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International Union of Basic and Clinical Pharmacology. XCI. Structure, Function, and Pharmacology of Acid-Sensing Ion Channels and the Epithelial Na⁺ Channel

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ABBREVIATIONS: AA, arachidonic acid; ASDN, aldosterone-sensitive distal nephron; ASIC, acid-sensing ion channel; ASL, airway surface liquid; BASIC, bile acid–sensitive ion channel; BDNF, brain-derived neurotrophic factor; CAP, channel activating protease; cASIC1, chicken ASIC1; CCD, cortical collecting duct; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CNS, central nervous system; CNT, connecting tubule; DEG, degenerin; DRG, dorsal root ganglion; ENaC, epithelial Na⁺ channel; ET-1, endothelin-1; GAS, Gly-Ala-Ser; GMQ, 2-guanidine-4-methylquinazoline; KO, knockout; LTP, long-term potentiation; MitTx, Mit-toxin; MS, multiple sclerosis; NAc, nucleus accumbens; NSAIDs, nonsteroidal anti-inflammatory drugs; PcTx1, Psalmotoxin1; PDB, protein data bank; pH₅₀, pH of half-maximal activation; PHA-1, pseudohypoaldosteronism type 1; PICK1, protein interacting with C kinase; PKA, protein kinase A; PKC, protein kinase C; PNS, peripheral nervous system; S3969, *N*-(2-hydroxyethyl)-4-methyl-2-(4-methyl-1*H*-indol-3-ylthio)pentanamide; SSD, steady-state desensitization; TM, transmembrane segment; V2R, vasopressin receptor type 2; WIN55,212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone.

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Abstract—The epithelial Na⁺ channel (ENaC) and the acid-sensing ion channels (ASICs) form subfamilies within the ENaC/degenerin family of Na⁺ channels. ENaC mediates transepithelial Na⁺ transport, thereby contributing to Na⁺ homeostasis and the maintenance of blood pressure and the airway surface liquid level. ASICs are H⁺-activated channels found in central and peripheral neurons, where their activation induces neuronal depolarization. ASICs are involved in pain sensation, the expression of fear, and neurodegeneration after ischemia, making them potentially interesting drug targets. This review summarizes the biophysical properties, cellular

functions, and physiologic and pathologic roles of the ASIC and ENaC subfamilies. The analysis of the homologies between ENaC and ASICs and the relation between functional and structural information shows many parallels between these channels, suggesting that some mechanisms that control channel activity are shared between ASICs and ENaC. The available crystal structures and the discovery of animal toxins acting on ASICs provide a unique opportunity to address the molecular mechanisms of ENaC and ASIC function to identify novel strategies for the modulation of these channels by pharmacologic ligands.

I. Introduction

The epithelial Na⁺ channel (ENaC) and acid-sensing ion channels (ASICs) represent the mammalian subfamilies of the ENaC/degenerin (DEG) family of ion channels that was discovered at the beginning of the nineties. ENaC has a well established role in Na⁺ reabsorption in the distal nephron, in the distal colon, and in the control of the liquid film on airway epithelia. ENaC is inhibited by the drugs amiloride and triamterene that are clinically used as potassium-sparing diuretics. ASICs are neuronal channels that were discovered based on their primary structure homology to ENaC. Many of their physiologic and pathologic roles, which include pain sensation, synaptic plasticity, expression of fear, and neurodegeneration after ischemia, have been elucidated in genetically modified animal models during the last years and make them potentially interesting drug targets. Several animal toxins act on ASICs. Currently, there are however no ASIC drugs in clinical use. Crystal structures of ASICs, but not other ENaC/DEG family members, have been published. We review here the physiologic and pathologic roles of ENaC and ASICs, their structural organization, functional properties, and regulation. Functional and structural parallels and differences are discussed in the perspective of ENaC and ASICs as targets for pharmacological ligands. Other recent reviews cover relevant aspects of ASIC and ENaC function in more detail than this review: physiologic and pathologic roles of ASICs (Wemmie et al., 2013), regulation of ASIC function (Chu et al., 2011), physiology and regulation of ENaC (Palmer et al., 2012; Rossier, 2014), and ENaC's role in cystic fibrosis (Hobbs et al., 2013). Information on ASIC and ENaC properties and pharmacology can be found in the IUPHAR/BPS Guide to PHARMACOLOGY (www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=118; and

www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=122, respectively).

II. Phylogenetic and Sequence Comparison

The phylogenetic tree in Fig. 1 is based mainly on sequences of ENaC and ASICs. Because this review focuses on the mammalian ENaC and ASICs, the large *Drosophila* and *Caenorhabditis elegans* subfamilies each are represented by only one member, pickpocket and MEC-4, respectively. In addition, the peptide-gated FaNaC of *Helix aspersa* is included. The four ENaC subunits are encoded by the genes SCNN1A–D, whereas the ASIC subunits are encoded by ACCN1–4. The mammalian hiNaC (human intestine Na⁺ channel) and its rat/mouse ortholog BLINaC (brain-liver-intestine amiloride-sensitive Na⁺ channel), whose names refer to their main sites of expression, have recently been renamed to BASIC for “bile acid-sensitive ion channel” after the discovery of their activation by bile acids (Wiemuth et al., 2012; Lefevre et al., 2014). BASIC has a slightly higher sequence homology to ASICs than to ENaC, is encoded by ACCN5, and also has been called ASIC5 despite the fact that it is not activated by protons and shares only ~30% sequence homology with ASICs. ENaC and ASIC genes have in the meantime been cloned from many different species. For clarity we include in this phylogenetic tree mostly the human and rat sequences and, in addition, some fish ASIC sequences, which were used for functional studies, chicken ASIC1, from which the published crystal structures are derived, as well as *Xenopus laevis* ENaC sequences. ASIC genes have different splice variants, leading to currently at least 8 mammalian subunits, ASIC1a and -1b; ASIC2a and -2b; ASIC3a, -3b, and -3c (only in humans, in other organisms there is only one ASIC3), and ASIC4. Because of the cloning history, most ASICs originally had different names and were later renamed (Kellenberger

Because ASICs are permeable to Na^+ , their activation leads to membrane depolarization, thus inducing action potentials in neurons (Deval et al., 2003; Vukicevic and Kellenberger, 2004; Poirot et al., 2006) (Fig. 2, A and B). ASICs, therefore, often have an activating effect on neurons. A strong ASIC-mediated depolarization, however, can inhibit an already actively signaling neuron (Vukicevic and Kellenberger, 2004). ASIC1a has a small permeability for Ca^{2+} in addition to its Na^+ permeability, and some of its cellular functions, as for example neurodegeneration after ischemic stroke (Xiong et al., 2004), are thought to depend on a Ca^{2+} entry into the cell (Waldmann et al., 1997b; Bassler et al., 2001).

2. Physiologic and Pathologic Roles.

a. Synaptic plasticity and learning. Disruption of the gene encoding ASIC1a in mice eliminates most of the acid-induced currents in central neurons. Consistent with the presence of ASIC1a in the postsynaptic membrane, long-term potentiation (LTP) was impaired in whole animal ASIC1a knockout mice (Wemmie et al., 2002). If the extracellular Mg^{2+} concentration was kept low in these experiments, LTP was normal in ASIC1a knockout animals, suggesting that the ASIC-mediated postsynaptic depolarization may increase NMDA receptor function by removing the Mg^{2+} block. Another study inhibited ASIC1a with Psalmotoxin1 (PcTx1) or used a nervous system-specific ASIC1 knockout approach based on the Cre/Lox system with the Nestin promoter, which leads to the loss of ASIC1 expression at approximately embryonic day 10.5 and, consequently, to the absence of ASIC1a mRNA and ASIC1a currents in brain neurons (Wu et al., 2013). In contrast to previous work with ASIC1a^{-/-} mice,

A. Acid-Sensing Ion Channels

ASIC subunits are preferentially expressed in the nervous system. ASIC1a, ASIC2a, ASIC2b, and ASIC4 are found

Fig. 2. Cellular functions. (A) Scheme illustrating the function of ASIC in a neuron. Activation of ASICs in the neuron by protons leads to Na^+ entry, inducing a depolarization that leads to the generation of action potentials by activating voltage-gated Na^+ and K^+ channels. (B) Current-clamp experiment from a hippocampal neuron in which activation of ASICs by acidification to pH 6.6 induces a depolarization and a series of action potentials. (C) Scheme illustrating the vectorial transepithelial transport of Na^+ in a principal cell of the renal cortical collecting duct.

hippocampal LTP was normal in this study after pharmacologic blockade or knockout of ASIC1a. In an earlier study it was reported that block of ASICs by amiloride did not affect postsynaptic currents in cultured hippocampal neurons (Alvarez de la Rosa et al., 2003). The nervous system-specific ASIC1a knockout mice showed no deficit in spatial memory (Wu et al., 2013), in contrast to the whole-animal ASIC1a knockout mice in which a mild deficit in spatial memory and a decreased eye blink conditioning were observed (Wemmie et al., 2002). The opposite findings between these studies with different ASIC1a knockout mice may be due to the different knockout strategies resulting in deletion of ASIC1a at the very beginning (Wemmie et al., 2002) or during embryonic development (Wu et al., 2013) or to differences in animal strains or conditions of experiments, e.g., the absence of drugs in LTP measurements from whole-animal knockouts and the use of a GABA_A receptor inhibitor in the corresponding experiments from nervous system-specific ASIC1a knockouts.

Two recent studies investigated the contribution of ASICs to synaptic plasticity in different areas of the mouse brain and showed that in nucleus accumbens (NAc) core neurons and lateral amygdala pyramidal neurons, a small fraction (~5%) of the evoked excitatory postsynaptic current amplitude is due to ASIC activation (Du et al., 2014; Kreple et al., 2014). Presynaptic stimulation transiently decreased the pH at spines and neighboring dendrites of lateral amygdala pyramidal neurons, consistent with proton release from synaptic vesicles (Du et al., 2014). This study also showed that LTP in the lateral amygdala depended on the presence of ASICs and the increase in proton concentration.

The NAc is involved in addiction-related learning and behavior. Surprisingly, ASIC1a in the NAc was found to inhibit cocaine-induced synaptic plasticity and cocaine-conditioned behavior in rodents (Kreple et al., 2014). In a cocaine-conditioned place preference test, ASIC1a^{-/-} mice showed greater preference for the cocaine-associated context than did wild-type control mice. Rats that overexpressed ASIC1a in the NAc after local injection of a viral vector, self-administered less cocaine than did control rats. Investigation of the cellular mechanisms underlying this effect of ASIC1a identified a small contribution of ASICs to the excitatory postsynaptic current, as mentioned above. However, the analysis also showed that loss of ASIC1a increased the dendritic spine density and the frequency of miniature EPSCs in the NAc, consistent with increased excitatory transmission. Glutamate receptors of the NAc are important for addiction-related behavior, and differences in the functional profile of glutamate receptors have been observed between NAc neurons of cocaine-naïve and of cocaine-withdrawn animals. Interestingly, this study showed that NAc neurons of cocaine-naïve ASIC1a^{-/-} mice displayed the glutamate receptor profile of cocaine-withdrawn wild-type animals, suggesting therefore that ASICs, by influencing the glutamate receptor properties, may prevent cocaine-associated changes in the composition of NAc synapses (Kreple et al., 2014). Together, these studies suggest that ASICs contribute to synaptic plasticity and that, depending on the brain area, they either promote or inhibit plasticity.

b. Fear and anxiety. Because ASIC1a expression is particularly high in the amygdala, a possible role in fear-related behavior was investigated. This showed

that whole-animal ASIC1a knockout mice displayed deficits in cue and context fear conditioning as well as in unconditioned fear behaviors (Wemmie et al., 2003, 2013; Coryell et al., 2007). The disruption of ASIC1a also produced antidepressant-like effects in several behavioral tests carried out with mice (Coryell et al., 2009). Overexpression of ASIC1a increased fear-related behavior (Wemmie et al., 2004). It was shown that the amygdala senses acidic pH and thereby evokes fear behavior (Ziemann et al., 2009). It is known that an increase in CO₂ levels induces fear behavior in humans and has the same effect in mice. Inhibition or disruption of ASIC1a function impaired CO₂-induced fear behavior (Ziemann et al., 2009). Localized expression of ASIC1a in the amygdala of ASIC1a knockout mice rescued the fear phenotype, demonstrating that ASIC1a of the amygdala contributes importantly to fear behavior. As mentioned above, it was also shown that presynaptic stimulation lowers the amygdala pH and activates ASICs in lateral amygdala pyramidal neurons (Du et al., 2014). Intriguingly, a recent study with rats showed that activation of ASIC1a in the basolateral amygdala suppressed anxiety-like behavior (Pidoplichko et al., 2014). Activation of ASICs by injection of ammonium in the basolateral amygdala decreased the time spent in the center of the open field in the open field test, whereas inhibition of ASIC1a by PcTx1 had the opposite effect. Ammonium injection also prolonged the latency before the rats entered the dark compartment in the light/dark box test. ASIC currents were present in both principal cells and interneurons of the basolateral amygdala in the rat, and ASIC activation led to a higher increase in spontaneous inhibitory postsynaptic currents than EPSCs in principal cells, indicating that the inhibitory effects of ASICs on principal cell signaling prevailed (Pidoplichko et al., 2014). Because most results obtained with knockout mice were confirmed with pharmacologic approaches, the opposite results obtained in these studies cannot be due to a role of ASICs in development. It is therefore most likely that species differences are the cause of these contradictory results, thus that involvement of ASICs in the basolateral amygdala signaling may be different between mice and rats. Currently, behavioral data from rats on the role of ASICs in fear behavior is very limited and it will be necessary to extend such studies to cue and context fear conditioning and to CO₂-induced fear to determine how different the role of ASICs in fear behavior is between mice and rats. In humans, single nucleotide polymorphisms in the noncoding region of the ASIC1a gene were found to be associated with panic disorder, amygdala volume, and fear-related reactions (Smoller et al., 2014). It would be interesting to identify the ASIC function that is changed in these variants.

c. Pain sensation. In a number of conditions involving pain, such as inflammation and ischemia, the tissue pH is lowered. The extracellular pH drops for

example in cardiac ischemia to 6.7 (Cobbe and Poole-Wilson, 1980). ASICs in sensory neurons are therefore candidate receptors for acidification inducing pain. ASIC1a and ASIC2 are also expressed in pain-processing areas of the CNS where they may additionally contribute to pain sensation (Wemmie et al., 2013). Studies on human volunteers showed that local injection in the skin or iontophoresis of acidic solutions induced pain that was prevented by amiloride and displayed a pH of half-maximal effect of ~6.5, thus close to the pH of half-maximal activation (pH₅₀) of ASIC1a and ASIC3 (Ugawa et al., 2002; Jones et al., 2004). Several animal studies provided strong evidence for the role of peripheral ASIC3 and ASIC1 in pain sensation. Specific knockdown of ASIC3 with intrathecally administered siRNA in rats prevented inflammation-induced heat hyperalgesia or flinching after local injection of irritating substances (Deval et al., 2008). The synthetic compound 2-guanidine-4-methylquinazoline (GMQ) activates ASIC3 at physiologic pH and inhibits other ASICs (Yu et al., 2010; Alijevic and Kellenberger, 2012). Local injection of GMQ in the mouse paw induced pain behavior that depended on the presence of ASIC3, further confirming the importance of ASIC3 (Yu et al., 2010). A number of studies has demonstrated a contribution of ASIC3 to inflammation- and chronic acidification-related forms of pain (Sluka et al., 2009). Injection of the ASIC activator Mit-toxin (MitTx) of the Texas coral snake venom in the mouse paw induced pain behavior that was decreased by ASIC1a disruption (Bohlen et al., 2011). Recently, the ASIC-inhibitory toxin mambalgins-1 from black mamba venom was shown to reduce pain behavior after peripheral injection due to inhibition of ASIC1b (Diochot et al., 2012).

