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# Structure, function, and pharmacology of acid-sensing ion channels (ASICs): focus on ASIC1a

## **Comments**

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## Review Article

# Structure, function, and pharmacology of acid-sensing ion channels (ASICs): focus on ASIC1a

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**Abstract:** Acid-sensing ion channels (ASICs) are H<sup>+</sup>-gated Na<sup>+</sup> channels, which are present in most, if not all, neurons. The typical ASIC current is transient and is elicited by a rapid drop in the extracellular pH. In the human genome, four genes for ASICs are present: *asic1* – 4. In this review, we will focus on ASIC1a, one of the key subunits in the central nervous system. We will describe the structure of this channel, a topic that has enormously profited from the recent elucidation of the first crystal structure of an ASIC. We will then relate the ASIC1 structure to current models of the gating mechanism of ASICs. Finally, we will review the pharmacology of ASIC1a. Advances in the pharmacological inhibition of individual ASIC currents have greatly contributed to our current knowledge of the functional roles of this channel in physiology, including learning, memory, and fear conditioning, and in pathophysiological states, including the neurodegeneration accompanying stroke, and axonal degeneration in autoimmune inflammation.

**Keywords:** Acidosis, desensitization, excitatory postsynaptic current, fear conditioning, ischemia, psalmotoxin

## Introduction

In 1981, Krishtal and Pidoplichko described for the first time a receptor for H<sup>+</sup> that carried inward Na<sup>+</sup> currents in mammalian sensory neurons [1, 2]. It was not until 1997 that the group of Lazdunski cloned a channel from rat brain that was directly activated by H<sup>+</sup> and carried an excitatory Na<sup>+</sup> current [3]. This channel, ASIC1a, was just the first member of a small family of H<sup>+</sup>-gated channels. Thanks to recent work in many laboratories, we now have a clearer picture of the physiological function of these H<sup>+</sup>-gated channels and there are clear indications that ASICs contribute to the outcome of several pathophysiological states.

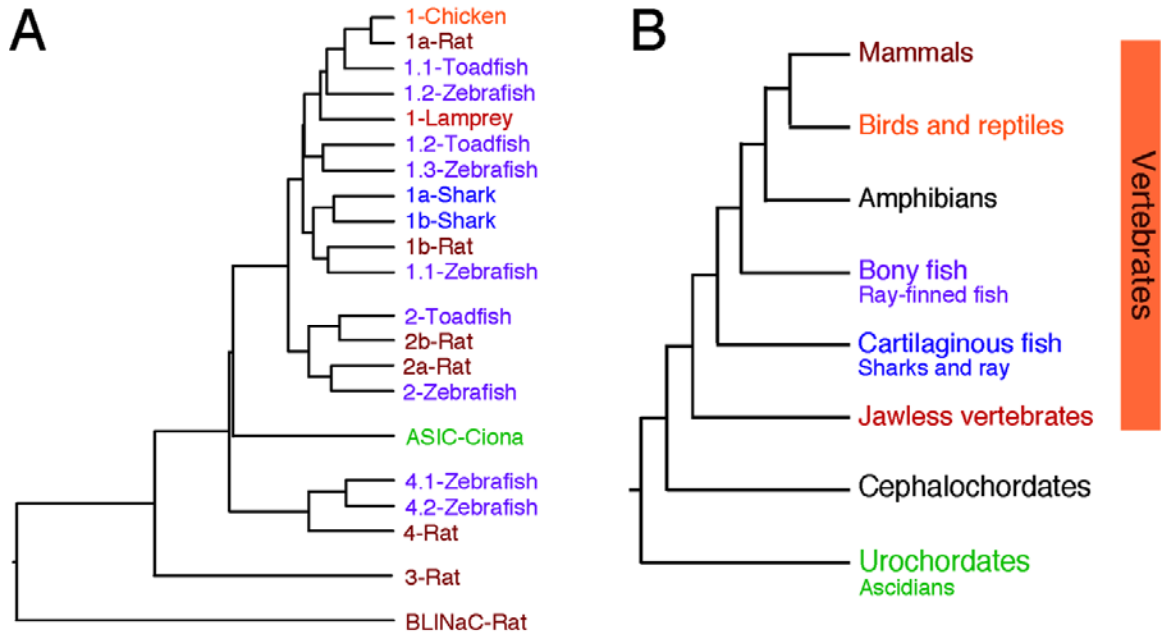
ASIC1a had been cloned by its homology to the amiloride-sensitive epithelial Na<sup>+</sup> channel (ENaC), with which it shares approximately 25% amino acid sequence identity [3]. Other homologues of ASICs include peptide-gated channels from polyps [4] and mollusks [5] and mechanosensitive channels (the degenerins or “DEG” channels) from *C. elegans* [6]. Thus, ASICs de-

fine a branch of the DEG/ENaC family of ion channels. Since the most ancient DEG/ENaC channels that have been characterized so far are peptide-gated channels from the cnidarian *Hydra*, the HyNaCs [4], and since HyNaCs are close relatives of ASICs within the DEG/ENaC gene family [4], it is conceivable that H<sup>+</sup>-gated ASICs evolved from peptide-gated channels.

So far, ASICs have been identified exclusively in chordates (**Figure 1**). A single ASIC gene is already found in the genome of *Ciona intestinalis* [7], an urochordate that belongs to the earliest branch in the chordate phylum. Moreover, ASICs have been cloned from a jawless vertebrate (lamprey) [8], from cartilaginous fish (shark) [8], from bony fish (toadfish and zebrafish) [9, 10], from chicken [8] and different mammals (mouse, rat, human) (**Figure 1**).

ASICs are defined by their sequence homology; functionally, some ASICs, like ASIC2b and ASIC4, are not sensitive to acid [11-13] and the function of those subunits is not always known. ASIC2b contributes to proton-sensing by forming

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**Figure 1:** A) Phylogenetic tree illustrating the relationship of selected ASICs. Note that a clear ASIC1 ortholog is present in all vertebrate lineages, whereas ASIC3 has so far only been identified in mammalian species. The urochordate *Ciona* contains a single *asic* gene [7], whereas in mammals the number of *asic* genes has increased to four. Amino acid sequences of selected ASICs were aligned and the tree for the cladogram established by neighbor joining using ClustalX (DNASTar software); sequences at the N- and C-termini had been deleted. The related brain-liver-intestine Na<sup>+</sup> channel (BLINaC) form rat [156] was included for comparison. B) Phylogenetic tree of the chordate phylum. Main chordate clades are shown. The color corresponds to the color of ASICs from organisms of these clades in (A). So far, no ASICs have been reported from cephalochordates and amphibians; their existence in these clades is, however, likely.

heteromeric channels with other ASICs [11]; the function of ASIC4, however, is unknown and current evidence suggests it is not involved in H<sup>+</sup>-sensing [12]. On the other hand, some DEG/ENaC members, which do not belong to the ASIC branch, are sensitive to H<sup>+</sup>. For example, acid-sensitive channel, degenerin-like (ACD-1) from *C. elegans*, which is most closely related to degenerins, is inhibited by intra- and extracellular acidification [14]. Another protein from *C. elegans* with sequence homology to DEG/ENaC channels has been dubbed ASIC-1 [15]. Since this channel does not belong to the ASIC branch of the DEG/ENaC family and functional expression, let alone H<sup>+</sup>-sensitivity, has not been described, this name is misleading and the protein should be renamed.

In the present review we will focus on ASIC1. ASIC1 is the most abundant ASIC subunit in the mammalian central nervous system (CNS). In addition, in the CNS it is the ASIC with the high-

est H<sup>+</sup>-affinity; ASIC3 is equally sensitive to H<sup>+</sup> but restricted to the peripheral nervous system (PNS) [16]. However, one recent study has reported expression of ASIC3 in rat hypothalamus [17]. Moreover, homomeric ASIC1a is the only ASIC that is slightly permeable to Ca<sup>2+</sup> [3, 18, 19]. The importance of ASIC1a in the CNS is underlined by the fact that it is apparently involved in a number of neurodegenerative diseases (see below). Finally, chicken ASIC1 is the only ASIC that has been crystallized [20] and the gating mechanism of ASIC1 has been elucidated in some detail. The ASIC1 gene gives rise to two variants – ASIC1a and ASIC1b [19, 21]. The splice variant ASIC1b is restricted to the PNS [21], like ASIC3, and its physiological functions are largely unknown. Moreover, although an ASIC1b gene is present in the human genome, clear evidence for an ASIC1b cDNA in humans is lacking. Therefore, we will discuss ASIC1b only where it sheds further light on the properties of ASIC1a.

