mutations in many residues could affect apparent proton sensitivity. Further understanding of the differences between proton-induced steady-state desensitization and activation may lend specific insight into the mechanisms of these two potentially therapeutically important gating states.

Mechanisms of proton-dependent gating. There is much to be determined on how proton binding to the extracellular domain triggers channel activation and desensitization. The acidic pocket is situated between the thumb and finger, and interactions between these domains have been proposed to be an important aspect of gating (71). Mutations that are calculated to destabilize this interaction reduce the apparent proton affinity of steady-state desensitization without profoundly affecting the pH\(_{50}\) for activation (90). Channel modulators can also affect steady-state desensitization specifically, or differentially affect the apparent proton affinity of steady-state desensitization and activation (50, 113, 129). Within this loop, proline residues create a sharp turn which allows tryptophan 288 to interact with tyrosine 72 within the wrist domain near transmembrane domain 1 (Fig. 3) (84).
Specific mutations of W288, P287, or Y72 can eliminate acid-dependent gating of ASICs (84, 95, 138). Additional conformational changes in the wrist region, both extracellular to TM1 and TM2, occur with channel gating and are important for normal channel activity (84, 95, 123, 138). It has been proposed that conformational changes in the wrist result in twisting or bending movement of the transmembrane domains to initiate channel gating (59, 86, 123, 137, 138). Multiple lines of evidence indicate that both TM1 and TM2 undergo a conformation change with gating (2, 86, 99). However, a defined twisting movement of TM2 (the transmembrane helix that predominantly lines the pore) has not been supported by accessibility studies of lamprey ASIC1 (86). Instead, data support a model in which activation involves a reduction in the tilt of the transmembrane helices which widens the external mouth and forms a narrowing deeper in the pore (possibly the selectivity filter) (86). A similar mechanism is utilized by P2X receptors which share multiple structural features and gating characteristics with ASICs (59, 80, 81).

The transition from the closed to open to desensitized state involves other conformation changes within the extracellular domain. Residues in the β1-β2 linker region in the palm, the β1 strand in the palm, the β11-β12 linker as well as the wrist domain affects the kinetics of opening and closing the pore (38, 43, 85, 87, 121). These events are manifested macroscopically as alterations in the rate of desensitization, proton dependence of activation, proton dependence of steady-state desensitization, or sustained channel opening (43, 85, 87, 121). Within the β1 region of the palm of ASIC3, E79 (E80 in cASIC1) becomes less accessible when the channel is in the desensitized state compared with the closed state (43). This supports the idea that this region undergoes a conformation change during gating. Similarly, amino acid 110 in shark ASIC1a (corresponding to A82 in cASIC1) in the β1-β2 linker is also more accessible when the channel is closed compared with desensitized (121). In addition, there appears to be tight apposition between this residue within the β1-β2 linker and a complement residue (corresponding to V413 in cASIC1) in the β11-β12 linker region when the channel is desensitized (121). Other residues within the β11-β12 linker region have also been shown to play a role in channel gating, particularly desensitization, supporting the idea that movement of these regions is critical to normal gating (82). Together these data suggest that gating, and desensitization in particular, involves movement of the palm domains. Mutations of residues within the contact region between the β-ball, finger, and upper palm domain also affect channel gating and surface expression of the channel. Specifically, residues within the β-ball to finger loop (residues 94–97, 101 in hASIC1a), the finger (163–164 in hASIC1a), and the palm to finger loop (224, 226, 228–229 in hASIC1a) are critical for normal activity (12). Further, evidence suggests that this region also undergoes a conformation change with gating (12). Thus, multiple regions of the extracellular domain move during channel gating. This idea is in agreement with other, more generalized, studies suggesting that there is a large conformational change in the extracellular domain in the transition from the closed to the desensitized state (6, 140).

Other modulators and inhibitors. Many modulators and inhibitors affect ASIC activity through interaction with the extracellular domain (34, 48, 51). For the purpose of this review, we focus on compounds in which there is some understanding into the structural domains of the channel important for modulator action. These modulators include zinc, reducing agents, proteases, peptide toxins, spermine, and neuropeptides (5, 7, 13, 28, 30, 33, 35, 37, 93, 102, 106, 108, 113, 114, 125).