Intrathecal injection of mambalgins-1 diminished pain behaviors due to inhibition of ASICs containing the subunits ASIC1a and/or ASIC2a. Previous work had shown that the main functional ASICs in the spinal cord were ASIC1a homomers and ASIC1a/2a heteromers (Baron et al., 2008), and that ASIC RNAs in the spinal cord were upregulated by peripheral inflammation (Duan et al., 2007). Administration to the CNS of the spider toxin PcTx1, a specific inhibitor of ASIC1a, diminished pain behavior, further demonstrating the important role of central ASIC1a in pain sensation (Duan et al., 2007; Mazzuca et al., 2007). A recent study showed that ASIC1a in the spinal cord is the target of brain-derived neurotrophic factor (BDNF) (Duan et al., 2012). BDNF increased ASIC1a cell surface expression by inducing phosphorylation of the N-terminal residue Ser25 via the phosphoinositide 3-kinase–protein kinase B cascade. Intrathecal injection of BDNF induced mechanical hyperalgesia, which was prevented by ASIC1a disruption (Duan et al., 2012).

Migraine is associated with tissue acidification in the dura. Functional changes of several types of ion channels may contribute to an altered state of neuronal

excitability of trigeminal afferent neurons innervating the dura (Yan and Dussor, 2014). The majority of rat dural afferents display ASIC currents, which are likely mediated by ASIC3-containing channels (Yan et al., 2011). Application of pH 5 synthetic interstitial fluid to the dura of rats induced facial and hind paw allodynia. Facial allodynia is often associated with migraine (Yan et al., 2011). Amiloride has been reported to inhibit cortical spreading depression in rodents, and improved aura and headache severity in 4 of 7 patients of a small clinical trial (Holland et al., 2012).

Together, these studies provide clear indications for nociceptive roles of central and peripheral ASICs. Surprisingly, two studies showed that mice in which all ASIC currents were suppressed displayed increased pain behavior (Mogil et al., 2005; Kang et al., 2012). Mice overexpressing a dominant-negative mutant ASIC3 subunit were more sensitive than wild-type controls to mechanical pain and chemical/inflammatory pain and developed stronger mechanical hypersensitivity after inflammation (Mogil et al., 2005). Triple knockout mice, in which the ASIC1, ASIC2, and ASIC3 genes were simultaneously disrupted, showed an increased behavioral sensitivity to mechanical stimuli and an increased mechanosensitivity of A-mechanoreceptors (Kang et al., 2012). This indicates that the role of ASICs in nociception is still not fully understood despite many important findings.

d. Mechanosensation. Because related channels in *C. elegans* are involved in mechanosensation, a similar possible role of ASICs was investigated in mammals. Currently there is evidence of ASIC expression in primary sensory neurons and the afferents of mechanoreceptors, obtained either by retrograde tracing combined with functional analysis of isolated sensory neurons or by immunohistochemistry (reviewed in Chen and Wong, 2013). Disruption of the expression of different ASICs produced defects in mechanotransduction in tissues such as the skin, the stomach, the colon, the cochlea, and arterial baroreceptors (reviewed in Chen and Wong, 2013). These effects were most clearly demonstrated in the gastrointestinal tract and in arterial baroreceptors. Mechanotransduction in the gastrointestinal system is important for the control of gastric coordination and emptying, colonic motility, and the sensation of pain. Single-fiber recordings on an in vitro vagus-gastroesophageal or colon preparation showed in ASIC1^{-/-} mice enhanced, in ASIC2^{-/-} mice mixed, and in ASIC3^{-/-} mice decreased mechanosensitivity in visceral mechanoreceptors (Jones et al., 2005; Page et al., 2005). Analysis of the digestive function showed that that ASIC1^{-/-} mice had an alteration in upper gastrointestinal functions manifested as a slower gastric emptying, whereas ASIC2^{-/-} mice showed a decrease in the number of fecal pellets per day, indicating a changed lower bowel function (Page et al., 2005).

Aortic baroreceptor neurons in the nodose ganglia and their terminals express ASIC2. ASIC2^{-/-} mice showed hypertension, increased sympathetic and decreased parasympathetic control of the circulation, and a decreased gain of the baroreflex (Lu et al., 2009). The impairment of the baroreflex in ASIC2^{-/-} mice suggests that ASIC2 is a determinant of the sensitivity of arterial baroreceptor and contributes to the autonomic control of the circulation.

So far it has not been possible to demonstrate mechanosensitivity of recombinantly expressed ASICs or of ASICs expressed in dorsal root ganglion (DRG) neurons (Drew et al., 2004). Therefore it is possible that ASICs need to form complexes with other proteins to be able to act as mechanosensors.

e. Neurodegenerative diseases. Calcium entry into neurons is thought to be the most important cause of neuronal injury after ischemic stroke. Because ischemic stroke induces a tissue acidification and ASIC1a is partially permeable to Ca²⁺, a possible contribution of ASIC1a to this toxicity was investigated. Disruption of the ASIC1a gene or inhibition of ASIC1a by PcTx1-containing spider venom reduced the infarct volume in an experimental stroke model by >60%, thus demonstrating an important contribution of ASIC1a to ischemic stroke-induced neurodegeneration (Xiong et al., 2004). Intracerebroventricular administration of PcTx1 up to 5 hours after transient middle cerebral artery occlusion reduced the infarct volume by >50% (Pignataro et al., 2007). Subsequent studies showed a protective effect of ASIC gene disruption or inhibition of ASIC function in a number of neurodegenerative diseases, including multiple sclerosis (MS), Huntington's, and Parkinson's disease (reviewed in Wemmie et al., 2013). The disruption of the ASIC1a gene reduced the clinical deficit and axonal degeneration in experimental autoimmune encephalomyelitis (Friesse et al., 2007). It was further shown that ASIC1a is upregulated in lesions from experimental autoimmune encephalomyelitis in mice and from MS patients (Vergo et al., 2011). Treatment with amiloride was neuroprotective in animal models and in human MS patients (Vergo et al., 2011; Arun et al., 2013). Interestingly, an association between the ASIC2 gene and MS has been shown in a remote human population in Sardinia (Bernardinelli et al., 2007).

f. Epileptic seizures. During epileptic seizures the brain pH is lowered, and it is known that acidosis can end seizures. Disruption of the ASIC1a gene increased the severity of seizures, and the opposite effect was observed with overexpression of ASIC1a (Ziemann et al., 2008). This study showed that ASIC1a expression is required for seizure termination by CO₂ inhalation. Because inhibitory interneurons in hippocampus display higher ASIC current amplitudes than pyramidal neurons it is possible that the interneuron-mediated inhibition ended the seizures (Ziemann et al., 2008). Observations of seizure inhibition by high concentrations

of amiloride (Ali et al., 2006; N'Gouemo, 2008; Luszczyk et al., 2009) appear to contradict the role of ASIC1a in seizure termination. Because of its hydrophilic nature, amiloride is expected not to cross the blood-brain barrier, and it is currently not clear whether it reaches the brain or whether it exerts anti-seizure effects by other mechanisms. Amiloride is not specific for ASICs, and if it reaches the brain its effect on seizures may well be due to inhibition of the Na^+/H^+ exchanger, which, by lowering the intracellular pH, may decrease epileptic activity (Ali et al., 2006; Luszczyk et al., 2009). Recently, a genetic study indicated an association between single nucleotide polymorphisms of ASIC1 and temporal lobe epilepsy (Lv et al., 2011). Whether these polymorphisms affected the expression and function of ASIC1a, however, were not investigated.

B. Epithelial Na^+ Channel

1. Function. According to the Koefoed-Johnsen-Ussing model of Na^+ absorbing epithelia, Na^+ enters the epithelial cell by facilitated diffusion across the apical membrane through a Na^+ -selective, amiloride-sensitive channel (Koefoed-Johnsen and Ussing, 1958; Palmer and Andersen, 2008) (Fig. 2C). This Na^+ entry across the apical membrane occurs along a favorable electrochemical gradient. The epithelial Na^+ channel ENaC was first identified by patch-clamp in the apical membrane of the collecting ducts from rat kidney (Palmer and Frindt, 1986). This epithelial Na^+ channel is spontaneously open at the cell surface and is characterized by a small conductance (5 pS at physiologic Na^+ concentrations), a half-saturation of ion conductance obtained at 70 mM Na^+ , and a selectivity for Na^+ over K^+ ions of >100 .

The primary structure of ENaC was solved by cloning of three homologous ENaC subunits, α , β and γ , that form the functional channel (Canessa et al., 1993, 1994b). Coexpression of the three $\alpha\beta\gamma$ ENaC subunits is required for maximal expression of ENaC-mediated Na^+ current (Firsov et al., 1996) and to reproduce the biophysical properties observed in the epithelial Na^+ channel of the cortical collecting duct (CCD). The three ENaC subunits α , β and γ are expressed and are detected at the protein level in the kidney, the lung, the colon, and in salivary glands. In skin, blood vessels, the eye, and heart, the presence of ENaC mRNAs was also reported (Mauro et al., 2002; Drummond et al., 2004; Krueger et al., 2012). The functional role of ENaC in these tissues is still a matter of debate, and no clear phenotype is associated with the loss of function of ENaC in these tissues in humans (Riepe, 2009).

In addition to the well characterized $\alpha\beta\gamma$ ENaC subunits, a δ subunit has been identified by cloning from a human kidney cDNA library (Waldmann et al., 1995). The δ subunit shows highest transcriptional expression levels in the ovaries, testis, pancreas, and brain. In heterologous cell expression systems, the δ subunit can

functionally substitute for the α subunit (Waldmann et al., 1995). The δ subunit has only been identified in humans, and nothing is known about its physiologic/pathologic role.

2. Kidney. Most of the filtered Na^+ (90%) is reabsorbed in the proximal tubule and the thick ascending limb of the nephron by antiporters or cotransporters, and only 3–5% of the filtered load of Na^+ is reabsorbed in the distal part. The ENaC subunit proteins are located at the apical membrane of principal cells lining the distal end of the distal convoluted tubule DCT2, the connecting tubule (CNT), and the collecting duct (Loffing et al., 2000a). This correlates with the presence of active ENaC channel detected by electrophysiologic techniques *ex vivo* in the CNT and CCD (Frindt and Palmer, 2004). The distal nephron segment is generally called the aldosterone-sensitive distal nephron (ASDN) because of the coexpression of ENaC channels with the mineralocorticoid receptor in this tubule segment (Malsure et al., 2014).

In the distal nephron and the renal collecting duct, ENaC is the only Na^+ -selective channel present at the apical membrane of the principal cells and constitutes the rate-limiting step for Na^+ reabsorption. The ENaC-mediated Na^+ absorption is under tight hormonal control to precisely match the amount of Na^+ excreted in the urine to the daily Na^+ intake to maintain a Na^+ balance. In principal cells, ENaC is coexpressed at the apical membrane with K^+ -selective channels named rat outer medulla K^+ (ROMK) channel (Palmer and Frindt, 2007) (Fig. 2C). ENaC and ROMK are electrically coupled, and when ENaC-mediated Na^+ absorption increases, the membrane depolarization provides an increase in electrical driving force for K^+ secretion across the apical membrane and an increase in renal excretion of K^+ .

The role of ENaC in the maintenance of Na^+ and K^+ homeostasis is best illustrated by two genetic syndromes associated with mutations in the ENaC genes leading to either a loss or a gain of function of the channel. Pseudohypoaldosteronism type-1 (PHA-1) is a rare disease of mineralocorticoid resistance associating hyponatremia, severe hyperkalemia, and metabolic acidosis with high levels of plasma aldosterone. Two forms of PHA-1 have been identified: an autosomal dominant form with usually mild symptoms restricted to the kidney that is associated with heterozygous mutations in the NR3C2 gene encoding for the mineralocorticoid receptor (Chang et al., 1996; Geller et al., 1998), and the generalized PHA-1 form that is also called autosomal recessive PHA-1. This second form of PHA-1 is a multisystem disorder characterized by salt wasting from the kidney, the colon, the sweat glands, and a reduced capacity to reabsorb Na^+ in the airways, leading to rhinorrhea, pulmonary congestion, and recurrent pulmonary infections. Mutations have been identified in the SCNN1A, SCNN1B, and SCNN1G

genes encoding the α , β , and γ ENaC subunits, leading to loss of function of ENaC (Chang et al., 1996). The mirror image of PHA-1 is Liddle syndrome (or pseudoaldosteronism), an autosomal dominant form of salt-sensitive hypertension associated with low plasma aldosterone, low plasma renin activity, hypokalemia, and metabolic alkalosis. In the early sixties, G. W. Liddle reported a case of pseudoaldosteronism and described the syndrome as “a disorder in which the renal tubules transport ions with such abnormal facility that the end result simulates that of a mineralocorticoid excess” (Liddle and Coppage, 1963). Blood pressure in these patients could be normalized with amiloride and dietary salt restriction, but the mineralocorticoid receptor antagonist spironolactone was not effective. The genetic defects associated with this syndrome are mutations in the last exon of the SCNN1B and SCNN1G genes encoding the β and γ ENaC subunits that delete a conserved proline-rich motif in the cytosolic C terminus (Shimkets et al., 1994). In vitro experiments could establish that the mutations associated with this syndrome are gain-of-function mutations, as postulated by G. W. Liddle, leading to an increase in the abundance and activity of ENaC at the cell surface (Schild et al., 1995; Firsov et al., 1996). The molecular mechanism leading to ENaC gain of function in Liddle syndrome is now well established. The proline-rich PPPxY motif in the C terminus of ENaC β and γ subunits that is mutated in this syndrome is involved in specific interactions with cytosolic proteins, such as the Nedd4-2 ubiquitin ligase, that tightly control the channel density at the cell surface (Rotin, 2000; Staub et al., 2000). The ENaC mutants lacking this proline-rich motif show a defect in channel internalization, leading to retention of active channels at the cell surface (Debonneville et al., 2001).