We will start with a short description of the biophysical properties of ASIC1a. We will then discuss current models of the gating mechanism of ASIC1a, review its pharmacology and will then briefly introduce what we know about the physiological functions of ASIC1a and its implications in the pathogenesis of neurodegenerative diseases. Most of the discussion will be on homomeric ASIC1a; it is known, however, that heteromers containing ASIC1a also mediate H<sup>+</sup>-gated currents in many neurons. In the CNS, homomeric ASIC1a and heteromeric ASIC1a/2a are the major ASICs [22-24].

### Biophysical properties of ASIC1a

In most studies, ASIC1a starts to open at pH 6.9 [3, 25]; current amplitude then increases with increasing H<sup>+</sup> concentrations down to pH 6.0, where ASIC current amplitude starts to saturate. The H<sup>+</sup>-concentration response curve can be fit by a Hill function and yields half-maximal activation at pH 6.5 with a Hill-coefficient of approximately 3 [3, 25]. Very recently, it has been shown that in neurons acutely dissociated from the amygdala, ASICs containing the ASIC1a subunit are even more sensitive to H<sup>+</sup>: channels opened at pH 7.2 [26]. Whether this extreme sensitivity to tiny variations in the H<sup>+</sup> concentrations is due to formation of heteromeric channels, or, more likely, to some modulation by accessory proteins or other unknown factors, awaits further studies.

ASIC1a desensitizes completely with a time constant of 1 - 2 sec [3, 19]. Complete desensitization means that ASIC1a cannot encode sustained acidification. It recovers from desensitization within a few seconds [25]. Desensitization of ASIC1a starts at pH values slightly below pH 7.4 and during prolonged acidification to pH values below 7.1 all ASIC channels are in the desensitized state [25]. Thus, ASIC1a can enter the desensitized state without apparent opening. This steady-state desensitization (SSD) can also be fit by a Hill function: for rat ASIC1a it is half-maximal at pH 7.25 and has a Hill coefficient of approximately 10 [25]; for mouse and human ASIC1a, it is half-maximal at pH 7.15 and 6.90, respectively [27]. Thus, SSD seems to be a process completely separate from activation, with a higher H<sup>+</sup> affinity.

This conclusion is not necessarily true, however.

At low H<sup>+</sup> concentrations (for rat, between pH 7.4 and 6.9) channels may open with a low probability. The unconcerted opening (and subsequent desensitization) of individual channels would not lead to visible currents in whole cell measurements and pass unnoticed. In agreement with such a scenario, SSD takes much longer than open state desensitization (70 sec, at pH 7.05, rather than 1 - 2 sec) [25]. In addition, in most mutagenesis studies where mutations changed the apparent H<sup>+</sup> affinity of ASIC1a, mutations also affected H<sup>+</sup> affinity of SSD [25], arguing that both processes are indeed intimately linked. Moreover, for the rapidly desensitizing ATP receptor P2X1, it has been shown that desensitization masks a higher ATP affinity of P2X1 activation [28, 29], suggesting that the "real" H<sup>+</sup> affinity of ASIC1a is that of SSD. The E79A substitution in ASIC3 is an example of a mutation that shifts the SSD curve to 10-fold lower H<sup>+</sup> concentrations without shifting the activation curve [30], suggesting that this mutation uncouples SSD from activation gating. However, since the same mutation also enhanced desensitization 4-fold [30], it is not excluded that the faster desensitization may mask a higher H<sup>+</sup> affinity of ASIC3 activation. In summary, whether ASIC1a channels can reach the desensitized state directly from the closed state and if so, to which extent this closed state desensitization contributes to SSD at slight acidification are open questions. Irrespective of this uncertainty, all observations suggest that ASIC1a is an exquisitely sensitive H<sup>+</sup> receptor; if H<sup>+</sup> affinity were only slightly higher, the channel would be chronically desensitized at rest.

Recently, it has been shown that the endogenous opioid peptides dynorphin A and big dynorphin shift the SSD curve of ASIC1a to lower H<sup>+</sup> concentrations limiting the impact of SSD [31]; probably dynorphin directly binds to the extracellular loop of ASIC1a. This result suggests that ASIC1 should be considered as a new nonopioid target for dynorphin action in the CNS.

For ASIC3 it has been shown that the curves of H<sup>+</sup> activation and SSD overlap and that in the window of overlap ASIC3 carries sustained currents [32]. This property endows ASIC3 with the capacity to encode also the sustained acidification in the window of overlap between pH 7.3 and 6.7. ASIC1a, however, carries no such window currents [32] and can, therefore, encode

only the acidic transients as they may occur during synaptic transmission.

Once gated open, the ASIC1a pore has a slight selectivity for Na<sup>+</sup> over K<sup>+</sup> ( $P_{Na}/P_K = 10$ ) [3, 19], so that at a membrane potential of -70 mV, which is much closer to the K<sup>+</sup> than the Na<sup>+</sup> equilibrium potential, ASIC1a mainly carries an excitatory inward current of Na<sup>+</sup>. In addition, homomeric ASIC1a is the only ASIC that has a slight permeability to Ca<sup>2+</sup> ( $P_{Na}/P_{Ca} = 15$ ) [18, 19, 33]. The fractional Ca<sup>2+</sup> current is, however, very small [34]. Therefore, the Ca<sup>2+</sup> influx through ASIC1a per se will significantly raise the intracellular Ca<sup>2+</sup> concentrations only in a microdomain close to the channel, if at all. Ca<sup>2+</sup> is a permeant blocker of ASIC1a, permeating through the channel but also blocking it [3, 35].

Finally, ASIC1a is not only gated by H<sup>+</sup> but also permeable to H<sup>+</sup> [3, 36]. Not unexpected, relative H<sup>+</sup> permeability is higher than Na<sup>+</sup> permeability ( $P_{Na}/P_H < 0.3$ ) [36] but due to the very small H<sup>+</sup> concentrations (1 μM at pH 6.0) relative to the Na<sup>+</sup> concentration (~100 mM), the fractional H<sup>+</sup> current will again be tiny. However, it could lead to slight acidification in a microdomain surrounding the intracellular mouth of the channel.

Tachyphylaxis is another peculiar feature of homomeric ASIC1a that is specific to this subtype [36]. Tachyphylaxis means that repeated activation of the channel leads to ever-smaller current amplitudes even though the time interval between individual activation is sufficient for recovery from normal desensitization. Thus, tachyphylaxis uncovers a second long-lived desensitized state, from which the channel may not recover [36]. Tachyphylaxis is linked to H<sup>+</sup> influx through ASIC1a and its Ca<sup>2+</sup> permeability [36]. Since tachyphylaxis is especially pronounced at pH < 6 [36], it is unclear to which extent it limits ASIC1a activity in vivo.

### The structure of an ASIC: pre-crystal era

ASICs consist of 500 to 560 amino acids. The primary structure of each ASIC subunit is characterized by two hydrophobic regions of approximately 20 amino acids, long enough to span the lipid bilayer, rather short termini (35 – 90 amino acids) and a large domain between the two hydrophobic domains (approximately 370 amino acids) (**Figure 2A**). The transmembrane topology

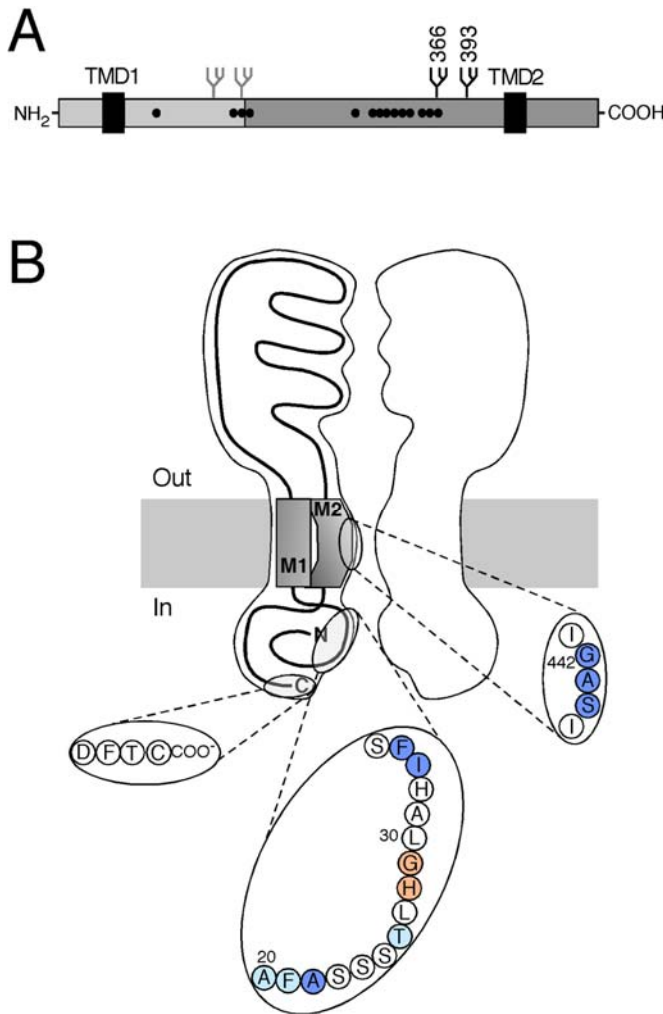
of the related ENaC had been established in three independent studies [37-39] and revealed that the two termini are located in the cytoplasm, whereas the domain between the two hydrophobic domains, each of which spans the membrane once, forms a large extracellular domain (ECD). It was confirmed that ASICs have the same transmembrane topology [40] (**Figure 2B**). The first exon, which is different between ASIC1a and ASIC1b, thus comprises the intracellular N-terminus, the first transmembrane domain (TMD) and approximately the first one-third of the ECD (**Figure 2A**).