The finger domain plays an important role in zinc and reducing agent-dependent modulation of ASICs. Low concentrations of zinc inhibit ASIC1a by decreasing apparent proton sensitivity of activation through a mechanism that requires K133 (K134 in cASIC1), a residue located at the beginning of α2 within the finger domain (Fig. 3B) (35). K133 is also involved in potentiation of hASIC1a activity by reducing agents (32, 33). Interestingly, residues within the finger domain are required for low-affinity zinc-mediated inhibition of ASIC1b (C149) and zinc-mediated potentiation of ASIC2a (H162) (13, 72). H162 (H164 in cASIC1) corresponds to a conserved histidine which is also one of the residues involved in the contact region between the β-ball, finger, and upper palm domain and is required for proper gating (Fig. 3B) (12). Although exactly how these residues contribute to modulation of ASIC activity is unknown, such results further indicate that the finger domain plays a critical role in ASIC gating and represents a potential target for modulator binding.

The finger domain also plays a prominent role in sensitivity to extracellular proteases. Trypsin and other proteases limit ASIC activity by dampening the proton response, changing ion permeation, and limiting RFamide (peptide terminating with the sequence arginine-phenylalanine-amide)-related neuropeptide modulation (see below) (37, 102, 125). Trypsin cleavage at R145 (R146 just following α2 in the finger domain in cASIC1) is responsible for attenuated rASIC1a activity (125). Further, matrix metalloproteinase cleaves rASIC1a at three sites (R145, K185, and K384) with cleavage at R145 producing the majority of the inhibitory activity (37). Thus, proteolytic cleavage within the finger domain can result in dramatic changes in ASIC gating further supporting the idea that the finger domain plays a critical role in ASIC activity.

The acidic pocket also plays a prominent role in ASIC modulation. One of the best studied ASIC inhibitors is the venom peptide psalmotoxin 1 (PcTx1) isolated from the West Indies tarantula Psalmopoeus cambridgei (53). PcTx1 is a 40 amino acid peptide with three disulfide bonds arranged in an inhibitor cysteine knot (ICK) pattern similar to spider and cone snail venoms (52, 53). Low nanomolar concentrations of PcTx1 can inhibit ASIC1a homomeric channels and ASIC1a/2b heteromeric channels by promoting steady-state desensitization (26, 28, 29). PcTx1 is not a classic “blocker” and does not inhibit ASIC activity by blocking the pore of the channel (28, 29). Instead, PcTx1 acts as a gating modifier toxin and inhibits ASIC1a and ASIC1a/2b channels by shifting the pH dependence of steady-state desensitization to more alkaline pHs such that the channels desensitize at basal pH 7.4 (28, 29, 115). PcTx1 also shifts the pH dependence of channel activation to more neutral pHs (28, 29). Thus, if PcTx1 is present at more basic basal pH and steady-state desensitization is not induced, PcTx1 can enhance ASIC1a activation: an effect in opposition to its normally inhibitory nature (29).