Genetic mouse models targeting ENaC genes have been generated, reproducing, completely or partially, the clinical features of these syndromes (Hummler et al., 1997; Pradervand et al., 2003). In addition, targeting the ENaC gene knockout (KO) to specific nephron segments revealed that the expression of ENaC in the collecting duct is not necessary for the maintenance of Na^+ and K^+ balance, suggesting that ENaC in more proximal parts of the nephron such as the CNT likely plays the most important role in this process (Rubera et al., 2003; Christensen et al., 2010).

3. Lungs. Airway epithelial cells reabsorb and secrete fluid to maintain an appropriate airway surface liquid (ASL) level and mucociliary clearance. Liquid movements across the airway epithelium depend on transepithelial ion transport, and the coordination of ENaC-mediated Na^+ absorption with Cl^- secretion is critical for the maintenance of ASL (Goralski et al., 2010). In the lung, ENaC subunits were detected at the mRNA level in the medium and small airways and in alveolar type II cells (Burch et al., 1995). α ENaC KO mice die shortly after birth, due at least in part to

a massive lung edema at birth, leading to a respiratory distress syndrome (Hummler et al., 1996). In contrast, an increase in Na^+ absorption by ENaC overexpression in a genetic mouse model leads to a cystic fibrosis lung phenotype (Mall et al., 2010). In airway epithelia of cystic fibrosis patients, the loss of cystic fibrosis transmembrane conductance regulator (CFTR) activity is accompanied by an increased ENaC activity resulting in Na^+ hyperabsorption, suggesting that ENaC upregulation may contribute to the cystic fibrosis lung phenotype (Mall et al., 2010; Hobbs et al., 2013). A cystic fibrosis-like lung phenotype, however, has not been consistently observed in Liddle patients. The ENaC activity is coupled to CFTR function in human sweat ducts, where it increases with CFTR activity; in sweat ducts of cystic fibrosis patients, the loss of CFTR activity is accompanied by reduced ENaC activity (Reddy et al., 1999).

4. Gastrointestinal Tract. As in the kidney, most of the Na^+ in the lumen of the gut is reabsorbed by electroneutral transporters. An electrogenic Na^+ reabsorption mediated by ENaC is restricted to the colon and the rectum. ENaC in the distal colon is under mineralocorticoid and glucocorticoid control: both aldosterone and dexamethasone control the abundance of β and γ but not α mRNA transcripts (Epple et al., 2000; Fuller et al., 2000). Mice lacking the α ENaC subunit in colonic superficial cells (SCNN1A-KO) are viable without perinatal lethality, in contrast to mice lacking α ENaC in the kidney, but show salt loss and mineralocorticoid resistance, indicating that Na^+ absorption in the kidney can compensate for the absence of ENaC-mediated Na^+ absorption in the colon (Malsure et al., 2014).

5. Sodium Sensor. Mammals perceive five different qualities of taste, bitter, sweet, umami, sour, and salty. Salt taste represents a warning signal to prevent consumption of hypersaline fluid or food and is also important to satisfy our salt appetite. The balance between these two antagonistic signals likely influences our daily sodium consumption, which is a major risk factor for developing hypertension. Different mechanisms are involved in salty taste sensation. One sodium-sensing system activated at high sodium concentrations (500 mM) is not specific for Na^+ and responds to a variety of salts. Another sensing system is activated by lower concentrations of Na^+ and inhibited by amiloride, suggesting the contribution of ENaC in salt taste. In humans, α , β , γ , and δ ENaC subunits are expressed in taste bud cells (Stahler et al., 2008). In mice lacking α ENaC in taste receptor cells, the highly sensitive response to Na^+ salt and the behavioral attraction to sodium salt were considerably reduced (Chandrashekar et al., 2010). These observations support an important physiologic role of ENaC in salt taste perception. Taste response to sodium is common among terrestrial vertebrates but is differentially sensitive to amiloride. In humans, for instance, the perception of saltiness to NaCl

or LiCl remained essentially unchanged by amiloride at concentrations up to 100 μM (see for review, Halpern, 1998). These observations are consistent with the contribution of different salt-sensing systems that may vary among vertebrates and possibly be dependent on factors such as the environment or the genetic background.

IV. Structural Aspects

A. Primary Structure and Subunit Topology

1. Subunit Organization of Epithelial Na^+ Channel and Acid-Sensing Ion Channels. All DEG/ENaC family members share a common membrane topology. ASIC and ENaC subunits are composed of two transmembrane segments, intracellular N and C termini, and a large extracellular loop that represents more than half of the mass of the channel protein (Canessa et al., 1994a) (Fig. 3A). Protease digestion and glycosylation site analysis of ENaC showed the presence of a large extracellular loop, comprising ~ 50 kDa, between the two transmembrane domains. It also indicated that the N and C termini are intracellular (Canessa et al., 1994a; Renard et al., 1994; Snyder et al., 1994). This subunit topology was confirmed in ASIC2a by analyzing engineered glycosylation sites and epitope accessibility to antibodies (Saugstad et al., 2004). The large extracellular domain has essential roles in the regulation and control of activity of ENaC and ASICs. It contains H^+ -sensing residues and is involved in the activation of ASICs. Its specific role in supporting Na^+ ion flow through the ENaC pore, however, remains unclear. The membrane topology was confirmed by the three-dimensional structures of chicken ASIC1 (cASIC1), the only ENaC/DEG family member that has successfully been crystallized so far.

The different crystal structures of cASIC1 show a channel composed of three subunits arranged around a central ion pore (Fig. 3A). In these structures, the intracellular channel domains are either truncated or, when present, not resolved (Table 1). The domain

arrangement of ASIC subunits has some resemblance with an upright forearm and a clenched hand. The forearm would correspond to the transmembrane part, the hand to the extracellular channel part, and the wrist would form the transmembrane-extracellular domain junction. Accordingly, the different extracellular domains have been labeled palm, knuckle, finger, β -ball, and thumb (Jasti et al., 2007) (Fig. 3, A and B).

The transmembrane helices are connected to β strands, forming the palm domain, that is arranged along the central vertical axis of the channel and constitutes the core of the extracellular domain (Jasti et al., 2007; Gonzales et al., 2009; Bacongus and Gouaux, 2012; Bacongus et al., 2014; Dawson et al., 2012). The knuckle and β -ball are located close to the central vertical axis at the upper end of the palm, while the finger and thumb are oriented toward the outside of the protein. The finger forms the upper external edge, whereas the thumb, a rigid structure formed by two α helices and stabilized by several disulfide bonds, is located below the finger and is connected by two loop structures to the lower part of the palm. One of these loops interacts with the top end of the first transmembrane segment via a structural element that has been named β -turn (Fig. 3A). The channel further contains four vestibules or cavities that are all aligned along the central vertical axis. From top to bottom these are the upper vestibule, the central vestibule, which is enclosed by the lower palm, the extracellular vestibule located just above the extracellular pore entry and finally the intracellular vestibule. Remarkably, the surfaces of the central and extracellular vestibules are electronegative, therefore attracting cations (Jasti et al., 2007; Gonzales et al., 2009).

2. Origin and Experimental Conditions of Different Crystal Structures. All currently published ASIC structures were obtained from the chicken ASIC1, which shares $\sim 90\%$ sequence identity with human and rat ASIC1a. The conditions of the different ASIC

Fig. 3. ASIC structure and ENaC model. (A) The different subdomains transmembrane segments (pink), palm (yellow), thumb (blue), finger (purple), β -ball (orange), and knuckle (turquoise) are indicated in one of the three ASIC subunits in the crystal structure obtained in complex with MitTx (Bacongus et al., 2014). The acidic pocket and the β -turn, as well as the four vestibules are indicated. (B) Schematic view of one ASIC subunit with indication of the domains in the same colors as in (A). (C) Structural alignment of an α ENaC model (turquoise) (Kashlan et al., 2011) and the cASIC1 desensitized structure 2QTS (yellow) (Jasti et al., 2007). All structural images were made with Chimera (Pettersen et al., 2004).

TABLE 1
ASIC structures

All crystal structures were obtained from chicken ASIC1. The structures **2QTS** and **3HGC** (**4NYK**) likely represent the desensitized state, whereas the other structures represent the open state. **2QTS**, **3S3W**, **4FZ0**, and **4FZ1** display a continuous TM2 segment, whereas in **4NYK** (reinterpretation of **3HGC**) and **4NTW** the TM2 is split in two parts by the selectivity filter GAS motif.

PDB ID	Construct	Complex with	pH	Resolution Å	Symmetry	Function	Reference
2QTS	N and C terminus truncated	—	5.6	1.9	~3-fold, except in TM part	Nonfunctional	Jasti et al., 2007
3HGC^a (4NYK)	C terminus truncated	—	6.5	3.0	3-fold	Functional	Gonzales et al., 2009
3S3X	N and C terminus truncated	PcTx1	5.5	3.0	~3-fold, except in TM part	Nonfunctional	Dawson et al., 2012
4FZ0	Truncation of 13 N-terminal and 63 C-terminal residues	PcTx1	5.5	2.8	~3-fold, except in TM part	Functional, Na ⁺ -selective and amiloride-inhibited at pH 5.5	Baconguis and Gouaux, 2012
4FZ1	“	PcTx1	7.25	3.3	3-fold	Functional, nonselective, and amiloride-resistant at pH 7.25	Baconguis and Gouaux, 2012
4NTW^b	“	MitTx	5.5	2.1	3-fold	Functional, Na ⁺ -selective, amiloride-inhibited	Baconguis et al., 2014

^a Electron density of this crystal was reinterpreted in the transmembrane segment, available under PDB ID **4NYK**; a structure was also obtained from these crystals soaked in Cs⁺.

^b Structures from this crystal were also obtained soaked in Cs⁺ (2.6 Å, **4NTY**) or in amiloride (2.3 Å, **4NTX**).

structures presently available are summarized in Table 1. The first published crystal ASIC structure and one of the PcTx1-cASIC1 structures were obtained from a truncated, nonfunctional construct (Jasti et al., 2007; Dawson et al., 2012). The other crystal structures were obtained from functional but still truncated constructs. ASICs exist in three functional states: closed, open, and desensitized. Two structures were obtained at acidic pH in the absence of gating modifiers (Protein Data Bank [PDB] ID **2QTS** and **3HGC/4NYK**) and their transmembrane region did not contain a continuous pore that would allow ion transport. Therefore, it was concluded that these structures likely represent the desensitized conformation of ASIC1 (Jasti et al., 2007; Gonzales et al., 2009). Recently, crystal structures of cASIC1 in complex with the gating modifiers PcTx1 or MitTx were published (Baconguis and Gouaux, 2012; Dawson et al., 2012; Baconguis et al., 2014). MitTx is an ASIC activator and PcTx1 had been known to be a gating modifier that shifts the pH dependence of activation and desensitization to more alkaline values, thereby inhibiting human and rat ASIC1a. However, PcTx1 opens cASIC1 at pH 7.4 and rat ASIC1b at slightly acidic pH (see section VI) (Chen et al., 2005, 2006; Samways et al., 2009). Both functional cASIC1-toxin complexes showed an incomplete desensitization at the pH used for crystallization. In contrast to the previous crystals, all toxin-bound cASIC1 structures contained a continuous open pore and therefore likely represent the channel in an open conformation. The cASIC1 crystal structures with the highest resolution were the first, putatively desensitized structure obtained at 1.9 Å and the open cASIC1-MitTx structure, resolved at 2.1 Å (Jasti et al., 2007; Baconguis et al., 2014). All structures showed generally a 3-fold symmetry except for the transmembrane domain, which was asymmetric in the first desensitized structure (**2QTS**) and the two cASIC1-PcTx1 structures obtained at pH 5.5 (**3X3X** and **4FZ0**).

3. Differences between the Open and the Desensitized Structure and between Different Published Structures. Comparison of the open PcTx1- and MitTx-complex structures with that of the desensitized state (PDB ID **4NYK**) showed no difference in conformation of the knuckle and upper palm domains, suggesting that these domains may act as a scaffold for the movement of other channel domains. Compared with the desensitized conformation, the lower palm and wrist of the open conformations undergo a rotation that increases the distance between the thumb and the palm domain of adjacent subunits, thus widening the central vestibule. In open ASIC structures the extracellular vestibule becomes wider than it is in the desensitized structures, and the “desensitization gate” forming an ~8 Å-thick occlusion in the upper half of the transmembrane part opens up (Baconguis and Gouaux, 2012; Grunder and Augustinowski, 2012; Baconguis et al., 2014).

The conformation of the ectodomain is the same in the two desensitized structures (**2QTS**, **3HGC**) and is similar between the open structures. In the recent cASIC1-MitTx structure obtained at high resolution, the transmembrane (TM)2 segment is separated into two parts at the level of the selectivity filter sequence Gly-Ala-Ser, which adapts a horizontal, extended conformation. Reinterpretation of the desensitized structure **3HGC** showed a similar break in helical structure. In contrast, the first desensitized structure (**2QTS**) and the cASIC1-PcTx1 complexes have continuous TM2 helices. It is currently not clear which of these pore structures is more realistic, although the cASIC1-MitTx structure may be favored because it has a higher resolution, shows a 3-fold symmetry, and seems to be compatible with known ASIC functions.

4. Difference and Similarities between the Acid-Sensing Ion Channels and Epithelial Na⁺ Channel Subunit Organization. In the extracellular domain, ENaC and ASIC1 show the highest sequence homology

in the palm and the β -ball (~35%). Although the sequence homology is lower in the peripheral domains finger, thumb, and knuckle, there is evidence for structural homology in most of these domains (Kashlan et al., 2011; Kashlan and Kleyman, 2011) (Fig. 3C). The predicted secondary structure of the ENaC thumb matches the crystal ASIC structure (although the $\alpha 5$ helices do not align well), and the Cys residues forming the 5 disulfide bonds of the thumb are conserved. The predicted secondary structure of the knuckle matches the ASIC structure moderately well. ENaC and ASIC sequences share the lowest homology in the finger domain. In addition, ENaC contains an ~80-residue insertion in this domain. The low homology between ENaC and ASIC finger domains may be at the origin of the different activation mechanisms of these two channels. The transmembrane segments TM1 and TM2 share ~20 and 40% homology, respectively, between ASIC and ENaC. A model of α ENaC has been constructed that, for the most part, is based on the cASIC1 structure 2QTS and in which the finger was modeled based on constraints derived from functional data and from secondary structure predictions (Kashlan et al., 2011) (Fig. 3C).