A large ECD is not specific to ASICs, but shared by all DEG/ENaC channels, although its size can vary. Homology between different ASICs is largest in the second two-thirds of the ECD, whereas some parts of the first one-third are characterized by considerable sequence divergence. All ASICs have 14 conserved cysteines in their ECD (**Figure 2A**), which likely stabilize the structure of the ECD by forming disulphide-bonds [41]. As almost every membrane protein, ASICs carry at least one N-glycan in the ECD. ASIC1a has two glycans in the distal part of the ECD, whereas ASIC1b carries four glycans: two in the distal part, which is identical to ASIC1a, and two additional glycans in the proximal part [42] (**Figure 2A**), which is different between ASIC1a and ASIC1b. It has been proposed that the two proximal glycans help in shaping the structure of the proximal ECD of ASIC1b [42], which may therefore be substantially different between ASIC1a and ASIC1b.

The number of subunits in a functional channel complex has been experimentally addressed in several studies, most of the studies focusing on ENaC and one study on FaNaC [43]. These studies came to conflicting results, most of them proposing tetramers [43-48] and some nonamers [49, 50]; one recent study found identical numbers of the three different ENaC subunits (α, β, γ), making a trimeric structure a minimal subunit composition [51].

Several studies, most on the ENaC [52-64] and some on ASICs [35, 65], suggested that the second TMD, especially the first, outer half contributes to the outer entrance and the selectivity filter of the ion pore (**Figure 2B**). ENaC, which is characterized by a high Na<sup>+</sup> selectivity of its pore ( $P_{Na}/P_K > 100$ ), selects ions by molecular sieving [54], largely excluding cations bigger

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**Figure 2:** A) Linear model of ASIC1a. The first one-third of the protein, which is different between ASIC1a and 1b, is indicated in light grey. The two hydrophobic domains are shown as black boxes. The position of the two glycosylation sites of ASIC1a are indicated by a branched symbol and the two additional glycosylation sites, which are specific for ASIC1b, are shown in grey. The 14 cysteines that are conserved in ASICs are shown as black dots; 10 of them cluster in one region. B) Model of the transmembrane topology of ASIC1a from the pre-crystal era. TMD2 faces the ion pore, the three residues (G-A-S) that probably form the ion-selective part of the molecular sieve [53, 55, 59] are magnified in the inset. Residues within the intracellular N-terminus that have been implicated in contributing to the internal pore of ASIC1a [65] or ASIC2 [66] are marked in the inset in dark and light blue, respectively. Amino acids in this region also determine Ca<sup>2+</sup>-permeability of ASIC1a [18]. The amino acid sequence from rat ASIC1a is shown. The two invariably conserved amino acids (H-G) in the cytoplasmic N-terminus that are involved in gating of ENaC [67, 68] are marked in orange. The four amino acids (D-F-T-C) at the cytoplasmic C-terminus that bind to the PDZ domain of PICK1 [69, 70] are also magnified in an inset.

than Na<sup>+</sup>. The narrowest part of the pore, representing the ion-selective part of this molecular sieve, is formed by three amino acids, which are in the middle of the second TMD [54, 56, 60]. In all ASICs, these three amino acids are (from N- to C-terminus or outside to inside) Gly-Ala-Ser (Gly<sup>442</sup>-Ser<sup>444</sup> in rat ASIC1a; **Figure 2B**), amino acids with small side chains. For ENaC, it was proposed that the side chains of these three amino acids do not point toward the lumen of the pore but rather towards subunit-subunit interfaces [61]. Thus, the backbone of the peptide would face the lumen of the selectivity filter and help to coordinate permeating ions; a similar situation is found in the P-loop of K-channels. The inner entrance to the ASIC pore is much less characterized [66].

Surprisingly, the second TMD is identical between ASIC1a and ASIC1b, yet ASIC1a is permeable to Ca<sup>2+</sup> whereas ASIC1b is not [19]. The

difference in Ca<sup>2+</sup>-selectivity has been attributed to the different intracellular N-terminus [19], suggesting that this part of the protein helps to shape the structure of the ion pore, perhaps by long-range allosteric effects. Other studies also demonstrate a role for the amino terminus in ion selectivity [67] and participation in the internal pore [66] of ASICs (**Figure 2B**).

Two amino acids (His-Gly) at the intracellular N-terminus (**Figure 2B**) are invariably conserved in all DEG/ENaC channels. Also their distance to the beginning of the first TMD (approximately 15 amino acids) is within one or two amino acids invariable, suggesting that these two amino acids have some crucial function for all DEG/ENaC channels. In ENaC, these two amino acids plus a few surrounding amino acids, which are also similar across the whole gene family, are crucial for the gating of this channel [68, 69], defining a "gating domain". It is likely that they

have a similar function in ASICs.

The function of the cytoplasmic C-terminus for ASICs is less well established. Actually the C-terminus can be quite short ( $\geq 40$  amino acids) in some ASICs. Although the ASIC C-terminus is not indispensable for channel activity (see below), it may be quite important for the physiological regulation of ASIC function. For example, ASIC1a interacts with its extreme C-terminus (**Figure 2B**) with the PDZ domain of the protein interacting with C kinase (PICK1) [70, 71]. No interactions have been directly demonstrated for ASIC1b, but since the C-terminus of ASIC1b is identical to that of ASIC1a, in principle at least, this subtype can also interact with PICK1. In addition to protein-protein interactions mediated by the C-terminus of ASIC1a, in dorsal root ganglion neurons ASIC1a physically interacts with its N-terminus with annexin light chain p11, increasing surface-expression of ASIC1a [72].

In summary, in the pre-crystal era we had a picture of ASICs being made of probably four subunits, each having an overall simple topology with two transmembrane domains, short cytoplasmic termini and a large ECD. Although it was clear that the ECD has a defined and probably intricate structure, what this structure would exactly look like was completely elusive.

### The structure of an ASIC: post-crystal era

A deletion mutant of chicken ASIC1 has been crystallized and the crystal structure has been resolved at a resolution of 1.9 Å [20]. Since rat and human ASIC1a are 90 % identical to chicken ASIC1, it is very likely that many structural details are conserved in mammalian ASIC1. Therefore and to have a common reference sequence, in this review all amino acids are numbered based on the sequence of rat ASIC1a. Initially, the chicken ASIC1 structure was obtained from a deletion mutant where most of the N- and C-termini had been deleted [20]. Subsequently, a functional channel, in which only the C-terminus was deleted but not the N-terminus with its essential "gating domain", had been crystallized at a lower resolution (3 Å) [73]. The main difference between the first crystal of a non-functional channel and the second crystal of a functional channel was the symmetric order of the two transmembrane domains in the functional channel [73]. Thus, the high-resolution structure revealed the precise

structure of the ECD, whereas the low-resolution structure added the structure of the transmembrane domains and the ion pore. Both proteins were crystallized at low pH (pH 5-6 and 6.5, respectively), where chicken ASIC1 is desensitized. Therefore, most likely both structures represent the desensitized conformation of the channel.

It came as a big surprise that chicken ASIC crystallized in units of two trimers [20]. Since crystals of the functional chicken ASIC also contained trimers as the basic unit [73], it is highly likely that functional ASICs, and probably all functional DEG/ENaC channels, are made of three subunits. In the meanwhile, a trimeric structure has been confirmed for ASIC1a by atomic force microscopy imaging [74].