PcTx1 folds tightly into the “knottin” fold pattern similar to other toxins that inhibit voltage-gated ion channels (52). However, PcTx1 is unique in this family in targeting ASIC1 channels and displays a novel collection of basic amino acids “K25-R26-R27-R28” that form a strong positive patch at the
surface of the toxin (52, 53). Since the discovery of PcTx1, it was hypothesized that these residues mediate ASIC1a-specific interaction (52, 53, 100, 104, 106, 108). Recent functional data confirm that R26 and R27 play a critical role in PcTx1 action (106). Excitingly, the use of chimeric ASIC channels composed of different regions of PcTx1-sensitive and -insensitive ASIC subunits, computer docking of PcTx1 to the crystal structure of cASIC1a, site-directed mutagenesis of ASIC1a, and a recent crystal structure of ASIC1a bound to PcTx1 indicate that PcTx1 binds within the acidic pocket of the channel, the same region that is thought to play a key role in pH-dependent gating (44, 100, 104, 106, 108, 112). Functionally, mutations of residues D349 (D350 in cASIC1 thumb and one of the residues involved in the carboxyl-carboxylate pairs) and E97 (E98 in cASIC1 in a loop connecting the β-ball and finger) dramatically reduce PcTx1 binding (Fig. 3, B and C) (108). Other residues such as E358 in the loop, E363 in the loop connecting β10 of the palm to the thumb, and F351 in the thumb also play a role in the response to PcTx1 (Fig. 3A) (108, 112). The recent crystal structure of PcTx1 bound to ASIC1a indicates that PcTx1 binding involves both a hydrophobic patch of the venom peptide which interacts with the thumb of ASIC1a and the basic cluster of residues of the venom peptide (R27 and R28 in particular) which extend into the acidic pocket of ASIC1a to form strong H-bonds (44). Of the residues in ASIC1a previously implicated in PcTx1 action, the new crystal structure supports a direct role for D350 and F351 in binding (44). The involvement of the acidic pocket in PcTx1 action further supports the role of the acidic pocket in pH-dependent gating of the channel, as PcTx1 shifts the pH dependence of both activation and desensitization to more basic pH values. In addition, amino acids from two adjacent subunits within the multimeric channel contribute to PcTx1 binding. This may explain why PcTx1 affects specific ASIC1-containing heteromeric channels such as ASIC1a and ASIC1a/2b, but not ASIC1a/2a (53).

Several other modulators are believed to also interact with the acidic pocket of ASIC1. The cation spermine enhances ASIC1a activity by slowing inactivation, shifting steady-state desensitization to more acidic pHs, and accelerating recovery from desensitization (9, 50). Spermine competes with the venom peptide PcTx1 for action and likely interacts within the acidic pocket of ASIC1 to alter channel gating (50). Modulation by spermine is disrupted by mutations of E219 (E220 in cASIC1 located within the palm domain in the acidic pocket and one residue involved in the carboxyl-carboxylate interaction) and E242 (E243 in cASIC1 within the acidic pocket) (Fig. 3, B and C) (50, 71). These residues are also important for steady-state desensitization, suggesting that these residues play a critical role in spermine modulation of steady-state desensitization (50).

Another set of modulators that may also bind to the acidic pocket are the endogenous opioid peptides big dynorphin and dynorphin A. Dynorphins reduce the apparent proton sensitivity of steady-state desensitization of ASIC1 through a mechanism that likely involves binding of the peptide to the extracellular domain of the channel (113). PcTx1 and big dynorphin compete for action on ASIC1a, suggesting that PcTx1 and dynorphin may share an interaction site or that the binding of one compound prevents the action of the other (113). Another set of neuropeptides, the RFamides, also shift the apparent sensitivity of ASIC steady-state desensitization to more acidic values. RFamides interact with the extracellular domain of ASICs to slow desensitization and reduce the apparent proton sensitivity of steady-state desensitization (7, 21, 30, 47, 93, 114). For some ASICs, RFamides can induce a robust sustained acid-dependent current (7, 30, 47, 133). Both mammalian RFamides and invertebrate RFamides can exert these effects, although smaller RFamides are generally more effective (7, 21, 30, 47, 93, 114). Calcium antagonizes and acidic pH enhances RFamide modulation (7, 30, 93). RFamide action is affected by specific amino acids within the loop region at the base of the thumb that links the β9 of the palm to the α4 of the thumb which is critical for translating signals in the extracellular environment to changes in the transmembrane domains (Fig. 3A) (84, 115, 138). Interestingly, alteration of these residues (A274, P285, K291, D298, L299) in hASIC1a does not seem to affect RFamide binding, but it compromises the ability of RFamide binding to alter desensitization (114). Although RFamides and dynorphins result in similar changes in channel gating characteristics, it is not yet clear whether they share a common binding site (113).