B. Subunit Stoichiometry

All published cASIC1 structures show the channel as a trimer (Jasti et al., 2007; Gonzales et al., 2009; Bacongus and Gouaux, 2012; Dawson et al., 2012; Bacongus et al., 2014). Recently, the counting of bleaching steps of fluorescently tagged mammalian ASIC1a and ASIC2a channels at the plasma membrane of *Xenopus* oocytes indicated that functional ASICs in a cellular environment are also trimers (Bartoi et al., 2014).

The subunit stoichiometry of the functional ENaC channel is still controversial. The total number of subunits that form the functional $\alpha\beta\gamma$ ENaC complex as reported in the literature varies from 3 to 9 subunits (Firsov et al., 1998; Kosari et al., 1998; Eskandari et al., 1999; Dijkink et al., 2002; Anantharam and Palmer, 2007; Carnally et al., 2008). This likely reflects methodological problems in evaluating the subunit stoichiometry of a channel as well as uncertainties in the interpretation of the experimental data. However, before the crystallization of ASIC1, a general consensus based on functional and biochemical observations from different laboratories agreed on a tetrameric subunit oligomerization of ENaC involving two α subunits, and one β and one γ subunit. Data leading to this finding include the quantification of the relative abundance of ENaC subunits at the cell surface per functional channel, the dominant or recessive effects of mutations in a single subunit on channel block by ligands or on channel conductance, functional analysis of ENaC subunit concatemers, and biochemical determination of the molecular mass of the ENaC channel complex (Firsov et al., 1998; Kosari et al., 1998; Dijkink et al., 2002; Anantharam and Palmer, 2007).

After the release of the three-dimensional structure of ASIC1, the ENaC/DEG field seems to have settled that ENaC is a trimer, a dogma that still needs solid confirmation by experimental evidence.

V. Channel Function and Regulation

A. Acid-Sensing Ion Channels

1. Channel Gating and Ion Selectivity. For a long time, protons were the only known activators of ASICs. Recently it was shown, however, that the synthetic compound GMQ activates ASIC3 at pH 7.4 (Yu et al., 2010). The spider toxin PcTx1 shifts ASIC pH dependencies, inhibiting ASIC1a under physiologic conditions and activating cASIC1 and rat ASIC1b at pH 7.4 and slightly acidic pH, respectively (Chen et al., 2005, 2006; Samways et al., 2009; Bacongus and Gouaux, 2012). The recently identified MitTx activates ASICs by a currently unknown mechanism (Bohlen et al., 2011). GMQ and the toxins are further discussed below (section VI). Currently it seems that protons are the only physiologic activators of ASICs.

a. Current kinetics. Acid-induced opening of homomultimeric ASICs is under most conditions transient, because it is rapidly followed by desensitization (Waldmann et al., 1997b) (Fig. 4A). This can be described by a kinetic model containing a closed, an open, and a desensitized state (Fig. 4B). Time constants of channel opening kinetics of 10 milliseconds or less have been measured for ASIC1a, ASIC1b, and ASIC3 under very rapid solution change conditions at pH ≤ 6 (Bassler et al., 2001; Sutherland et al., 2001; Li et al., 2010b). In most of the published functional ASIC studies, the observed kinetics of channel opening are probably limited by the speed of the solution change. Desensitization is fastest in ASIC3 and slowest in ASIC2a (Fig. 4A; Table 2). ASIC3 activation by pH ~ 7 and by pH < 5 induces a sustained current component in addition to the transient current (Waldmann et al., 1997a; Yagi et al., 2006). Sustained currents were also observed with ASIC heteromers (Lingueglia et al., 1997; Benson et al., 2002). They may mediate ASIC function in slow and long-lasting pH changes. Human ASIC3 is not only activated by extracellular acidification. Changes to a pH more alkaline than the physiologic values can induce a sustained ASIC3 current (Delaunay et al., 2012).

b. Ion selectivity. Transient ASIC currents are Na⁺ selective with a Na⁺/K⁺ permeability ratio of ~ 10 (Table 2) (Kellenberger and Schild, 2002). Homomeric ASIC1a and heteromeric ASIC1a/2b have in addition a low permeability to Ca²⁺ (Na⁺/Ca²⁺ permeability ratio between 1.8 and 18.5, depending on study), which is likely important for some of their physiologic and pathologic roles (Waldmann et al., 1997b; Bassler et al., 2001; Samways et al., 2009; Sherwood et al., 2011). Although ASICs are also permeable to protons with an estimated H⁺/Na⁺ permeability ratio of < 5 for

ASIC1a (Chen and Grunder, 2007), the contribution of H^+ to the ASIC current amplitude, under most conditions, is negligible because the H^+ concentration is orders of magnitude lower than the Na^+ concentration.

c. pH dependence. From pH-response curves of ASIC currents a pH_{50} can be determined (Fig. 4C, shown for ASIC1a). pH_{50} values of ~ 6.5 have been measured for ASIC1a and ASIC3, indicating that these channels are activated by acidification to values just below pH 7. ASIC1b has a pH_{50} of ~ 6.1 , whereas ASIC2a, with a pH_{50} of ~ 4.5 , requires much higher proton concentrations for activation (Table 2). If ASICs are exposed to a moderately acidic pH they can desensitize without apparent opening, thus passing directly from the closed to the desensitized state (Waldmann et al., 1997b). This process that is analogous to closed-state inactivation of voltage-gated Na^+ channels is called steady-state desensitization (SSD). The steep part of its pH dependence is found for ASIC1a, -1b, and -3 at slightly more acidic pH than physiologic values (Fig. 4C; Table 2). Changes in this pH dependence or in the basal pH can therefore affect the availability of ASICs for opening. A small overlap of the activation and the SSD

curves in ASIC3 is at the origin of the small sustained current at pH 7 (Yagi et al., 2006). ASIC activity is also modulated by intracellular pH. It was shown that the amplitude of ASIC currents in mouse cortical neurons was decreased by intracellular acidification due to an alkaline shift of the pH dependence of SSD and an acidic shift of the pH dependence of activation (Wang et al., 2006).

d. Coupling of activation and steady-state desensitization. Mutations affecting the pH dependence of activation change in many cases the pH dependence of SSD as well (Sherwood and Askwith, 2008; Bargeton and Kellenberger, 2010; Liechti et al., 2010), indicating that activation and desensitization are structurally coupled. Further support for a coupling of these two transitions comes from the observations that changes in the extracellular Ca^{2+} concentration, as well as application of PcTx1 or cleavage by proteases shift the pH dependence of both activation and SSD in ASIC1a (Babini et al., 2002; Poirrot et al., 2004; Chen et al., 2006).

2. Regulation. ASIC function is regulated by many different modulators such as Ca^{2+} , Zn^{2+} , redox reagents, free radicals, arachidonic acid (AA), kinases, and proteases, which are likely physiologically relevant.

Fig. 4. Biophysical properties of ENaC and ASICs. (A) Representative current traces of ASIC1a, ASIC2a, and ASIC3 induced by extracellular acidification as indicated (whole-cell patch-clamp at -60 mV of CHO cells expressing the indicated ASIC subtype). (B) Gating scheme illustrating the simplest kinetic model of ASIC gating, containing a closed, an open, and a desensitized state. (C) SSD and activation curve of ASIC1a. For the SSD curve, cells were exposed for 60 seconds to the indicated conditioning pH before the fraction of nondesensitized channels was measured by a 5-second acidification to pH 6. This response was normalized to that obtained with a conditioning pH 7.6, at which no desensitization occurs. For the activation curve, the normalized current amplitude is plotted as a function of the stimulation pH. (D) Illustration of a macroscopic ENaC current, appearing when the inhibitor amiloride is removed. The experiment was done at -80 mV from $\alpha\beta\gamma$ ENaC-expressing *Xenopus* oocytes, the amiloride concentration was 10μ M. (E) Top, ENaC single-channel current, obtained from outside-out patches from *Xenopus* oocytes at 100 mM LiCl and -130 mV. Bottom, gating scheme of ENaC.

TABLE 2
ASIC and ENaC properties

Data are from many different studies and reviews (Sutherland et al., 2001; Hesselager et al., 2004; Poirot et al., 2004; Blanchard and Kellenberger, 2011; Alijevic and Kellenberger, 2012; Wemmie et al., 2013). pH dependence and kinetics of ASICs vary between subtypes and also between orthologs of the same subtypes. Typical values are indicated here. For more information, see the relevant chapters.

	IC ₅₀ Amiloride Inhibition ^a	Na ⁺ /K ⁺ Permeability Ratio	pH ₅₀ Activation	pH ₅₀ Steady-State Desensitization	Desensitization Time Constant (at pH Close to pH ₅₀)	Most Important Sites of Expression
	μM				s	
ASIC1a	10	6–13	6.2–6.6	~7.2	~0.4	CNS, PNS
ASIC1b	21	~3	5.9–6.3	~6.7	~0.9	PNS
ASIC2a	28	~10	4.0–4.9	~5.6	~1.4	CNS, PNS
ASIC3	60–100	~12	6.4–6.7	~7.1	~0.3	PNS
ENaC $\alpha\beta\gamma$	0.1	≥100	N.A.	N.A.	N.A.	Kidney, colon, lung

N.A., not applicable; pH₅₀, pH of half-maximal desensitization.

^aFor transient ASIC current component.

We discuss here the most important modulators of ASIC function and provide an overview in Tables 3 and 4. For a more exhaustive discussion of ASIC modulation see also Chu et al. (2011) and Wemmie et al. (2013).

a. Regulation by protein-protein interactions. As many other membrane proteins, ASICs interact with other proteins, some of which have been identified in the last years, as shown in Table 3 (Wemmie et al., 2006). ASICs were shown to interact mostly with scaffolding proteins and with other ion channels. PDZ binding domains at the cytoplasmic C terminus were involved in many of these interactions. In many cases, these interactions affect the cell surface expression or localization of ASICs, their regulation, or their gating. Currently, most of these interactions have been shown in cellular systems and their in vivo relevance is not known.

b. Regulation by ions, small molecules, and proteins.

i. Calcium and Other Divalent or Polyvalent Cations.

The extracellular Ca²⁺ concentration affects ASIC pH dependence in a way that suggests a competition between Ca²⁺ and H⁺ for a common binding site. It has been shown that increasing the extracellular Ca²⁺ concentration in the range of low micromolars to 10 mM shifts the pH dependence of activation to more acidic values in ASIC1a and ASIC3 (Babini et al., 2002; Immke and McCleskey, 2003). A similar Ca²⁺-dependent shift has also been shown for the pH dependence of SSD in ASIC1a and ASIC1b, and other divalent or polyvalent cations (Mg²⁺, Ba²⁺, spermine) had similar effects as Ca²⁺ (Babini et al., 2002). The spermine-induced increase in neuronal injury in animal stroke models might be due to such a mechanism (Duan et al., 2011). In addition to its effects on gating, extracellular Ca²⁺ also inhibits maximal currents by a pore block with an IC₅₀ of the order of millimolars. The decrease of the unitary conductance by Ca²⁺ has been demonstrated in ASIC1a, ASIC2a, and ASIC3 (de Wille and Bassilana, 2001; Immke and McCleskey, 2003; Paukert et al., 2004; Zhang et al., 2006). Depending on the pH conditions, an increase in extracellular Ca²⁺ concentration can therefore either increase current amplitudes due to an acidic shift of the SSD pH dependence or decrease them due to the shift of

the activation pH dependence and the pore block (Waldmann et al., 1997b; Babini et al., 2002; Wu et al., 2004). The Ca²⁺ blocking site in ASIC1a is formed by E426 and D433 of the extracellular pore entry (Paukert et al., 2004) (cASIC1 numbering, Fig. 6B). Its localization in other ASICs is not known. The Ca²⁺ binding site mediating the shift in pH dependence has not been identified yet. The Ca²⁺ and H⁺ dependence of ASIC3 currents is compatible with a mechanism of H⁺-induced opening of ASIC3 by relieving a constitutive pore block by Ca²⁺ (Immke and McCleskey, 2003). However, two articles strongly indicate that ASIC1a is not opened by this mechanism. After mutation of the residues forming the Ca²⁺ binding site in the ASIC1a pore, the mutant channels were not constitutively open but still activated by protons (Paukert et al., 2004). In toadfish ASIC1, lowering of the Ca²⁺ concentration progressively opened more channels and did not show properties associated with a pore block, such as, e.g., an increase in the mean open time (Zhang et al., 2006).