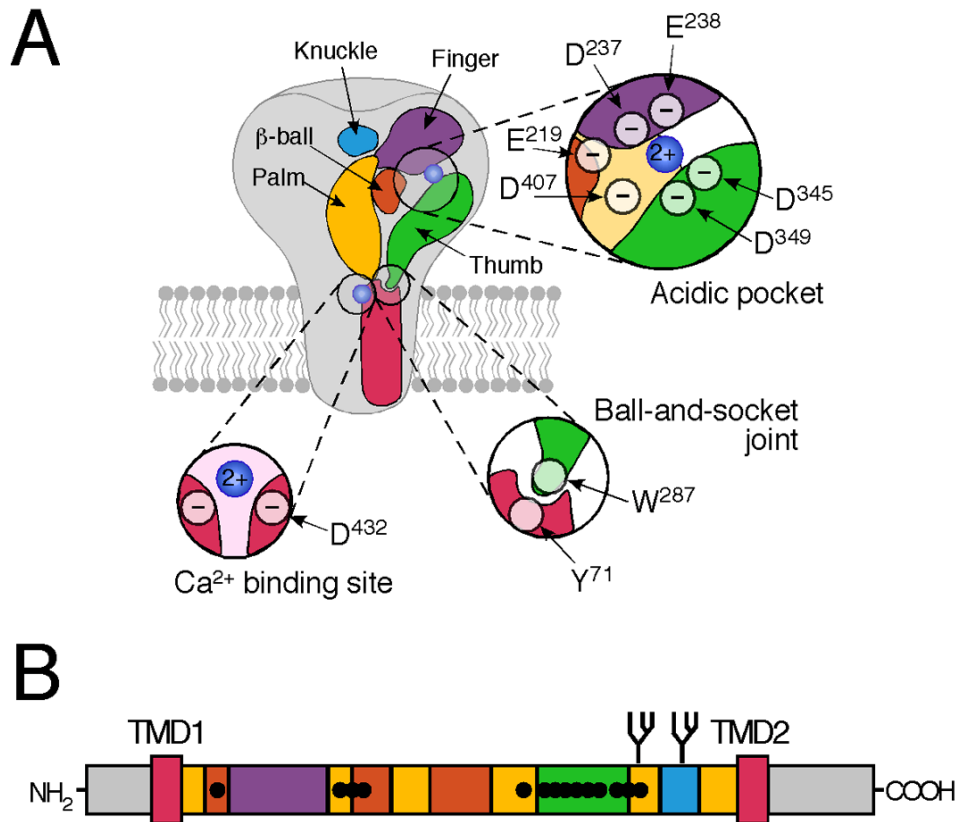
The ECD of each subunit resembles a clenched hand, which is linked to the TMDs via an apparently flexible wrist [20]. The hand domain can be divided in five subdomains: palm, finger, thumb, and knuckle domains and a  $\beta$ -ball domain (**Figure 3A**). The contacts between the subunits are in particular between different palm domains and the palm domain of one subunit and the thumb domain of another. Prominent subunit interactions are 1) hydrogen bonds between Asp<sup>78</sup> (all numbers referring to rat ASIC1a) of one subunit and His<sup>73</sup> and Gln<sup>420</sup> of an adjacent subunit, 2) interactions between the loops of  $\beta$ -strands in the palm domain and the C terminus of helix  $\alpha 5$  of the thumb domain of an adjacent subunit, and 3) interactions between helix  $\alpha 6$  of the knuckle that is enveloped by a concave surface on the neighboring finger.

Most of the disulphide bonds localize to the thumb (**Figure 3B**), giving it a rigid structure. Since the thumb domain has contact with the wrist junction via a conserved Trp (Trp<sup>287</sup>) residue (**Figure 3A**), its rigid structure may serve to faithfully transduce conformational changes to the transmembrane ion pore [20].

A peculiar feature of the structure was the clustering of several acidic amino acids within a small space, the so-called acidic pocket. This pocket is far (45 Å) from the TMDs and is formed by intra-subunit contacts between the thumb, the  $\beta$ -ball and the finger domains, together with residues from the palm domain of an adjacent subunit [20] (**Figure 3A**). The acidic pocket contains three pairs of acidic residues



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**Figure 3:** A) Model of the transmembrane topology of ASIC1a from the post-crystal era (based on reference [19]). Three features of the structure and their crucial amino acids are magnified in the insets: the acidic pocket, the ball-and-socket joint and the  $\text{Ca}^{2+}$  binding site in the ion pore. Binding of a  $\text{Ca}^{2+}$  ion in the acidic pocket is hypothetical. Note that all amino acids are numbered based on the sequence of rat ASIC1a. B) Linear model of ASIC1a highlighting the relation of the primary sequence to the five subdomains of the ECD. It becomes apparent that the  $\alpha$ -helices of the finger, thumb and knuckle subdomains are contained within contiguous stretches of the polypeptide chain, whereas the palm and  $\beta$ -ball domains are formed by  $\beta$ -sheets that are scattered over the polypeptide chain. Those  $\beta$ -sheets connect the finger, thumb and knuckle subdomains. Note also that most of the cysteines (black dots) cluster within the thumb subdomain (green), giving it a rigid structure. The structure of the cytoplasmic termini is unresolved.

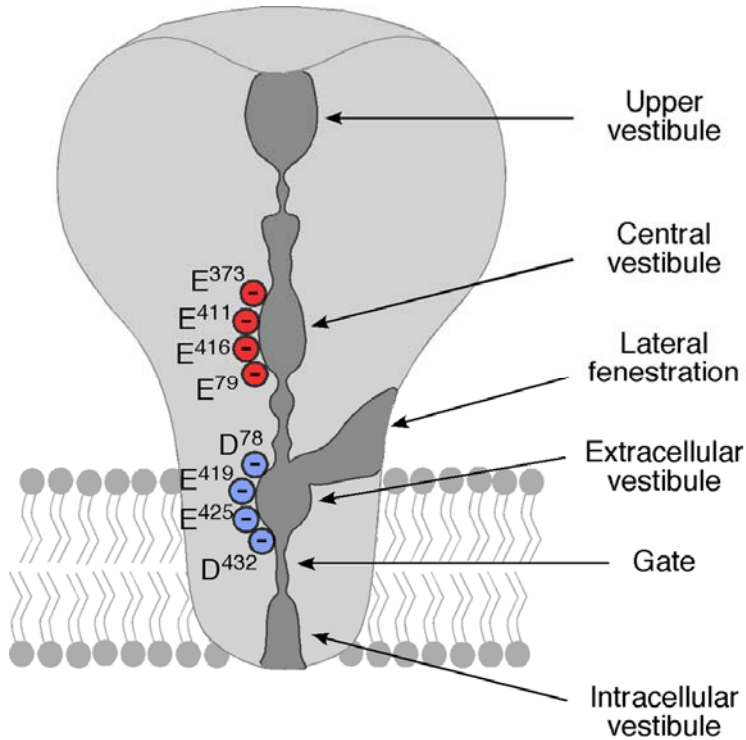
(glutamate or aspartate), which form carboxyl-carboxylate interactions. The distance of the carboxyl groups is so small (2.8 – 3.0 Å; [20]) that at least one of the carboxyl groups of each pair must be protonated. This means that the pKa value of one residue of each pair is several units larger than that of isolated Asp or Glu residues.

TMD1 and TMD2 of the three subunits form  $\alpha$ -helices [73]. Whereas in the crystal of the non-functional protein TMDs were asymmetric, in the crystal of the functional channel they are symmetric, providing the structure of the ion pore in the desensitized conformation. The TMD2 helices are closest to the three-fold axis,

are tilted by  $\sim 50^\circ$  with respect to the membrane and cross each other in the middle of the membrane bilayer [73]. Thus, in line with previous functional studies [52-65], TMD2 helices probably line the ion pore. The TMD1 helices make most contacts with the lipid bilayer but also with TMD2 helices of the same subunit and the TMD1 and TMD2 helices of adjacent subunits.

In the desensitized conformation, two cation-binding sites have been revealed by soaking the crystal in solutions containing  $\text{Cs}^+$ . In both sites,  $\text{Cs}^+$  is coordinated by six ligands arranged in a trigonal antiprism geometry. At site 1,  $\text{Cs}^+$  is coordinated by three side chain carboxyl groups of Asp<sup>432</sup>. At site 2, ligands consist of either

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**Figure 4:** Model of ASIC1a showing the three vestibules in the ECD and the lateral access to the extracellular vestibule via a lateral fenestration (based on reference [72]). Acidic amino acids that line the central and extracellular vestibules are indicated; they are numbered based on the sequence of rat ASIC1a. The negative charge of their side chains could attract cations to the vestibules.

main-chain carbonyl oxygen atoms from Gly<sup>431</sup> or side chain oxygen atoms from Asp<sup>432</sup>. Moreover, it was suggested that main-chain carbonyl oxygen atoms, derived mainly from glycine residues, also coordinate permeant ions in the open channel. Thus, G<sup>431</sup> and G<sup>435</sup> could build a third ion-binding site in the open conformation of the channel [73]. Other candidates for glycines involved in ion coordination are G<sup>438</sup> and G<sup>442</sup> [73]. G<sup>442</sup> is the first of the three residues proposed by earlier studies to form the selectivity filter [54, 56, 60] (**Figure 2B**).