Alternate activation mechanisms. Recently, novel mechanisms of ASIC activation have been identified. ASIC3 can be activated at physiological pH (7.4) by the synthetic compound 2-guanidine-4-methylquinazoline (GMQ), the endogenous compound agmatine, and related chemicals (141). ASIC3 becomes activated by GMQ, but little to no desensitization is induced, resulting in constitutive GMQ-dependent currents (141). GMQ interacts with E79 (E80 in β1 of the palm domain of cASIC1), E423 (E417 in β12 of the palm domain cASIC1a), L77 (L78 in β1 of the palm domain of cASIC1), and R376 (R370 in the β10 of the palm domain), which are located within a shallow depression near the base of the extracellular domain formed by residues of the palm domain (Fig. 3A) (71, 141, 142). Interestingly, this cavity is at the edges of β1 and β12, whose linkers have been shown to play a critical role in ASIC desensitization and production of sustained acid-dependent current with altered ion selectivity (82, 85, 121). Further, E79 of ASIC3 has already been linked to alterations in desensitization (see above) (43). DTNB (5,5'-dithiobis-2-nitrobenzoic acid) modification of E79C mutants of ASIC3 also results in channel activation (141). Interestingly, the classical nonspecific inhibitor of ASICs, amiloride, can activate ASIC3 channels through interaction with this GMQ-binding region (3, 88). Thus, this “non-proton” ligand-sensing domain can induce channel activation at neutral pH in response to multiple compounds (88). The currents induced by GMQ have a different ion selectivity compared with those evoked with protons alone, suggesting that binding of GMQ or activation by GMQ results in changes to the transmembrane domains which are different from those encountered with protons alone (141). This form of activation is further distinguished from proton-induced activation in that mutations that disrupt critical carboxyl-carboxylate proton-binding residues within the acidic pocket (and reduce apparent proton sensitivity) spare GMQ-mediated activation (141). GMQ-mediated activation is not, however, entirely independent of protons or calcium. Low pH potentiates GMQ-mediated activation and, conversely, GMQ potentiates proton response (141). Similarly, low calcium facilitates and high calcium limits GMQ-mediated activation much like the effect of calcium upon proton-dependent activation (141). Together
these results suggest that ASIC3 can act as a multifactorial sensor (protons, calcium, and GMQ-related compounds). In support of this idea, agmatine (an endogenous GMQ-like compound) can synergize with other ASIC modulators such as arachidonic acid and hyperosmolality to enhance ASIC3 current (89). These results suggest that this region is a critical site for binding of new activators and other modulators that affect channel activation, and represents a potentially new therapeutic target for the development of ASIC inhibitors. To date, ASIC1 and ASIC2 channels have not been activated by GMQ and related compounds (141), but, the existence of such a mechanism of ASIC activation is exciting and suggests that other, non-proton ligands for other ASICs may exist in vivo.

A second alternative activation mechanism for ASICs was recently discovered. A heteromeric coral snake toxin called MitTx can activate ASIC1a channels at neutral pH (20). MitTx is a complex of two toxins: a Kunitz-type toxin MitTx-a and a phospholipase-A2-like toxin MitTx-b (20). MitTx induces large sustained amiloride-sensitive currents from ASIC1a and ASIC1b as well as ASIC3 (at approximately 100-fold higher concentration). The currents evoked from ASIC1a lack desensitization and are slow to reverse after removal of the toxin (20). MitTx-evoked currents from ASIC3 display a slower onset and faster washout compared with ASIC1 channels. ASIC2a channels are weakly affected by MitTx at neutral pH (20). MitTx, however, does substantially potentiate proton-induced activation of ASIC2a. MitTx and PcTx1 compete, suggesting that there is functional occlusion of toxin action or the toxins share a binding site (20). The interplay between protons, calcium, and MitTx is not known. Yet, the fact that MitTx can induce currents from ASICs at neutral pH provides another line of evidence to suggest that other endogenous ligands for ASICs may exist and await discovery.

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AUTHOR CONTRIBUTIONS

T.W.S. and C.C.A. interpreted the results of the experiments; T.W.S. and C.C.A. drafted the manuscript; T.W.S., E.N.F., and C.C.A. edited and revised the manuscript; T.W.S., E.N.F., and C.C.A. approved the final version of the manuscript; E.N.F. prepared the figures.

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Review

ASIC STRUCTURE AND FUNCTION

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