Anaerobic metabolism during ischemia can lead to extracellular lactate concentrations of 12–20 mM (normally ~1 mM (Schurr, 2002)). At these concentrations, lactate potentiated ASIC1a and ASIC3 currents, as well as ASIC currents of PNS and CNS neurons (Immke and McCleskey, 2001; Allen and Attwell, 2002). Lactate is a weak chelator of Ca²⁺ and other divalent cations. It appears that the induced reduction of free Ca²⁺ concentration is sufficient to increase the ASIC currents (Immke and McCleskey, 2001).

ii. Zinc. ASIC1a is inhibited by nanomolar concentrations of Zn²⁺, whereas micromolar concentrations are needed to inhibit ASIC1b and ASIC3 (Chu et al., 2004; Poirot et al., 2006; Jiang et al., 2010, 2011). The inhibition of ASIC1b and ASIC3 is pH-independent, whereas an acidic shift of the pH dependence of activation contributes to the Zn²⁺ inhibition of ASIC1a. Interestingly, H⁺-induced currents mediated by ASIC2-containing channels are potentiated by Zn²⁺ due to an alkaline shift in the pH dependence of activation (Baron et al., 2001). Residues important for the effects of Zn²⁺ were identified in the finger of ASIC1a and ASIC1b and in the finger and thumb of ASIC2a (Baron et al., 2001;

TABLE 3
ASIC protein interaction partners

Interaction Partner	ASIC Subtypes Concerned	Site of interaction on ASIC (C or N Terminus) ^a	Consequences	System	References
A kinase-anchoring protein (AKAP) 79/150 and calcineurin	1a, 2a	ASIC2a: N & C, ASIC1a: C	AKAP 150 binding increases ASIC currents; calcineurin decreases ASIC currents.	Transfected cortical neurons or CHO cells	Chai et al., 2007
Channel-interacting PDZ domain protein (CIPP)	3	C, PDZ	Enhances peak current amplitude of ASIC3	Transfected COS cells	Anzai et al., 2002
PICK1	1a, 2a, 2b	C, PDZ	Affects localization of ASIC1a and allows ASIC2a current increase by PKC. Allows current increase of ASIC2b/3 channels by PICK1-dependent PKC phosphorylation of ASIC3.	Mainly recombinant expression systems	Baron et al., 2002; Duggan et al., 2002; Hruska-Hageman et al., 2002; Deval et al., 2004
Lin7B	3	C, PDZ	Increases ASIC3 cell surface expression and current amplitude	Transfected CHO cells	Hruska-Hageman et al., 2004
MAGI-1b (membrane-associated guanylate kinase)	3	C, PDZ ^b	Increases ASIC3 current amplitude	Transfected CHO cells	Hruska-Hageman et al., 2004
PIST	3	C, PDZ ^b	Increases ASIC3 current amplitude	Transfected CHO cells	Hruska-Hageman et al., 2004
PSD95	3	C	Reduces ASIC3 cell surface expression and current amplitude	Transfected CHO cells	Hruska-Hageman et al., 2004; Eshcol et al., 2008; Zha et al., 2009
Stomatin	1, 2, 3	NR	Inhibits ASIC3 current amplitude and accelerates ASIC2a desensitization		Price et al., 2004
Stomatin-like proteins (STOML)	1, 2, 3	NR	ASIC- and STOML-subtype-dependent, inhibition of current amplitude and acceleration of desensitization	Transfected cells, neurons	Lapatsina et al., 2012; Kozlenkov et al., 2014
Na ⁺ /H ⁺ exchanger regulatory factor (NHERF)	3	C, PDZ ^b	Increases ASIC current amplitude	Various cell lines	Deval et al., 2006
Annexin II light chain p11.1	1a	N	Increases cell surface expression and current amplitude	DRG neurons and transfected cells	Donier et al., 2005
α -Actinin	1a	C	Effect on ASIC1a current amplitude and properties, depending on actinin subtype	Cell lines and neurons	Schnizler et al., 2009
High-conductance Ca ²⁺ - and voltage-activated K ⁺ (BK) channel	1a	NR	Reciprocal current inhibition	Transfected cells and neurons	Petroff et al., 2008, 2012
P2X channels	3	NR	ATP sensitizes ASIC3 for activation by protons	Neurons and transfected cells	Birdsong et al., 2010

NR, not resolved

^a"PDZ" in this column indicates that the interacting protein binds to the ASIC PDZ binding domain.

^bASIC3 PDZ binding domain is different from that of ASIC1 and ASIC2. These proteins did not bind to ASIC1 or ASIC2.

Chu et al., 2004; Jiang et al., 2011). Lys134, which is critical for ASIC1a inhibition by Zn²⁺, is also involved in the redox regulation of ASIC1a (Chu et al., 2006; Cho and Askwith, 2007).

iii. Neuropeptides. The short peptide FMRFamide activated the ENaC/DEG family member FaNaC (Lingueglia et al., 1995). In ASICs, dynorphins and the shorter RFamide peptides shift the pH dependence of SSD to more acidic values. The RFamide peptides also slow the kinetics of desensitization and induce a sustained current in some ASICs (Askwith et al., 2000; Lingueglia et al., 2006; Sherwood and Askwith, 2009; Sherwood et al., 2012). FMRFamide and structurally related peptides are abundant in the invertebrate nervous system where they function as neurotransmitters and modulators. Related peptides are also found in mammals and exert most of their effects by binding to

G protein-coupled receptors. These peptides affect the function of ASIC1 and ASIC3, but not ASIC2. The EC₅₀ of dynorphin A and the RFamide peptides for ASIC modulation are on the order of 10–50 μ M, whereas big dynorphin shifts the SSD of ASIC1a with an EC₅₀ of ~30 nM (Lingueglia et al., 2006; Sherwood and Askwith, 2009). FMRFamide modulates the function of ASICs directly, although the exact binding site is still not known. Residues in the loop linking β 9 of the palm to α 4 of the thumb are involved in the effects of RFamide peptides on ASIC1a, although they do not form their binding site (Sherwood and Askwith, 2008). A competition with PcTx1 may suggest that dynorphins bind to the acidic pocket (Sherwood et al., 2012).

iv. Redox Reagents and Free Radicals. ASIC modulation by reducing and oxidizing reagents, mostly studied with recombinant ASIC1a or in neurons, showed

TABLE 4
ASIC modulators

Interacting proteins, toxins, and pharmacological agents are not included in this table. Only most directly-linked references are indicated. For more exhaustive indication of references see text.

Class	Example	Effect	Site of Action	References
Divalent cations	Ca^{2+} , Ba^{2+} , Mg^{2+}	Acidic shift of pH dependence of activation and SSD Pore block	Not known for shift of pH dependence Acidic residues in the pore entry for pore block of ASIC1a	Babini et al., 2002; Immke and McCleskey, 2003; Paukert et al., 2004
	Zn^{2+}	Inhibits ASIC1a, ASIC1b, and ASIC3	Inhibitory site on the finger domain of ASIC1a	Chu et al., 2004
		Potentiates ASIC2-containing channels	Potentiating sites in thumb and finger of ASIC2a	Baron et al., 2001
Polyamines	Spermine	Acidic shift of pH dependence of SSD, leading to a current increase	Extracellular	Babini et al., 2002; Duan et al., 2011
	Agmatine	Activates ASIC3	Involvement of palm ^a	Li et al., 2010c
Redox reagents and free radicals	H_2O_2 , dithiothreitol, nitric oxide	Peak current increase by reducing agents and nitric oxide, inhibition by oxidizing agents	Nitric oxide acts extracellularly	Andrey et al., 2005; Chu et al., 2006; Cadiou et al., 2007; Cho and Askwith, 2007
Neuropeptides	FMRFamide, dynorphin	Shift the pH dependence of SSD to more acidic values Slow the kinetics of desensitization and may induce a sustained current These effects are observed with ASIC1 and ASIC3 but not ASIC2a	Extracellular	Askwith et al., 2000; Sherwood and Askwith, 2009
GPCR activators	Serotonin, cannabinoid receptor agonists	Depending on the compound, potentiation or inhibition	Effects via GPCR or directly on ASICs	Qiu et al., 2012; Wang et al., 2013
Proteases	Trypsin, tissue kallikrein	Acidic shift the pH dependence of activation and SSD of ASIC1a	Trypsin cleaves ASIC1a in the finger domain	Poirot et al., 2004; Vukicevic et al., 2006; Su et al., 2011
Protein kinases	PKA, PKC	Affecting expression and function of ASICs	Intracellular phosphorylation sites	Baron et al., 2002; Leonard et al., 2003; Deval et al., 2004; Gao et al., 2005
Other	Arachidonic acid	Increases the peak currents and induces in some ASIC types a sustained current	Not known	Allen and Attwell, 2002; Smith et al., 2007
	Lactic acid	Increases ASIC currents, most likely by chelating extracellular divalent cations	Probably not acting directly on ASICs	Immke and McCleskey, 2001
	ATP	Increases pH sensitivity of ASIC3	Probably through P2X receptors	Birdsong et al., 2010

GPCR, G protein-coupled receptor; PKC, protein kinase C.

^aOne study concluded that the related GMQ binds to the palm of ASIC3, whereas another study showed that mutation of a putative binding site residue affected only one aspect of GMQ function (section VI.A.1.b).

a current increase (up to ~3-fold) by reducing reagents and an inhibition by oxidizing reagents (Andrey et al., 2005; Chu et al., 2006; Cho and Askwith, 2007). Depending on the conditions, the effects of reducing agents were in some cases only slowly reversible. The peak current increase by reducing agents was due to an alkaline shift of the pH dependence of activation in recombinant ASIC1a and in ASICs of mouse cortical neurons (Chu et al., 2006; Cho and Askwith, 2007). In recombinant ASIC1a and ASICs from trigeminal neurons, reducing agents also slowed the desensitization kinetics (Andrey et al., 2005; Cho and Askwith, 2007). There remains a controversy about the subunit specificity of ASIC redox modulation and about the

involvement of heavy metal chelation in the effects of reducing agents. One study showed that only ASIC1a is redox-modulated and that chelation of Zn^{2+} is not involved (Chu et al., 2006), whereas another study provided evidence of redox modulation of all homomeric ASIC currents and showed that dithiothreitol had no additional effect after application of a heavy metal chelator (Cho and Askwith, 2007).

The inflammatory mediator nitric oxide (NO) regulates protein function either by an indirect mechanism involving the production of cGMP or directly by S-nitrosylation of Cys residues, which can lead to disulfide bond formation. NO donors increased peak current amplitudes of all homomeric ASICs by up to ~2.5-fold.

For ASIC1a, it was shown that this was due to an alkaline shift in the pH dependence of activation. NO acted directly on the ASIC ectodomain; however, the site of action is not known. Topical application of the NO donor glyceryl trinitrate increased acid-induced pain in human volunteers (Cadiou et al., 2007).

v. Arachidonic Acid. AA is a metabolite of membrane phospholipids produced by phospholipase A₂. Its concentration is increased in ischemia and inflammation and it has been shown to modulate the function of several channel types (Chu et al., 2011). AA potentiated ASIC currents by up to ~2.5-fold in neurons of the CNS and the PNS and of recombinant ASIC1a and ASIC3 and in many cases increased the sustained current fraction (Allen and Attwell, 2002; Smith et al., 2007). It was shown that the AA-induced current increase of ASIC3 is due to an alkaline shift of the pH dependence of activation (Deval et al., 2008).

vi. G Protein-Coupled Receptors. Serotonin and other inflammatory mediators, such as nerve growth factor, increase ASIC mRNA levels (Mamet et al., 2002). Serotonin or subtype-specific 5-HT receptor agonists also potentiated ASIC currents in sensory neurons (Deval et al., 2004), either through a direct action on ASIC3 (Wang et al., 2013) or via activation of 5-HT₂ receptors (Qiu et al., 2012). Both serotonin and the 5-HT₂ agonist α -methyl-5-HT increased acid-induced nociceptive responses.

The cannabinoid receptor agonist WIN55,212-2 [(*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone] inhibited ASIC currents of DRG neurons by up to ~50% with an IC₅₀ of ~10 nM, without shifting the ASIC pH dependence (Liu et al., 2012). This inhibition was mediated by cannabinoid receptor type 1.

vii. Proteases. Stroke, brain injury, and other pathologic conditions can compromise the integrity of the blood-brain barrier and allow blood-derived proteases to access the CNS (Gingrich and Traynelis, 2000). Serine proteases modulate the function of various ion channels, including the ASICs (Poirot et al., 2004) and ENaC (see section V.B.2). Trypsin and other serine proteases shift the pH dependence of SSD and activation of ASIC1a to more acidic values. This decreases ASIC currents under physiologic pH conditions but increases the current amplitudes in situations of constitutively lowered pH such as ischemia. The current modulation is due to cleavage of the ASIC1a protein in the finger, at Arg146 (Fig. 5B) (Vukicevic et al., 2006), in the same subdomain as the cleavage of α and γ ENaC. Exposure of ASIC1a to trypsin reduced the relative Ca²⁺ permeability (Neaga et al., 2005), which may reduce the cellular toxicity of prolonged ASIC activity. Tissue kallikrein that was shown to cleave ASIC1a, but not ASIC2a, protected ASIC1a-expressing cells from acidosis-induced injury (Su et al., 2011). This study did not test whether cleavage changed the functional properties of ASIC1a;

however, the protective effect is likely due to a changed function of ASIC1a.

viii. Kinases. The protein kinase C (PKC) activator 1-oleyl-2-acetyl-sn-glycerol potentiates ASIC2a currents only in the presence of protein interacting with C kinase 1 (PICK1) (Baron et al., 2002). PICK1 interaction sites were found on ASIC1a, -2a, and 2b (Baron et al., 2002; Duggan et al., 2002; Hruska-Hageman et al., 2002; Deval et al., 2004). The PKC modulation of ASIC2a currents requires the presence of the PDZ domain of PICK1 and the PDZ binding domain on the C terminus of ASIC2a and involves phosphorylation of residue T39 in the cytoplasmic N terminus. A phorbol ester activating PKC enhances ASIC2b/3 currents by inducing an alkaline shift in their pH dependence of activation (Deval et al., 2004). The presence of PICK1 and its binding site on the ASIC2b C terminus are required for this effect, which involves phosphorylation of ASIC3 residues. The basal ASIC3 expression in sensory neurons depends on the activation of neurotrophic tyrosine kinase receptor type 1 by nerve growth factor, which in turn leads to activation of the phospholipase C/PKC pathway (Mamet et al., 2003). In contrast to ASIC2, ASIC1a is not phosphorylated by PKC, but by protein kinase A (Leonard et al., 2003). Protein kinase A phosphorylates residue S479 of the C terminus and disrupts the binding of PICK1, thereby affecting the localization of ASIC1a.

In global ischemia, intracellular Ca²⁺ concentrations are increased because of, among other mechanisms, the activity of NMDA receptors, leading to activation of the calcium/calmodulin-dependent protein kinase II that phosphorylates the C-terminal residues S478 and S479 of ASIC1a (Gao et al., 2005). This sensitizes the channel to protons and thus potentiates its currents.

B. Epithelial Na⁺ Channel

ENaC is constitutively active at the cell surface and transits between open and closed conformations with an average open probability of ~0.5 (Palmer and Frindt, 1996) (Fig. 4, D and E). During its short lifetime at the cell surface, the channel evolves between different gating modes of high and low open probability before its internalization (Garty and Palmer, 1997; Shimkets et al., 1997; Kleyman et al., 2001; Palmer et al., 2012).

1. Intrinsic Regulation. Na⁺ ions constitute one of the different factors that directly modulate ENaC at the cell surface and affect ENaC activity by two mechanisms. The so-called Na⁺ feedback inhibition is a decrease in ENaC activity upon an increase in cytosolic Na⁺ concentration due to the rapid entry of Na⁺ ions into the cell. This inhibition develops over a time course of minutes and is due to a decrease in both the number of ENaC channels at the cell surface and to a reduction of their open probability (Frindt et al., 1993, 1995; Anantharam et al., 2006). Na⁺ self-inhibition refers to a rapid decrease in ENaC activity after an

increase in extracellular Na^+ concentration (Fuchs et al., 1977; Horisberger and Chraïbi, 2004; Bize and Horisberger, 2007). These regulatory processes tend to limit the Na^+ entry into a single cell to optimize Na^+ absorption along the aldosterone-sensitive distal nephron.