In the desensitized conformation, three vestibules can be discerned in the ECD, along the central axis of the channel [73] (**Figure 4**). The upper vestibule is in direct contact with the extracellular solution, whereas the central and the extracellular vestibule, which is situated at the height of the outer surface of the membrane, are separated from each other and the upper vestibule by constrictions. If these constrictions would be wider in the open channel, they would form a central tunnel, constituting a pathway for ions to the pore. Cations would be concentrated within the vestibules along the central tunnel by several acidic residues, clustered in the central and extracellular vestibules (**Figure 4**). In addition to this putative central pathway for ions, in the desensitized conformation, there is direct

access to the extracellular vestibule through three lateral fenestrations at the wrist of the ECD.

Even with the lateral access pathway to the extracellular vestibule, ions cannot permeate through the pore because, in the desensitized conformation, TMD2 helices cross each other in the middle of the membrane bilayer. Thus, the helices apparently change their conformation during desensitization, constricting the ion pore. This constriction therefore constitutes the desensitization gate – a physical block of the ion pore (**Figure 4**). Close packing of the three TMD2 helices at the constriction is made possible by Gly<sup>435</sup>.

The speed of the desensitization is determined by three amino acids in the proximal ectodomain (S<sup>83</sup>-T<sup>85</sup>) [10]. Moreover, a residue in the immediate vicinity (E<sup>79</sup>) is modified by cysteine-modifying reagents in the closed but not the desensitized conformation [30], suggesting that this residue undergoes a conformation change during desensitization gating of the channel. Together these results suggest that the loop encompassing residues E<sup>79</sup>-T<sup>85</sup>, which localizes between the palm and the  $\beta$ -ball domains, undergoes a conformation change during desensitization gating that determines the speed of

desensitization.

In summary, the crystal structure of chicken ASIC1 provides an invaluable picture of the intricate three-dimensional structure of the ECD and the ion pore. It suggests models for ion permeation and selectivity in these channels and, as we will see, it also suggests models for H<sup>+</sup>-gating of ASICs. One has to keep in mind, however, that the chicken ASIC1 structure is a snapshot of the desensitized state of the channel. A clear picture of the conformational changes accompanying ASIC gating needs additional crystal structures of, ideally, the closed and the open states. In the absence of these structures, the first crystal structure can nevertheless guide future structure-function studies and greatly refine models of ASIC gating.

### How to gate an ASIC by H<sup>+</sup>

In principle, titration of a single amino acid of an ASIC, for example in the ECD, could induce a conformational change, opening the ion pore. Current models of ASIC gating, however, invoke a more complicated process to explain ASIC gating.

An initial observation providing a clue to ASIC gating came from the observation that extracellular Ca<sup>2+</sup> ions modulate the apparent H<sup>+</sup> affinity of ASIC1: high [Ca<sup>2+</sup>]<sub>e</sub> decreases apparent H<sup>+</sup> affinity (higher H<sup>+</sup> concentrations are needed to open the pore) and low [Ca<sup>2+</sup>]<sub>e</sub> increases apparent H<sup>+</sup> affinity (lower H<sup>+</sup> concentrations are needed to open the pore) [25]. To explain these findings, it was proposed that Ca<sup>2+</sup> stabilizes the closed state of the channel and that H<sup>+</sup> displaces Ca<sup>2+</sup> from its binding site, effectively competing with Ca<sup>2+</sup> for this binding site [25]. This model was then extended for ASIC3 by proposing that a Ca<sup>2+</sup> ion in fact blocks the open ASIC pore. According to this model, H<sup>+</sup> would gate an ASIC open by unblocking the ion pore with no accompanying conformational change [75].

Substitution of D<sup>432</sup> and E<sup>425</sup> at the N-terminal end of TMD2 by neutral amino acids disrupted pore block of ASIC1a by Ca<sup>2+</sup> [35]. The crystal structure of cASIC1 confirmed that D<sup>432</sup> and E<sup>425</sup> are accessible from the aqueous phase within the extracellular vestibule; moreover, D<sup>432</sup> forms a putative binding site for cations at the bottom of the extracellular vestibule [73]

(**Figure 3A**), suggesting that substitution of D<sup>432</sup> and E<sup>425</sup> disrupted the Ca<sup>2+</sup> binding site in the outer pore of ASIC1a. In addition, voltage clamp fluorometric measurements showed that E425 undergoes a significant reorganization following extracellular acidification [76], in agreement with the idea that such a movement is associated with relief of Ca<sup>2+</sup> blockade. This Ca<sup>2+</sup> binding site mutant, therefore, allowed testing of the pore block model. According to this model, disruption of the Ca<sup>2+</sup> binding site should have three effects: first and most importantly, the ion pore should be constitutively open, second, the channel should be no longer gated by H<sup>+</sup> and third, Ca<sup>2+</sup> should no longer modulate the apparent affinity for H<sup>+</sup>. All three predicted effects were not observed: mutant channels were not constitutively open, they were still gated by H<sup>+</sup> and their apparent H<sup>+</sup> affinity was still modulated by Ca<sup>2+</sup> [35]. These results effectively rule out the possibility that pore block by Ca<sup>2+</sup> can fully explain gating of ASIC1. Examination of single channel recordings of ASIC1 from toadfish also argued against the Ca<sup>2+</sup> pore block model and favored an allosteric model to explain ASIC gating [77]. Moreover, modulation by a spider toxin (psalmotoxin 1, see below) of chimeras between ASIC1a and 1b suggested the existence of partially-liganded open states of ASIC1 [78], a finding, which is in agreement with a Monod-Wyman-Changeux model for cooperativity in allosteric proteins.

Channels with the Ca<sup>2+</sup> binding site mutations (D<sup>432</sup> and E<sup>425</sup>) had, however, reduced affinity for H<sup>+</sup>, a smaller Hill co-efficient and modulation by Ca<sup>2+</sup> was less pronounced [35], suggesting that pore block by Ca<sup>2+</sup> contributes to ASIC gating. This interpretation suggests the existence of two Ca<sup>2+</sup> binding sites involved in ASIC gating: one at the outer entrance to the ion pore, crucial determinants are D<sup>432</sup> and E<sup>425</sup>, and another one at a different location within the ECD. The acidic pocket is suspicious of such a second putative Ca<sup>2+</sup> binding site within the ECD [20]. Pairs of acidic amino acids within the acidic pocket are D<sup>237</sup>-D<sup>349</sup>, E<sup>238</sup>-D<sup>345</sup>, and E<sup>219</sup>-D<sup>407</sup> (**Figure 3A**); E<sup>79</sup>-E<sup>416</sup> defines a fourth pair outside the acidic pocket [20].

Since the crystal probably represents the structure of the desensitized conformation, it has been hypothesized that, in the closed state, the acidic pocket held a Ca<sup>2+</sup> ion that was displaced upon acidification when one of the acidic resi-

dues of each pair became protonated [20]. This is an attractive and plausible hypothesis that is supported by the experimental finding that E<sup>79</sup>, which is in an acidic pair, is inaccessible for cysteine-modifying reagents in the desensitized but not the closed state [30]. Moreover, molecular simulations of cation binding to the structure of chicken ASIC1 revealed that Ca<sup>2+</sup> binds to the acidic pocket, but only when all three pairs of acidic residues are charged, and that H<sup>+</sup> binding inhibits Ca<sup>2+</sup> binding at this site [79]. Evidence for cation binding was especially strong for the two pairs D<sup>237</sup>-D<sup>349</sup> and E<sup>238</sup>-D<sup>345</sup> [79]. Individual mutations of amino acids of the acidic pocket have rather modest effects on ASIC gating [20, 80], however, and even combined substitution of all four amino acids from the two critical pairs (D<sup>237</sup>-D<sup>349</sup>, E<sup>238</sup>-D<sup>345</sup>) did not disrupt proton gating of an ASIC [81]. These results suggest that there are further determinants of ASIC gating. Indeed molecular simulations suggested the surface residue pairs D<sup>298</sup>-D<sup>331</sup> and E<sup>338</sup>-E<sup>342</sup> as additional potential candidates for H<sup>+</sup> sensing sites of ASIC1a [79]; these sites are not conserved in other H<sup>+</sup>-sensitive ASICs (e.g. ASIC3), however, suggesting that if they have a role in ASIC gating it must be a rather modulatory role.

In addition to titration of acidic residues within Ca<sup>2+</sup>-binding sites, H<sup>+</sup> may also titrate amino acids that contribute to open gating of an ASIC by an allosteric mechanism. Indeed some years ago, a histidine that is highly conserved in ASICs has already been shown to be important for H<sup>+</sup> gating of ASIC2a [82]. ASIC1a has two adjacent histidines at the corresponding position (H<sup>72</sup>/H<sup>73</sup>) and in a large screen for amino acids involved in gating of ASIC1a, these same two histidines were found to be indispensable for ASIC1a gating [80], confirming the importance of these His residues. In this screen, D<sup>78</sup> and E<sup>79</sup> were also found to be important for ASIC1a gating, but only when combined with the Ca<sup>2+</sup> binding site mutations in the pore (D<sup>432</sup> and E<sup>425</sup>) [80]. Interestingly, in the desensitized conformation, D<sup>78</sup> forms a hydrogen bond with H<sup>73</sup> [20] and E<sup>79</sup> forms a carboxyl-carboxylate pair with E<sup>416</sup>, supporting an important function of these two residues. Molecular simulations suggested that the E<sup>79</sup>-E<sup>416</sup> pair, in contrast to the pairs from the acidic pocket (D<sup>237</sup>-D<sup>349</sup>, E<sup>238</sup>-D<sup>345</sup>, and E<sup>219</sup>-D<sup>407</sup>), preferentially binds Na<sup>+</sup> over Ca<sup>2+</sup> ions [79], suggesting a role in ion permeation for E<sup>79</sup>. Although their exact role is still unknown, collectively current evidence suggests

a crucial role for H<sup>72</sup>/H<sup>73</sup>, D<sup>78</sup>, and E<sup>79</sup> in ASIC gating. Indeed the presence of these amino acids has been successfully used to predict H<sup>+</sup>-sensitivity of ASIC1b from shark [83].

Several studies used chimeric approaches to identify the regions that are responsible for differential H<sup>+</sup> sensitivity of different ASICs. Such regions do not necessarily contain crucial H<sup>+</sup> binding sites, however. In one such approach with chimeras between rat ASIC1a and 1b, two amino acids (K<sup>105</sup>N<sup>106</sup>) that localize to the finger domain were identified where substitutions convert the relatively high apparent H<sup>+</sup> affinity of ASIC1a into the lower affinity of ASIC1b [25]. Molecular simulation also suggested that the finger domain moves during gating [79]. In another study with chimeras between rat ASIC1a and 2a, two larger regions (from amino acid 87 - 197 and from 323 - 431) were identified that specify the different H<sup>+</sup> sensitivities of these two ASICs [84]. ASIC2a has a rather low H<sup>+</sup> sensitivity, however, explaining the larger regions necessary to swap sensitivities.

Based on current evidence it has been proposed [80] that upon acidification, H<sup>73</sup> (and perhaps H<sup>72</sup>) gets protonated, which induces a conformational change, facilitating the displacement of Ca<sup>2+</sup> ions from its binding sites in the pore and the acidic pocket. Concomitantly, charged residues of the Ca<sup>2+</sup> binding sites get protonated and form the carboxyl-carboxylate pairs. The conformational change is probably accompanied by a movement of the palm domain relative to the thumb domain. Movement of the thumb domain is then likely transferred to the TMDs to open the ion pore. Voltage clamp fluorometry indeed suggests that the pre-TMD2 region changes its conformation upon acidification [76]. The crucial role of the thumb domain in transferring the conformational changes of the ECD to the TMDs is suggested by the close contact of Tyr<sup>71</sup> and Trp<sup>287</sup> (at the base of the thumb in immediate contact to the wrist junction) in the cASIC crystal [20]. It has been proposed that these two residues form a ball-and-socket joint to transduce conformational changes of the ECD to the TMDs [20] (**Figure 3A**). Moreover, the importance of the interaction between Y<sup>71</sup> and W<sup>287</sup> for ASIC gating has recently been experimentally confirmed [81]. In addition, amino acid changes in the region around W<sup>287</sup> determine the differential H<sup>+</sup> sensitivity of SSD of mouse and human ASIC1a [27].

## Acid-sensing ion channels

**Table 1:** Inhibitory constants ( $IC_{50}$ ) of different inhibitors at homomeric ASIC1a and ASIC currents in cultured neurons. ND, not determined

Inhibitor	$IC_{50}$ at ASIC1a [ $\mu$ M]	$IC_{50}$ at ASIC in neurons [ $\mu$ M]	Reference
Zn <sup>2+</sup>	0.007	~0.015	[87]
Cu <sup>2+</sup>	ND	~50	[90]
Pb <sup>2+</sup>	~5	~10	[91]
Cd <sup>2+</sup>	no inhibition	ND	[92]
Ni <sup>2+</sup>	~600	ND	[92]
PcTx1	~0.001 (at pH 7.4)	ND	[95]
amiloride	10	10 - 50	[3]
nafamostat	~15	ND	[121]
ibuprofen	~350	ND	[122]
flurbiprofen	~350	ND	[122]
A-317567	ND	2 - 30	[123]
DAPI	ND	3	[124]
pentamidine	ND	40	[124]
hydroxystilbamidine	ND	1.5	[124]
diminazene	2 - 3	0.3	[124]

Desensitization would then be accompanied by a movement of the loop encompassing residues E<sup>79</sup>-T<sup>85</sup>, which is probably accompanied by rotation of the adjacent residues D<sup>78</sup> towards H<sup>73</sup> and E<sup>79</sup> towards E<sup>416</sup>, and finally by constriction of the ion pore in the middle of TMD2. This is a tentative and hypothetical model. What is quite clear is that ASIC gating critically involves several residues scattered over the ECD, with residues at the wrist junction having a central role.

### Pharmacology of ASIC1

The pharmacology of ASIC inhibitors and their therapeutic potentials have been reviewed previously [85-87]. Here we summarize recent findings from the view of structure-function relationships.

Chemically, there are three classes of ASIC inhibitors: metal ions, polypeptide toxins, and small-molecule inhibitors (table 1). Besides Ca<sup>2+</sup> and Mg<sup>2+</sup>, a number of divalent and trivalent metal ions inhibit ASICs. PcTx1 is a cysteine-rich high-affinity polypeptide toxin purified from a tarantula venom. Amiloride, A-317567, nafamostat and diarylamidines are examples for synthetic, small molecule ASIC inhibitors.

### Metal ions

Currently zinc, copper, lead, nickel, and cadmium have been documented to affect ASIC1a. Zinc (Zn<sup>2+</sup>), an endogenous trace element, inhibits homomeric ASIC1a and heteromeric ASIC1a/2a with nanomolar affinity, without affecting currents mediated by homomeric ASIC1b, ASIC2a, or ASIC3 [88]. In agreement with high-affinity Zn<sup>2+</sup>-inhibition of the major ASICs in the CNS (ASIC1a and ASIC1a/2a), chelation of trace amounts of Zn<sup>2+</sup> by TPEN, a Zn<sup>2+</sup>/Fe<sup>2+</sup> chelator with low affinity for Ca<sup>2+</sup>, potentiates approximately two-fold ASIC currents in cultured mouse cortical neurons [88]. Likewise, sulfhydryl-containing reducing agents like dithiothreitol potentiate ASIC currents in hippocampal neurons, mainly by chelating metal ions that tonically inhibit ASIC1a and ASIC1a/2a [89]. Homomeric ASIC1a is more strongly inhibited by Zn<sup>2+</sup> than heteromeric ASIC1a/2a [88]. At much higher (micromolar) concentrations, Zn<sup>2+</sup> has an opposite effect and potentiates currents through homomeric ASIC2a and heteromeric ASIC1a/2a ( $EC_{50}$ : ~100  $\mu$ M), but not through homomeric ASIC1a [82]. In addition, such high concentrations of Zn<sup>2+</sup> slow the decay time constant of ASIC currents in hippocampal neurons [90]. High-affinity Zn<sup>2+</sup>-block depends on K<sup>133</sup> in

the ECD of ASIC1a [88] whereas Zn<sup>2+</sup>-potentiation depends on H<sup>162</sup> and H<sup>339</sup> in the ECD of ASIC2a [82].

Micromolar concentrations of copper (Cu<sup>2+</sup>) inhibit ASIC currents in cultured hypothalamic neurons (IC<sub>50</sub>: ~50 μM) [91] and the neurotoxic metal ion lead (Pb<sup>2+</sup>) strongly and reversibly blocks ASIC1a-containing homo- and heteromeric channels (IC<sub>50</sub>: ~5 μM) [92]. Nickel (Ni<sup>2+</sup>) and cadmium (Cd<sup>2+</sup>) affect some ASIC currents at high concentrations (1 mM) [93]. At this concentration, Ni<sup>2+</sup> inhibits homomeric ASIC1a and heteromeric ASIC1a/2a [93]. In contrast, Cd<sup>2+</sup>, up to 1 mM, does not affect homomeric ASIC1a currents [93]. Since Cd<sup>2+</sup> blocks voltage-gated calcium channels [94, 95], it can be used as a pharmacological tool to distinguish between ASIC1a and voltage-gated calcium channels as a source of acid-evoked calcium influx in neurons.