In addition, intracellular perfusion of SH-oxidizing agents or a decrease in intracellular pH also reduces ENaC activity (Chalfant et al., 1999; Kellenberger et al., 2005), but the physiologic relevance of these processes is less clear. The cytosolic N terminus of ENaC subunits contains a conserved HG motif and Cys residues that modulate channel gating (Grunder et al., 1997; Kellenberger et al., 2005). The importance of the cytosolic amino terminus as regulatory domain of ENaC was first evidenced by a mutation in the HG motif of ENaC associated PHA-1 (Chang et al., 1996). The ENaC channels carrying mutations in the HG motif show altered gating properties characterized by abnormally long closures and short channel openings corresponding to a loss of function mutation.

2. Serine Proteases. The extracellular domain between the two transmembrane helices represents more than half of the mass of ENaC/DEG proteins. A similar feature is found in the P2X channel family (Gonzales et al., 2009). Serine proteases regulate ENaC function by proteolytic cleavage. In vitro studies showed that soluble proteases, such as trypsin or chymotrypsin, potentiate ENaC activity, and functional cloning approaches allowed the identification of membrane-bound serine proteases as ENaC partners involved in channel activation (Vallet et al., 1997; Vuagniaux et al., 2000). Extracellular serine proteases stimulate ENaC directly by increasing the open probability independently of a G protein-coupled receptor (Chraïbi et al., 1998). Among these proteases, the first identified was the mouse CAP-1 (channel activating protease-1), an ortholog of the human prostaticin (Vallet et al., 1997). Subsequently, mouse CAP-2, an ortholog of the human transmembrane protease serine 4 (TRPMSS4), and mouse CAP-3 (ortholog of the human matriptase or epithin) were shown to stimulate ENaC to a similar extent (Vuagniaux et al., 2002; Andreassen et al., 2006). Later, the identification of a cleavage sequence for the furin-like proprotein convertase in the extracellular domain of the α and γ ENaC subunits suggested a possible role of furin in ENaC cleavage, ENaC sorting, and the control of ENaC activity at the cell surface (Hughey et al., 2004; Bruns et al., 2007). However, ENaC regulation by serine proteases appears quite complex, because cleavage of γ ENaC by furin is not absolutely required for channel activation (Harris et al., 2008), suggesting a redundancy of serine proteases in ENaC activation. Proteases cleave α and γ ENaC at defined sites in the finger domain (Hughey et al., 2003; Carattino et al., 2008) (Fig. 5C). It has been proposed that a double cleavage in the α and γ subunits removes an inhibitory peptide (26 residues in

α and 43 residues in γ ENaC), resulting in the activation of ENaC (Sheng et al., 2006; Bruns et al., 2007).

3. Hormonal Regulation. The activity of the channel at the cell surface is tightly regulated by hormones. They control the expression of active ENaC at the cell surface by interfering at different steps of the biosynthetic pathway such as transcription, translation, or membrane trafficking. Ultimately, these hormones are essential for the maintenance of Na^+ homeostasis and the control of blood pressure.

Aldosterone is the major regulator of ENaC in the ASDN. Aldosterone increases the synthesis of α ENaC but not of β or γ ENaC in the kidney, in contrast to the colon where aldosterone or dexamethasone increase the synthesis of β and γ only (Masilamani et al., 1999; Epple et al., 2000; Fuller et al., 2000; Loffing et al., 2000b). This increase in the synthesis of α ENaC is accompanied by the redistribution of the ENaC subunits from an intracellular pool to the apical membrane of principal cells. Interestingly this also correlates with the presence of cleaved α and γ ENaC subunits at the cell surface (Frindt and Palmer, 2009b). The increase in the abundance of α ENaC contributes to, but is not sufficient for, the increase in aldosterone-induced net Na^+ transport (Frindt et al., 2008), suggesting the contribution of other processes essential for the upregulation of ENaC by aldosterone.

Binding of aldosterone to the mineralocorticoid receptor allows the dimerization of the receptor, its migration to the nucleus, and the binding to hormone-responsive elements on the DNA for the induction of transcriptional responses. Aldosterone-induced proteins likely play a role in the redistribution of ENaC to the cell surface by controlling the exocytic pathway. These proteins include SGK1 (serum- and glucocorticoid-regulated kinase 1), the deubiquitylation enzyme USP2-45 and GILZ (glucocorticoid-induced leucine zipper) (Soundararajan et al., 2007). These proteins likely work in concert with the ubiquitin ligase Nedd4-2 that is involved in the endocytic pathway. No evidence supports a limiting role of these proteins for the aldosterone effect on ENaC, because mouse models with complete knockout of the genes encoding these proteins (Nedd4-2, SGK, USP2-45, GILZ) failed to suppress the response of ENaC-mediated renal Na^+ absorption to aldosterone (Fejes-Toth et al., 2008; Suarez et al., 2012; Pouly et al., 2013; Ronzaud et al., 2013). The complete picture of the aldosterone signaling pathway that controls ENaC-mediated Na^+ absorption remains therefore largely unknown.

Dexamethasone upregulates the abundance of different Na^+ transporters in the kidney and stimulates ENaC-mediated current in the principal cells of the CCD. Dexamethasone has additive effects on the abundance of α ENaC when coadministered with aldosterone in rats, indicating that dexamethasone regulates ENaC synthesis by occupying the glucocorticoid receptors

(Frindt and Palmer, 2012). This glucocorticoid-dependent increase in α ENaC synthesis is not associated with an increase in ENaC activity at the cell surface, further suggesting that still unidentified regulatory proteins, and not as previously thought the intracellular abundance of ENaC proteins, are rate-limiting for addressing ENaC channels to the apical membrane of principal cells, (Frindt and Palmer, 2012).

A recent report showed that ENaC activity in the late distal convoluted tubule and the connecting tubule is independent of aldosterone, suggesting the presence of other hormones involved in ENaC regulation (Nesterov et al., 2012). Angiotensin II upregulated the activity of the Na^+/Cl^- cotransporter at the apical membrane of the distal tubule (San-Cristobal et al., 2009) and also of ENaC in the distal nephron ex vivo (Mamenko et al., 2012; Sun et al., 2012). Endothelin-1 (ET-1) is a potent vasoconstrictor that regulates systemic blood pressure. In CCD perfused ex vivo, ET-1 significantly inhibited Na^+ absorption; the ENaC-mediated Na^+ absorption was restored by an ET_A receptor inhibitor, suggesting that ET-1 downregulates ENaC (Lynch et al., 2013). However, deletion of the ET-1 receptor ET_A in the kidney of mice did not affect blood pressure and resulted in a modest volume expansion (Stuart et al., 2012), indicating that ET_A receptors in the nephron have little effect on Na^+ absorption by the kidney and on blood pressure. In several cellular models, vasopressin or cAMP agonists stimulated ENaC activity (Schafer and Troutman, 1990; Kleyman et al., 1994; Auberson et al., 2003; Snyder et al., 2004). Vasopressin and aldosterone have synergistic effects on ENaC-mediated Na^+ absorption in the collecting duct. The vasopressin-induced upregulation of ENaC is mediated by vasopressin receptor type 2 (V2R). In humans, vasopressin induced an increase in urine osmolality and a reduced Na^+ excretion, which is likely due to an increased ENaC-dependent Na^+ absorption mediated by stimulation of V2R (Bankir et al., 2005).

C. Structure-Function Relationship of Acid-Sensing Ion Channels and Epithelial Na^+ Channel

Initial information on structure-function aspects of degenerin and ENaC was obtained from mutagenesis screens on *C. elegans*, and the identification of human disease mutations on ENaC.

ENaC and ASIC activity is temperature-dependent (Askwith et al., 2001; Chraïbi and Horisberger, 2002; Neelands et al., 2010; Blanchard and Kellenberger, 2011). This suggests that conformational changes are part of the mechanisms that control ENaC and ASIC activity and likely transmit binding events in the ectodomain to the channel gate. Fluorescence studies have indeed shown evidence for rapid conformational changes in ASIC1a (Passero et al., 2009; Bonifacio et al., 2014). For detailed discussions of ASIC structure-function aspects, see also Grunder and Chen (2010) and Sherwood et al. (2012). An insightful overview of

degenerin structure-function aspects is given by Eastwood and Goodman (2012).

1. The Acidic Pocket and Other Proton-Sensing Sites of Acid-Sensing Ion Channels. The acidic pocket, located between the thumb, the finger, and the β -ball is highly negatively charged and contains several pairs of acidic side chains (Jasti et al., 2007) (Fig. 5A). Because mutation of such acidic residues led to acidic shifts in the pH dependence of activation, it was proposed that the acidic pocket forms the pH sensor that controls ASIC activity (Jasti et al., 2007; Li et al., 2009; Sherwood et al., 2009). It was suggested that, in the closed conformation, the acidic residues are more distant from each other than in the crystal structures that likely represent the open or desensitized conformation. Protonation would allow the thumb to approach the finger and β -ball, and this movement would induce activation and possibly desensitization. Mutations in the acidic pocket that decreased the interaction energy between the thumb and the finger or the β -ball tended to shift the pH dependence of activation to more acidic values, suggesting that channel gating is facilitated by attractive forces between these subdomains (Yang et al., 2009). The mutagenesis and functional analysis of conserved charged residues of ASIC1a identified acidic residues and His residues outside the acidic pocket in the finger and the extracellular vestibule, whose mutation affected the pH dependence of activation (Paukert et al., 2008). This analysis emphasized the role of residues in the extracellular vestibule and pore entry for ASIC function. Another study calculated the pK_a values of Asp, Glu, and His residues in ASIC1a based on the crystal structure, and functionally analyzed the role of residues with pK_a values in the gating range of ASIC1a (Liechti et al., 2010). This approach identified putative pH-sensing residues for activation or desensitization in the acidic pocket and the palm. The role of palm residues in activation was recently confirmed (Krauson et al., 2013).

2. The Finger—A Determinant of Subfamily-Specific Activation Mechanisms? Residue Asp108 of the $\alpha 1$ finger helix is critical for activation of ASIC1a and ASIC3, possibly by building a salt bridge with basic finger residues (Paukert et al., 2008; Yang et al., 2012), and protease cleavage next to Arg146 of the $\alpha 2$ helix shifts the ASIC1a pH dependence to more acidic values (Vukicevic et al., 2006) (Fig. 5B). Note that we use throughout this review the amino acid numbering of cASIC1 for ASIC1a and ASIC1 to facilitate comparison between different orthologs. Residues involved in ASIC1a and -1b inhibition and ASIC2a potentiation by Zn^{2+} are located at different positions within the finger. The finger is covalently linked to the β -ball and the palm at a site containing residues Asn97, His164, and Gln226. This interaction site is highly conserved, sensitive to mutations, and undergoes conformational changes during ASIC activity (Bargeton and Kellenberger, 2010)

Fig. 5. Acidic pocket and finger. The color code for subdomains is the same as in Fig. 3. (A) Acidic pocket, showing acidic side chains of the thumb, finger, loop and β -ball. (B) The three α helices of a finger domain are indicated. The knuckle of a neighboring subunit is highlighted in dark gray. D108, involved in pH-dependent activation of ASIC1a and ASIC3; K134, important for Zn^{2+} inhibition of ASIC1a; H164, a residue involved in the enhancement of ASIC2a currents by Zn^{2+} , localized with N97 and Q226 in the interaction domain between the finger, the β -ball, and the palm. ASIC1b-C149, important for Zn^{2+} inhibition of ASIC1b, is located at the position homologous to G163 (not shown); R146, ASIC1a cleavage site by trypsin. (A and B) Based on the 2QTS structure; (C) α ENaC structural model, indicating the location of furin cleavage sites (F1 α and F2 α) and homologous position of cleavage sites in γ ENaC by furin (F1 γ), CAP-1, plasmin (Plas.), and elastase (Elas.). The peptide of α ENaC located between the two furin sites and therefore cleaved by furin is highlighted in black.

(Fig. 5B). Experiments with fluorophore-quencher pairing and voltage-clamp fluorometry suggest that the upper finger helix $\alpha 2$ and the β -ball move away from each other upon channel activation, because acidification induced a fluorescence signal increase of fluorophores placed in the $\alpha 2$ finger helix and depended on the quenching by the β -ball residue Trp234 (Fig. 5B) (Bonifacio et al., 2014).

ENaC is activated by diverse cleaving events in the α and γ subunits (see section V.B.2) (Kleyman et al., 2009; Rossier and Stutts, 2009). Two cleavage sites for furin are present in the finger domain of α ENaC and one in the finger of γ ENaC (Fig. 5C). Elastase, plasmin, and prostaticin can cleave γ ENaC C terminus to the furin cleavage site.

3. The Palm. The palm forms a β strand-rich scaffold at the central part of the channel, reaching from the transmembrane segments up to the knuckle (Fig. 3, A and B). The lower part of the palm, formed by the two short β strands 1 and 12 connected to the TM segments and by the two long β strands 9 and 10, encloses the central vestibule (Figs. 3A and 6A). The $\beta 1$ – $\beta 2$ loop, connecting the lower palm to the β -ball, is critical for the kinetics of ASIC1 desensitization from the open state (Coric et al., 2003). This study showed that the desensitization kinetics are 25-fold faster in toadfish compared with rat ASIC1a and that this

difference is due to three residues in the $\beta 1$ – $\beta 2$ loop, Ser-Gln-Leu in rat ASIC1a, located just at the beginning of a stretch of residues that are highly conserved in the ENaC/DEG family. The crystal structure indicated that the $\beta 1$ – $\beta 2$ loop is located close to the $\beta 11$ – $\beta 12$ loop. Several studies have shown that these loops interact with each other and codetermine the current kinetics and that mutations in this region can induce sustained opening (Li et al., 2010a,b; Springauf et al., 2011; Roy et al., 2013). Comparison of the open and desensitized crystal structures indicated a 180° rotation of the peptide bond between Ser84 and Gln85 and a swapping of two critical side chains in the $\beta 11$ – $\beta 12$ loop, Leu414 and Asn415 (Baconguis and Gouaux, 2012). Every second residue of the lower palm β sheet surrounding the central vestibule points into the central cavity. Glu417 is a proton-sensing residue for activation (Fig. 6A) (Liechti et al., 2010; Krauson et al., 2013). Several residues of the lower palm are important for ASIC3 activation by GMQ (see section VI.A.1.b) (Yu et al., 2010; Alijevic and Kellenberger, 2012).