### *Polypeptide toxins*

Psalmotoxin 1 (PcTx1) is a polypeptide toxin isolated from the venom of the South American tarantula *Psalmopoeus cambridgei* and specifically inhibits homomeric ASIC1a with an IC<sub>50</sub> of ~ 1 nM. PcTx1 does not inhibit other ASIC subtypes or voltage-gated ion channels [96] and has already proven to be a useful pharmacological tool for probing the functions of ASIC1a in vivo [97-100]. PcTx1 consists of 40 amino acids, containing 6 cysteines that form three disulfide bridges. The solution structure of PcTx1 has been determined [101] and, interestingly, PcTx1 is structurally similar to a class of gating modifier toxins of voltage-gated ion channels, such as Hanatoxin 1 [101]. Like Hanatoxin 1, PcTx1 has a canonical inhibitor cysteine knot (ICK) scaffold, which possesses an embedded ring formed by two disulfide bonds and their connecting backbone segments which is threaded by a third disulfide bond [101, 102]. Spider toxins most often modify the voltage dependence of voltage-gated ion channels [103-106] or slow their inactivation kinetics [107, 108]. PcTx1 inhibits ASIC1a in a similar manner: it increases the apparent ligand affinity of ASIC1a [109]. Since ASIC1a is activated by H<sup>+</sup> at concentrations that are only slightly higher than the resting H<sup>+</sup> concentrations, this increase in H<sup>+</sup> affinity is sufficient to transfer ASIC1a channels into the desensitized state. Since ASIC1 has been implicated in the neurodegeneration accompanying

ischemic stroke and since it contributes to axonal degeneration in autoimmune inflammation, this finding implies a new approach to intervene with ASIC1a activity: to pharmacologically facilitate its steady-state desensitization by slightly increasing its H<sup>+</sup> affinity. Such an approach is hampered, however, by the comparatively low H<sup>+</sup> affinity of SSD of human ASIC1a [27].

PcTx1 does not inhibit ASIC1b but rather increases the proton affinity of ASIC1b and shifts its pH-activation curve to higher pH values. PcTx1 directly opens ASIC1b under slightly acidic conditions. In addition, PcTx1 significantly slows the desensitization process of ASIC1b, but not of ASIC1a. These data strongly support a model of state dependent binding, i.e. PcTx1 has different affinities for the closed, open, and desensitized states of the channels. For ASIC1a, it stabilizes the desensitized state promoting SSD, whereas for ASIC1b, it stabilizes the open state promoting channel opening [78].

First hints on the PcTx1 binding site came from mutagenesis studies, in which chimeras between ASIC1a and ASIC2a and/or 1b were assessed for binding of radiolabelled PcTx1 [110] or for PcTx1-inhibition of proton-gated currents [78, 110]. One of these studies identified amino acids 167-185 in the ECD of ASIC1a that determines a high PcTx1 affinity of the desensitized state of ASIC1a [78]. Another study identified the same region plus a second region (amino acids 271-369) in the ECD [110]. The involvement of both domains in PcTx1 binding was recently confirmed by computational docking simulations [111, 112], which revealed that PcTx1 binds to a pocket in the ECD of ASIC1a which is formed by four structural motifs: the two stretches identified with the chimeric channels (amino acids 167-185 and the distal part of stretch 271-369), an additional region comprising amino acids 113-129, and E<sup>219</sup> [111, 112]. Moreover, molecular docking revealed that PcTx1 binds at the interface of two subunits and that the domains constituting the high-affinity binding site are located on two adjacent subunits [111, 112]. The crucial role of the distal part of the stretch from amino acids 271-369 was recently confirmed by the finding that the F350L substitution (F352 in human ASIC1a) completely eliminated the modulation of ASIC1a by PcTx1 [84]. Since PcTx1 competes with dynorphin for binding to ASIC1a [31], the bind-

ing site for dynorphin may overlap with the PcTx1 binding site.

Recently, it was demonstrated that PcTx1 strongly activates chicken ASIC1a even at neutral pH [34]. Therefore, PcTx1 functions as an allosteric agonist for cASIC1, providing a novel tool for elucidating the gating mechanism of cASIC1. This finding also provides an explanation why a tarantula venom contains a toxin that targets ASIC1. From an evolutionary point of view, PcTx1 may have been generated by the tarantula as an activator rather than as an inhibitor of ASICs so as to ward off its potential predators (such as birds) because activating ASICs would cause pain or inflammation. Similar examples from nature are vanillotoxins that are contained within the venom of the same tarantula species (*Psalmopoeus cambridgei*). Vanillotoxins share high homology and a conserved cysteine bridge pattern with PcTx1 and directly open TRPV1 channels [113] to cause pain as a defending strategy. The result that PcTx1 directly gates chicken or rat ASIC1, thus, supports the idea that ASICs are involved in pain perception. Therefore, blocking ASIC1 may have beneficial effects in relieving pain. Indeed it has been shown that PcTx1 has potent analgesic properties against thermal, mechanical, chemical, inflammatory and neuropathic pain in rodents [100].

### *Small-molecule inhibitors*

The diuretic amiloride is an open channel blocker plugging the ion pore [35] and is viewed as a prototype blocker of ASIC channels. Amiloride blocks ASIC1a currents with an  $IC_{50}$  of 10  $\mu$ M [16, 114]. It is not very selective and inhibits, among others, ENaC, T-type calcium channels, and the sodium-proton exchanger [86]. In peripheral sensory neurons, amiloride has been used to suppress acid-induced pain [115, 116], whereas in CNS neurons, it reduces acid-mediated ischemic neuronal injury and axonal degeneration [97, 98]. Both effects are potentially mediated by the inhibition of ASICs.

Nafamostat, a synthetic, competitive serine protease inhibitor that is clinically used for the treatment of disseminated intravascular coagulation and acute pancreatitis [117-121], has recently been identified as an inhibitor of ASICs [122]. In contrast to amiloride, nafamostat inhibits ENaC ( $IC_{50}$ : ~1 mM) less potently than

ASICs ( $IC_{50}$ : ~2 – 70  $\mu$ M, depending on the subtype). Non-steroidal anti-inflammatory drugs, such as diclofenac and ibuprofen, directly inhibit ASICs in nociceptors but with relatively low potency [123]. Ibuprofen and its analogue flurbiprofen inhibit ASIC1a with an  $IC_{50}$  of 350  $\mu$ M, whereas diclofenac inhibits ASIC3 ( $IC_{50}$ : 100  $\mu$ M) but not ASIC1a [123]. A-317567, a novel non-diuretic blocker, inhibits three distinct types of native ASIC currents of acutely dissociated adult rat dorsal root ganglion (DRG) neurons [124].

Through a small scale screen, anti-protozoal diarylamidines, including diminazene, hydroxystilbamidine (HSB), and pentamidine were recently identified as potent inhibitors of ASICs in primary cultured hippocampal neurons as well as of ASICs over-expressed in CHO cells or in *Xenopus* oocytes [125]. Diarylamidines have been widely used for the treatment of protozoal diseases such as trypanosomiasis and leishmaniasis since the 1930s [126-128]. Diarylamidines such as diminazene are lead compounds that point to a new route to identify new potent and selective inhibitors of ASICs. Diminazene is the most potent small molecule inhibitor of ASICs known so far ( $IC_{50}$ : 0.3  $\mu$ M, measured in cultured hippocampal neurons). It has distinct potency on different ASIC subtypes. It blocks homomeric ASIC currents expressed in CHO cells with a rank order of potency 1b > 3 > 2a > 1a. Intriguingly, anti-parasitic diarylamidines comprise more than 30 members and the family number is rapidly increasing due to medical needs in the treatment of pneumocystosis, African trypanosomiasis and leishmaniasis. Thus, it is feasible to select these compounds from the chemical family of diarylamidines to test their inhibitory effects on ASICs and to possibly identify new selective and potent ASIC blockers with therapeutic potential.

Comparison of nafamostat with diarylamidines reveals that both are dications which are governed by amidine/guandine groups at their termini, with variable bulk (like benzene rings) on the outer edge of the molecule linked by molecular bridges of varying length and composition. Their difference lies in that nafamostat has a guanidine group at one terminus and an amidine group at the other, while diarylamidines have amidine groups at both ends. It is not surprising that dicationic diarylamidines or nafamostat block ASIC potently as the ectodomain of

ASICs has a large number of negatively charged amino acids [80].

A “patchdock” computation technique was used to dock nafamostat, diminazene, HSB and pentamidine, respectively, to the ASIC1 crystal structure and to analyze possible binding domains of these compounds [125]. This analysis suggests that these dicationic compounds likely bind to a groove formed by part of the  $\beta$ -ball and palm domains of ASICs [125]. Specifically, residues in a stretch from F<sup>174</sup> to L<sup>207</sup> shape this groove [125]. Thus, this domain partly overlaps with the PxTc1 binding site. So far, there is no selective small-molecule inhibitor for ASICs, mainly because the potency of the known inhibitors is relatively low. Further structure-function studies and larger screens based on the cASIC1 crystal structure and the common template of diarylamidines may lead to the identification of more potent and subtype-specific blockers of ASICs.

Regarding the pharmacological classification of known ASIC inhibitors, they fall into three classes: gating modifiers (PcTx1), non-competitive antagonists (probably diminazene and nafamostat) and un-competitive antagonists (pore blocker amiloride). Ca<sup>2+</sup> and Mg<sup>2+</sup> might be competitive antagonists.

### Physiological functions and pathophysiology of ASIC1

ASICs are predominantly, though not exclusively, expressed throughout the PNS and CNS. In the CNS, ASIC1a is abundantly present in the hippocampus, cortex, striatum, olfactory bulb and elsewhere, with a particularly high expression in the amygdala [129]. Low pH-induced ASIC currents are detected in virtually every cultured neuron from spinal dorsal horn [130], cortex [97], and hippocampus [22, 23]. In contrast, ASIC currents are not detectable in glial cells [131, 132] and we were unable to detect ASIC currents by puff-application of pH 5.8 to microglia in acute cortical slices (X.C., unpublished). However, the presence of ASIC-like currents has been documented in cells of the oligodendrocyte lineage [133, 134] and in glioblastoma cells [132].

What is the function of ASICs in central neurons? Synaptic vesicles are acidic [135] and it is conceivable that the release of the content of the synaptic vesicles sufficiently acidifies the

synaptic cleft [136] to activate ASICs. In agreement with such a scenario, in transfected cultured hippocampal neurons, ASIC1a has a synaptic pattern of distribution and co-localizes with the postsynaptic marker PSD95 but not with the axonal marker dephospho tau-1 [137]. ASIC1a transfected into hippocampal slices also has somatodendritic localization and is present in most spines [138]. Moreover, ASIC2, which can tether to PSD95 (in contrast to ASIC1a), facilitates ASIC1a localization at dendritic spines by direct association with ASIC1a [139]. In addition, there is evidence that ASIC1a modulates the density of dendritic spines [138]. The presumably postsynaptic location of ASIC1a together with its Na<sup>+</sup>-selectivity, thus, suggests that ASIC1a contributes to the excitatory postsynaptic current in many neurons.

In agreement with this hypothesis, knock out of the *asic1* gene in mice reduces excitatory postsynaptic potentials during high-frequency electric stimulation and impairs long-term potentiation (LTP) at Schaffer collateral-CA1 synapses in the hippocampus [137]. In contrast, baseline synaptic transmission is normal in those mice [137]. It is plausible that ASIC1a facilitates neuronal depolarization especially during high frequency stimulation and thus may promote the opening of NMDA receptors, activation of which is a central feature of CA1 LTP. However, the exact role of ASIC1a in LTP is not fully understood. Behaviorally, ASIC1a null mice display defective spatial learning and defective cerebellum-dependent eyeblink conditioning [137].

Consistent with the robust expression of ASIC1a in lateral and basolateral nuclei of the amygdala and in the hippocampus [129, 137, 140], mice lacking ASIC1 also exhibit deficits in multiple fear-related behaviors including unconditioned fear, and the acquisition and retention of conditioned fear [129, 140]. Conversely, overexpressing ASIC1a throughout the brain in a transgenic mouse enhances context fear conditioning [141]. Restoring ASIC1a function specifically in the amygdala of ASIC1 knockout mice with a viral vector rescues fear memory but not unconditioned fear responses [142]. Finally, ASIC1a was recently shown to be a chemosensor in the amygdala detecting carbon dioxide and acidosis that elicits fear behavior [26]. In line with an important role in the fear circuit, genetically disrupting or pharmacologically inhibiting ASIC1 has antidepressant-like effects [143], establishing ASIC1a as a new possible



target to combat depression.

Somewhat contradictory to its excitatory role, some studies point to an inhibitory role of ASIC1a. One recent study suggested that ASIC1a could exert some protective effects in the brain under epileptic conditions [99]. This study combined genetic, pharmacological, and electrophysiological approaches with animal behavioral analysis to examine how ASIC1a works under epileptic conditions. It was found that ASIC1a facilitates termination of seizures rather than causing neuronal over-excitation [99]. ASIC1a reduces seizure severity, shortens seizure duration, and promotes seizure termination [99]. Transgenic over-expression of ASIC1a had beneficial effects without affecting seizure threshold, while ASIC1a ablation had opposite effects and increased seizure severity and reduced postictal depression of epilepsy and thus increased seizure-caused mortality [99]. Thus, there is solid evidence that ASIC1a mediates the antiepileptic effects of extracellular acidosis during epilepsy. But how can an excitatory channel dampen neuronal over-excitation? To explain this paradox, a mechanistic model was proposed that activation of ASIC1a in inhibitory interneurons of the hippocampus, which have larger ASIC currents than excitatory pyramidal neurons [99], could increase the inhibitory tone and thereby attenuate epileptic activities [99]. Thus, according to this model, ASIC1a is an excitatory ionotropic receptor, but its predominant expression in inhibitory neurons would increase the inhibitory tone in a network during ASIC1a activation.

A similar model may explain increased (rather than reduced) pain sensitivity in mice with a loss of ASIC function. A dominant-negative ASIC3 subunit assembles with other ASIC subunits and transgenic expression of this subunit leads to a complete loss of ASIC-like currents to pH 5.0 stimulation in DRG neurons [144]. Unexpectedly, in these transgenic mice sensitivity to mechanic and inflammatory pain is increased rather than reduced [144]. Similarly, ASIC3 null mice show enhanced behavioral responses to high-intensity nociceptive stimuli [145]. This increased pain sensitivity may again be explained by the precise location of ASICs within the pain pathway.

But there are also observations suggesting that ASICs might down-regulate excitability of individual neurons. For example, presynaptic release

probability is enhanced in hippocampal neurons from ASIC1 null mice [146]. Moreover, in the retina ASIC2a negatively modulates rod phototransduction and protects the retina from light-induced degeneration [147]. ASIC1a, however, is a positive modulator of cone phototransduction and adaptation [148]. Although the exact role of different ASICs in signal transduction in the retina is unknown, these studies support the possibility that ASICs, under certain circumstances, might function as negative regulators instead of depolarizing elements in the course of synaptic transmission.

In addition to its roles in fear conditioning and seizure termination, ASIC1a apparently gets activated during the course of a number of neuropathological syndromes such as brain ischemia, autoimmune inflammation, or inflammation-associated pain. A prolonged acidosis is common to all these syndromes; how completely desensitizing ASIC1a gets activated during prolonged acidosis is unknown. Since extracellular acidosis increases neuronal cell calcium by activating ASIC1a [33, 138], ASIC1a activation might enhance brain injury, for example during the ischemia associated with stroke. Indeed ASIC1a was found to have a significant detrimental effect on neuronal survival under ischemic conditions [97]. In the focal ischemia model, intracerebroventricular injection of ASIC1a blockers (amiloride, PcTx1) or knockout of the ASIC1a gene protects the brain from ischemic injury [97]. Furthermore, post-ischemic administration of PcTx1 was found to be neuroprotective against brain ischemic injury in the transient middle cerebral artery occlusion model [149]. In addition and as mentioned before, there is evidence that ASIC1a functionally couples to NMDA receptors. Consequently, activation of ASIC1a may exacerbate neuronal death during ischemia also by coupling to NMDA receptors [150]. These findings identify ASIC1a as an emerging non-classical excitotoxic therapeutic target for combating stroke [151].

In a mouse model for experimental autoimmune encephalomyelitis (EAE), ASIC1a mRNA is up-regulated and genetic disruption of ASIC1 or pharmacological inhibition by amiloride ameliorates disease severity and axonal loss in EAE mice [98]. Axonal degeneration may be initiated by activation of ASIC1a localized to dendrites or cell bodies or alternatively, ASIC1a may be abnormally distributed to demyelinated axons in inflamed EAE lesions [98].

In the PNS, ASIC1a is expressed together with other ASICs (ASIC1b, 2a, 2b, and 3) in sensory neurons of the dorsal root ganglion (DRG) and trigeminal ganglion (TG) [123, 152, 153]. Moreover, homomeric ASIC1a and heteromeric ASIC1a/2a are the predominant ASICs in neurons of the spinal dorsal horn [130, 154]. During inflammation, ASIC1a expression increases in the DRGs [123, 155] and the spinal horn [114, 154]. Increased ASIC1a expression most likely contributes to enhanced pain during inflammation [100, 156]. In agreement with this hypothesis, spinal infusion of PcTx1 or ASIC1a antisense oligonucleotides attenuated thermal and mechanical hypersensitivity [154], suggesting that ASIC1a contributes to central sensitization.

In summary, in the last decade we learned a lot about the roles of ASIC1a in the central nervous system and in neuropathological conditions. Many aspects of its action remain mysterious, however, suggesting that the study of ASIC1a will lead to further exciting discoveries in the future.

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