The Glu80 side chain is oriented toward the central cavity (Fig. 6A). When mutated to Cys, this residue of ASIC3 was better accessible to small hydrophilic sulfhydryl reagents in the closed compared with the desensitized state, consistent with a conformational change of the palm upon desensitization (Cushman

Fig. 6. Palm and transmembrane domain. (A) The lower palm domain encloses the central vestibule. Its β -strands 1 and 12 are connected to the transmembrane segments, whereas the lower ends of β -strands 9 and 10 are connected via linker regions to the thumb. One of these linkers contains the β -turn (W288) interacting with Y72 of TM1. Based on 4NYK structure. (B and C) View of the pore in side view (B) and seen from the bottom (C) in the desensitized channel (left, PDB ID 4NYK) and the MitTx-bound open channel (right, PDB ID 4NTW). Only two subunits are shown in (B). The calcium binding site residues in ASIC1a (E426, D433), the DEG site (G432), the amiloride binding site (G439), and the "GAS" residues G443, A444, and S445 of the selectivity filter are indicated. *, Gly residue.

et al., 2007). In line with these observations, functional studies on ASIC1a provided evidence for a closing of the lower palm domain around the central vestibule during desensitization (Liechti et al., 2010; Della Vecchia et al., 2013; Roy et al., 2013). As mentioned above, a closing of the palm during the open desensitized transition was also evident from the comparison of putative desensitized and open crystal structures of chicken ASIC1 (Baconguis and Gouaux, 2012). Molecular dynamics simulations showed that desensitization-impairing mutations changed the palm conformation and prevented its closing movement (Roy et al., 2013). Together, this demonstrates the important role of the lower palm and adjacent loops for desensitization. The constriction below the central cavity at the level of Leu78 is not changed between the open and the

desensitized structure. Leu78 forms a contact point between the three subunits and may serve as a pivotal point that allows transmission of palm movements to the pore. The closing movement of the palm may lead through a rotation around residues Leu78 to pore closing.

4. The β -Turn and the Extracellular Vestibule—at the Interface between the Extracellular and Transmembrane Domains. The β -turn is located in the loop connecting the palm β strand 9 with the thumb helix $\alpha 5$ and points down toward the upper end of the TM1. Trp288 of the β -turn interacts with Tyr72 of TM1 (Jasti et al., 2007) (Fig. 6, A and B). It had been suggested that this interaction may transmit conformational changes of the acidic pocket toward the channel gates. Consistent with this hypothesis, mutations that disrupted this hydrophobic interaction led to nonfunctional channels (Li

et al., 2009). Mutations of the interacting residue in either the β -turn or the TM1 lowered the expression of the channels at the cell surface and rendered the plasma membrane–resident mutants nonfunctional (Jing et al., 2011). Normal mode analysis suggested the movements of β -turn residues to be highly correlated with movements of the upper TM1 (Yang et al., 2009).

The extracellular vestibule is surrounded by the flexible wrist that connects the palm to the transmembrane pore region and is located at the same level as the β -turn. It contains three large fenestrations that most likely form the extracellular access route for cations to the channel pore. Functional studies identified two His residues in the extracellular vestibule, His73 and His74, as well as Asp79 as critical for pH-dependent ASIC1a activity (Paukert et al., 2008). Mutations of these residues did not affect the Ca^{2+} dependence of gating, suggesting that they are not Ca^{2+} binding sites. His74 is oriented toward Asp79 of the neighboring subunit (Fig. 6A). This intersubunit interaction likely stabilizes protonation of one of the two residues.

5. The Transmembrane Domain.

a. Components of the channel pore. Studies examining the accessibility of specific residues in the transmembrane helices of ENaC indicated that the TM2 lines the channel pore and identified residues oriented toward the pore lumen (Schild et al., 1997; Snyder et al., 1999; Sheng et al., 2001, 2005). Crystal structures of cASIC1 in complex with PcTx1 or MitTx confirmed that the TM2 segments line the pore. The cASIC1 construct used for cocrystallization with PcTx1 showed in the presence of high concentrations of PcTx1 a nonselective, amiloride-resistant conductance at pH 7.25 and a Na^+ -selective, amiloride-sensitive current at pH 5.5 (Baconguis and Gouaux, 2012). Accordingly, the structure obtained at pH 7.25 showed a very wide pore and that obtained at pH 5.5, a narrower open pore. These structures contained continuous TM1 and TM2 α helices. In contrast, the TM2 of the cASIC1-MitTx structure is split in two parts (TM2a and -b) by the Gly-Ala-Ser (GAS) selectivity filter motif, which forms a horizontal extended structure (Fig. 6, B and C). The GAS site corresponds to the G/S-x-S selectivity filter identified in ENaC (see section V.C.5.d). The horizontal GAS segment places the lower TM2 α helix (TM2b) in the continuation of the upper TM2 helix (TM2a) of a neighboring subunit. The narrowest part of the nonselective cASIC1-PcTx1 pore was ~ 10 Å wide. The Na^+ -selective cASIC1-PcTx1 pore was asymmetric, its section showing an elliptical shape, and was narrowest in its lower half, with dimensions of $4\text{--}5 \times 7\text{--}10$ Å. In the cASIC1-MitTx structure the transmembrane channel part is symmetrical and has similar dimensions as the pH 5.5 cASIC1-PcTx1 structure, except for wider intra- and extracellular openings (Baconguis et al., 2014).

b. The desensitization and closing gate. The desensitized structure identified the “desensitization gate,” an

~ 8 Å-thick constriction in the upper part of the pore at the level of residues Asp433-Gly436 (Gonzales et al., 2009). A functional study used measurements of reaction rates of sulfhydryl reagents to engineered Cys residues to locate the closing gate (Li et al., 2011a). These experiments were carried out in the lamprey ASIC1, which does not desensitize. They identified a constriction in the closed ASIC between Gly432 and Gln437, corresponding likely to the closing gate, which therefore appears to be at the same location as the desensitization gate (Fig. 6B). The open channel was not accessible to the sulfhydryl reagents between residues Leu440 and Ile446, consistent with the presence of the selectivity filter.

c. The degenerin residue and the amiloride binding site. Gly432 constitutes the DEG site, a conserved small residue in all ENaC/DEG channels, whose replacement by larger residues induces constitutive activity in most family members, leading to degeneration of sensory neurons in *C. elegans* (Eastwood and Goodman, 2012). Substitution of the DEG residue induced in ASIC2a, besides a constitutive current, a slowing of desensitization and an altered pH dependence of the H^+ -induced current (Adams et al., 1998; Champigny et al., 1998). The DEG site of ASIC2a is solvent accessible only in open channels (Adams et al., 1998). Its replacement by large residues may keep the pore open. Mutation to large residues or introduction of large adducts to engineered Cys residues at the DEG position of α , β or γ ENaC subunits locked the channel in an open conformation (Snyder et al., 2000; Kellenberger et al., 2002). In the crystal structure, the DEG site is on the side of the TM2 helix that faces the pore lumen.

A recent study connected ASIC1a subunits via modification of engineered Cys residues at position 430—hence one helical turn higher up than the DEG site—with a photosensitive linker whose conformation could be switched by light from *cis* to *trans*. The *cis*-to-*trans* isomerization pushed the upper ends of the TM2 α helix apart, thereby opening the channel (Browne et al., 2014). This likely mimics the movement of the TM2 helices needed for channel opening that is normally induced by complex conformational changes of the ectodomain.

The amiloride binding site of ENaC is at the position corresponding to Gly439 (Schild et al., 1997), which has been confirmed as functional amiloride binding site in ASICs (Adams et al., 1999; Alijevic and Kellenberger, 2012). The G439 backbone carbonyls point into the pore lumen in the cASIC1-MitTx structure.

d. The selectivity filter. As mentioned, ENaC conducts Na^+ ions at rates 100- to 1000-fold higher than K^+ . Substitution of the C-terminal Ser residue of the conserved G/S-x-S motif in the lower part of the TM2 (Ser589 in α ENaC) with large residues reduced the high selectivity of ENaC for Na^+ ions considerably (Kellenberger et al., 1999a). The likely explanation for this observation is that the mutations of α ENaC-S589

enlarge the pore diameter, allowing large cations, such as K^+ or ammonium, to permeate the ENaC pore (Kellenberger et al., 2001; Takeda et al., 2007). These and related studies identified the ion selectivity filter as the narrowest part of the ENaC channel pore, allowing almost exclusively monovalent cations of the size of Na^+ to pass through the channel pore (Kellenberger et al., 1999b; Snyder et al., 1999; Sheng et al., 2000). The ENaC selectivity filter corresponds to the GAS motif in ASIC1. In ASIC1, this stretch is part of the narrowest region in the open pore (Baconguis and Gouaux, 2012; Baconguis et al., 2014). In the cASIC1-MitTx structure the GAS motifs of the three subunits split the TM2 α helices in two and form a horizontal belt. The carbonyl oxygens of Gly443 point into the pore lumen and form a binding site for permeant ions. At this level, the pore radius is 3.6 Å, close to the radius of a hydrated Na^+ ion (3.8 Å) (Baconguis et al., 2014; Mahler and Persson, 2012). The authors suggest therefore that the selectivity filter functions as a barrier, achieving selectivity by discrimination of hydrated ions. The side chain of Ser445 interacts with residues of the TM2a helix of a neighboring subunit, stabilizing the continuity of the separated TM2 helix (Fig. 6, B and C). In the cASIC1-PcTx1 structures in contrast, the GAS motif is part of the continuous TM2 α helix, with the side chain of Ser445 pointing to the adjacent TM1 (Baconguis and Gouaux, 2012).

In the cASIC1-PcTx1 structures a single cation binding site in the pore was shown at the extracellular pore entry close to Asp433. The cASIC1-MitTx structure showed cations at the same level and, in addition, one cation in the center of the selectivity filter. Functional studies demonstrated that the GAS segment of ASICs is important for ion selectivity and permeation. Mutation of Gly443 of one single subunit within the trimer decreased the unitary conductance and affected ion selectivity (Li et al., 2011a). Mutation of either Leu440 or Ala444 changed the Na^+/K^+ permeability ratio (Yang et al., 2009). Together this suggests that the location of the selectivity filter is conserved between ENaC and ASICs. The TM2 is the best-conserved domain between ASICs and ENaC, and several residues involved in ion selectivity are conserved between ENaC and ASICs. Functional studies indicated, however, that ENaC has a 10- to 100-fold higher Na^+/K^+ selectivity than ASICs and needs to transport completely dehydrated ions to ensure its high selectivity. This functional difference may be due to the small differences in primary structure of the selectivity filter between ENaC and ASICs or to a different relative positioning of the TM2 domains.

6. Cytoplasmic N and C Termini. It was shown that the cytoplasmic N terminus codetermines the selectivity between monovalent cations in ASIC2 and ASIC3 (Coscoy et al., 1999), whereas the analogous domain in ASIC1 contributes to the Ca^{2+} permeability (Bassler

et al., 2001). Accessibility studies have shown that residues of the ASIC1a N terminus likely contribute to the intracellular pore vestibule (Pfister et al., 2006). The conserved HG motif, whose mutations in ENaC are at the origin of PHA-1 in humans by decreasing ENaC open probability and cell surface expression (see section V.B.1), is located directly downstream of the residues involved in ASIC selectivity.

The cytoplasmic C termini of ENaC and ASIC contain phosphorylation and protein-protein interaction sites. The motifs of ENaC β and γ subunits, which bind Nedd4-2 and are mutated or deleted in Liddle syndrome (see section III.B.2) are located at a distance of 15 residues from the C terminus. ASIC subunits contain PDZ binding domains formed by the last four residues, which are involved in protein-protein interactions (Wemmie et al., 2006) (Table 3).

7. A Model of Acid-Sensing Ion Channel Gating. The crystal structures, together with state-dependent accessibility, mutagenesis, and voltage-clamp fluorometry studies, suggest the following steps leading to opening and desensitization, illustrated in Fig. 7. Upon acidification, which protonates residues in different domains, such as the finger, the acidic pocket, the

Fig. 7. A model of ASIC gating. Schematic view of an ASIC trimer, showing the domains of one subunit in color. The contours of the domains of a second subunit are shown as dashed lines, whereas the third subunit is omitted for clarity. Proposed protonation sites and conformational changes are only shown in the colored subunit. The main protonation sites are shown as " H^+ ." Red arrows indicate the direction of proposed conformational changes associated with ASIC activation. Black arrows indicate the direction of proposed conformational changes associated with ASIC desensitization. Proposed conformational changes in the wrist are not shown, because their direction is not known. The domains are colored as follows: finger (purple), thumb (blue), knuckle (turquoise), β -ball (orange), palm (yellow), transmembrane segments (red).

palm, and the wrist (Paukert et al., 2008; Liechti et al., 2010; Krauson et al., 2013), rapid movements are observed in the finger and the extracellular vestibule, with the finger $\alpha 2$ helix and the β -ball moving away from each other (Passero et al., 2009; Bonifacio et al., 2014) and likely the thumb helices approaching the β -ball and the finger (Jasti et al., 2007). It is currently not clear whether the finger movements are transmitted to the pore via the outer structures (thumb and palm-thumb loops) or more centrally via the palm to the extracellular vestibule. These conformational changes lead together with contributions of the palm and the wrist to the opening of the closing gate (Li et al., 2011a), which is located between Gly432 and Gln437. Slower movements in the palm (Bonifacio et al., 2014), detected as closing movement of the lower palm around the central vestibule in accessibility studies in the closed-to-desensitized transition (Cushman et al., 2007; Liechti et al., 2010; Della Vecchia et al., 2013; Roy et al., 2013) and documented as a narrower central vestibule in the desensitized compared with the open ASIC structure (Bacongus and Gouaux, 2012; Bacongus et al., 2014), are associated with desensitization. The closing movement of the palm leads to the closing of the desensitization gate, preventing ion conduction (Bacongus and Gouaux, 2012).

VI. Pharmacology

A. Acid-Sensing Ion Channels

1. Small molecules.

a. Acid-sensing ion channel inhibitors.

i. *Amiloride*. Amiloride (Fig. 8) inhibits the transient ASIC currents with IC_{50} values of the order of 10–100 μ M, thus with a 100- to 1000-fold lower potency than its potency for ENaC block (Table 2) (Kellenberger and Schild, 2002). The sustained component of the ASIC3 current is only partially inhibited or even increased by amiloride (de Weille et al., 1998; Babinski et al., 1999; Yagi et al., 2006; Li et al., 2011b). Amiloride induces a conformational change in ASIC2a

that exposes the “DEG site” (located in the pore entry; Fig. 6B) to the extracellular solution, in addition to blocking the pore (Adams et al., 1999). Together these observations suggest that amiloride binds to two different sites in ASICs. The homologous residue to the ENaC blocking site (Schild et al., 1997) is also necessary for inhibition of ASIC1a, ASIC2a, and ASIC3 (Adams et al., 1999; Alijevic and Kellenberger, 2012) (Fig. 6B). The location of the modulatory amiloride binding site is not known; however, it was shown that residues of the palm are necessary for the amiloride-induced current increase in ASIC3 (Li et al., 2011b). In the open MitTx-cASIC1 structure, amiloride was found in the acidic pocket when the crystallization was carried out in the presence of the drug, suggesting that the modulatory site may be in the acidic pocket (Bacongus et al., 2014). Although amiloride is far from being an ideal ASIC inhibitor, because at the micromolar concentrations needed to inhibit ASICs it also inhibits ENaC, T-type calcium channels, and the sodium-proton exchanger (Kleyman and Cragoe, 1988), it has been successfully used in several studies targeting ASIC functions. Cortical spreading depression, occurring during migraine aura, is associated with failure of brain ion homeostasis and the release of various agents, as e.g., protons and AA, which activate or enhance ASIC function. Amiloride blocked cortical spreading depression in rodents and reduced aura and headache symptoms in human patients (Holland et al., 2012). Furthermore, a recent study suggested that amiloride has a neuroprotective action on MS patients (Arun et al., 2013). A search for amiloride derivatives with increased potency on ASIC3 resulted in the identification of several substitutions of the NH₂ group at position 5 of the pyrazine ring that increased the potency by 10-fold (Kuduk et al., 2009b).

ii. *Nonsteroidal Anti-Inflammatory Drugs*. Inflammation induces a large increase in ASIC mRNA levels in DRGs, which is prevented by administration of the nonsteroidal anti-inflammatory drugs (NSAIDs)

Fig. 8. ENaC/DEG channel modulators and inhibitors. The chemical structures of GMQ, A-317567, amiloride, and benzamil are shown.

aspirin, diclofenac, and ibuprofen at therapeutic doses (Voilley et al., 2001). In addition, ASIC currents are inhibited by NSAIDs with IC_{50} values in the order of hundreds of micromolars to several millimolars (Voilley et al., 2001; Dorofeeva et al., 2008), thus with 100- to 1000-fold lower potency than the inhibition of cyclooxygenases by these drugs (Warner et al., 1999). It is therefore likely that the inhibition of ASIC expression, but not the experimentally observed inhibition of ASIC currents, may contribute to the clinical effects of NSAIDs.

iii. Other Small-Molecule Inhibitors. A-317567 is structurally unrelated to amiloride (Fig. 8) and inhibits ASIC currents in DRG neurons with IC_{50} values of 2–30 μ M (Dube et al., 2005). In contrast to amiloride, A-317567 inhibits transient and sustained ASIC currents with similar potency. In animal pain models, A-317567 was more potent than amiloride. Subsequently, a series of indol amidines was tested for inhibition of ASIC3, identifying compounds inhibiting ASIC3 with an $IC_{50} < 1 \mu$ M that partially reversed mechanical hypersensitivity after paw inflammation (Kuduk et al., 2009a). Different anti-protozoal diarylamidines inhibited ASICs with IC_{50} of 0.3–38 μ M (Chen et al., 2010). Nafomastat, a clinically used serine protease inhibitor (Fujii and Hitomi, 1981), inhibits ASIC currents, including the sustained component of the ASIC3 current, with an IC_{50} of ~2–70 μ M (Ugawa et al., 2007). More recently, the screening of a fragment library and subsequent optimization yielded 2-aminopyridine derivatives inhibiting ASIC3, with an IC_{50} of ~3 μ M (Wolkenberg et al., 2011). ASIC currents are inhibited by the local anesthetics tetracaine and lidocaine with IC_{50} values of ~10 mM and by the K^+ channel inhibitor 4-aminopyridine with IC_{50} values ≥ 1 mM (Lin et al., 2011; Boiko et al., 2013; Leng et al., 2013).

b. The acid-sensing ion channel modulator 2-guanidine-4-methylquinazoline. GMQ induces persistent ASIC3 currents when applied at pH 7.4 and induces pain-related behavior in wild-type but not ASIC3^{-/-} mice (Yu et al., 2010). GMQ, which shares some structural similarity with amiloride (Fig. 8), was the first discovered ASIC activator other than protons. It was shown that mild acidosis and reduction of extracellular Ca^{2+} concentration enhance the GMQ-induced currents. Several residues in the palm domain are critical for the effects of GMQ (Yu et al., 2011). Covalent modification of one of these residues, Glu79, is sufficient for ASIC3 activation, suggesting that GMQ may bind to the central cavity formed by the lower palm domains (Yu et al., 2010). The potency of GMQ for ASIC3 activation is low with an EC_{50} of ~1 mM at physiologic Ca^{2+} concentrations. It was later shown that, in addition to the synthetic GMQ, the arginine metabolites agmatine and arcaine have qualitatively similar effects on ASIC3 (Li et al., 2010c). Agmatine levels are increased during trauma, infection, and cancer. In concert with other endogenous ASIC modulators, such as AA, agmatine

may affect ASIC function under physiologic or pathologic conditions. Agmatine injection induced pain-related behaviors in mice. A recent study addressed the mechanisms of action of GMQ and showed that this compound induces an acidic shift of the pH dependence of SSD and an alkaline shift of the pH dependence of activation of ASIC3, creating a window current at physiologic pH (Alijevic and Kellenberger, 2012). Intriguingly, mutation of Glu79, previously shown to be critical for ASIC3 activation by GMQ at physiologic pH, prevented the shift of the SSD, but not of the activation pH dependence, suggesting that this residue may rather be involved in the effect of GMQ than being part of the GMQ binding site (Alijevic and Kellenberger, 2012). Deletion of a residue in the lower palm—thus in the same domain—of β ENaC suppressed the effect of the ENaC activator S3969 [N-(2-hydroxyethyl)-4-methyl-2-(4-methyl-1H-indol-3-ylthio)pentanamide] (Lu et al., 2008). GMQ also affected the pH dependence of ASIC subtypes other than ASIC3—mostly by shifts to more acidic pH—which, however, did not induce currents at physiologic pH (Alijevic and Kellenberger, 2012). At higher concentrations GMQ inhibited ASIC currents by a pore block.

2. Toxins. Venom toxins acting on ASICs have importantly contributed to the understanding of physiologic and pathologic functions of ASICs (Baron et al., 2013). The most important ASIC-targeting toxins are the spider toxin Psalmotoxin1 (PcTx1) (Escoubas et al., 2000), the sea anemone toxin APETx2 (Diochot et al., 2004), and the snake toxins Mambalgins 1–3 (Diochot et al., 2012) and MitTx (Bohlen et al., 2011). APETx2 and Mambalgins are inhibitors, MitTx is an activator of ASICs, and PcTx1 acts depending on the ASIC subtype and the pH conditions either as an inhibitor or an activator. PcTx1 and Mambalgins, the two toxins for which the mechanism of action has been elucidated, are gating modifiers. The properties of these toxins are summarized in Table 5. Other ASIC-targeting toxins with lower potency or incomplete inhibition are known, such as the sea anemone toxins Ugr 9-1 that inhibit ASIC3 currents with an IC_{50} of ~10 μ M (Osmakov et al., 2013) and PhcTx1, whose inhibition of DRG neuron ASIC currents is incomplete (~40%) with an IC_{50} of ~100 nM (Rodriguez et al., 2014). These two toxins will not be further discussed here. All of these ASIC-targeting toxins are minor venom components, and their use for the organism producing them is not clear, except for MitTx that induces pain.

a. Psalmotoxin1. PcTx1 of the spider *Psalmopoeus cambridgei* inhibits homomeric ASIC1a and heteromeric ASIC1a/2b with nanomolar potency (Escoubas et al., 2000; Sherwood et al., 2011). The structure of PcTx1 was solved by NMR (Escoubas et al., 2003; Saez et al., 2011) and later by X-ray crystallography as part of the cASIC1-PcTx1 complex (Bacongus and Gouaux, 2012; Dawson et al., 2012). Composed of a three-stranded antiparallel β sheet, it is folded

TABLE 5
Properties of ASIC-targeting toxins

	Psalmotoxin 1	APETx2	Mit-Toxin (act)	Mambalgin-1 ^a
Origin	<i>Psalmopoeus cambridgei</i>	<i>Anthopleura elegantissima</i>	<i>Micrurus tener tener</i>	<i>Dendroaspis polylepis polylepis</i>
Fold	Inhibitor cystine knot	All-β toxin	Kunitz type (MitTx-α) and phospholipase A ₂ -like protein (MitTx-β)	Three-finger toxin
Number of amino acids	40	42		57
ASIC target	ASIC1a ASIC1a/2b	ASIC3 ASIC3-containing heteromers Na _v 1.8	Dimer of MitTx-α: 60 MitTx-β: 119 All homomeric ASICs	ASIC1a and ASIC1b and many ASIC1a-containing heteromers
Typical IC ₅₀ or EC ₅₀	ASIC1b (act) cASIC1 (act) r/hASIC1a: 0.4–13 nM rASIC1a/3b: 3 nM rASIC1b: 100 nM cASIC1: 189 nM	rASIC3: 37–63 nM rASIC2b/3: 117 nM rASIC1a/3: 2 μM TTX-R Na _v : 2.6 μM	rASIC1a: 9 nM rASIC1b: 23 nM rASIC3: 830 nM	rASIC1a: 55 nM rASIC1b: 192 nM rASIC1a/2a: 246 nM
Binding site	Acidic pocket/thumb helix	Unknown	Wrist, palm, and thumb	Acidic pocket
Effect	Inhibition/activation	Inhibition	Activation or potentiation (ASIC2a)	Inhibition
Mechanism	Alkaline shift in the SSD (→inhibition) and/or activation pH dependence (→ activation)	Unknown	Unknown	Acidic shift of the pH dependence of activation

act, activation.
^aMambalgin-2 from *Dendroaspis polylepis polylepis* and Mambalgin-3 from *Dendroaspis angusticeps* differ each in a single residue from Mambalgin-1 (Baron et al., 2013). For references, see text.

according to the inhibitor cystine knot motif. The charge distribution of PcTx1 shows an electrostatic anisotropy that can be represented by a dipole moment. PcTx1 binds to the acidic pockets of ASIC in a stoichiometry of three PcTx1 molecules per channel trimer (Fig. 9A). A hydrophobic patch of PcTx1 (pink in Fig. 9A) binds to the α5 thumb helix, and a basic cluster (Arg27, Arg28, light green) extends deeply into the acidic pocket (Baconguis and Gouaux, 2012; Dawson et al., 2012). The functional importance of several of the interacting residues on the toxin and the channel had previously been demonstrated by site-directed mutagenesis (Salinas et al., 2006; Saez et al., 2011). PcTx1 shifts the ASIC1a pH dependence of SSD and to a smaller extent that of

activation to more alkaline values and thereby desensitizes the channel at pH 7.4 (Chen et al., 2005). At higher concentrations, PcTx1 also binds to ASIC1b, on which it also induces alkaline shifts in pH dependence. Because SSD occurs at more acidic pH in ASIC1b compared with ASIC1a and the shift induced by PcTx1 is small in ASIC1b, PcTx1 does not inhibit ASIC1b under physiologic pH conditions but in contrast enhances ASIC1b currents at mildly acidic pH (Chen et al., 2006). Chicken ASIC1 is activated by PcTx1 at pH 7.4 (Samways et al., 2009; Baconguis and Gouaux, 2012), suggesting that PcTx1 induces an alkaline shift of the cASIC1 activation pH dependence. This has not, however, been shown so far.

Fig. 9. Complexes of ASIC1 with PcTx1 and MitTx. The structural images show all three subunits of the ASIC trimer with either three PcTx1 molecules or three MitTxα+β molecules bound (one of them, in the back, is only faintly visible). Toxins are shown in surface representation. (A) cASIC1 with bound PcTx1 (PDB ID 4FZ1) (Baconguis and Gouaux, 2012). The basic cluster of PcTx1 protruding deep into the acidic pocket of ASIC1 is shown in light green, and the hydrophobic patch, interacting with the α5 thumb helix is highlighted in pink. (B) cASIC with bound MitTx (PDB ID 4NTW) (Baconguis et al., 2014). Residue F14 of MitTxα, interacting with the palm domain, is shown in pink, and K16 that is inserted between the β-turn and the upper end of TM1 is shown in dark blue.

b. APETx2. This toxin of the sea anemone *Anthopleura elegantissima* inhibits ASIC3 and ASIC3-containing heteromers with IC_{50} values ranging from 63 nM to 2 μ M (Diochot et al., 2004). APETx2 shares 64% sequence identity with the HERG channel inhibitor APETx1 and 25–35% identity with several K_v - and Na_v -targeting toxins (Diochot et al., 2004). On the basis of the solution structure of the native APETx2 peptide and on comparison with related toxins, it was suggested that the channel-interacting surface may be constituted by basic and aromatic residues (Chagot et al., 2005). One of these aromatic residues, Phe15, was recently shown to be important for ASIC3 inhibition (Anangi et al., 2012). The site of action of APETx2 is not known. On the basis of the presence of the basic-aromatic toxin surface patch it has been suggested that APETx2 may bind as PcTx1 to the acidic pocket (Baron et al., 2013). APETx2 is not selective for ASICs and inhibits voltage-gated Na^+ currents in DRG neurons with an IC_{50} of 2.6 μ M, recombinant $Na_v1.8$ (IC_{50} of 55 nM or 19 μ M, depending on the study), and $Na_v1.2$ (IC_{50} of 114 nM) (Blanchard et al., 2012; Peigneur et al., 2012).

c. Mambalgins. Mambalgin-1 and -2 from black mamba and Mambalgin-3 from green mamba differ only in one amino acid from each other and inhibit ASIC1a, ASIC1b, and ASIC1a-containing heteromers with IC_{50} values ranging from 11 to 250 nM (Diochot et al., 2012; Baron et al., 2013). Modeling of the three-dimensional structure of Mambalgins and its determination by NMR (Schroeder et al., 2014) showed that they form a new subfamily of three-finger toxins. The peptide surface contains a hydrophobic patch and several clusters of basic or acidic residues. Combination of mutagenesis and functional analysis showed that Mambalgin-2 binds in the acidic pocket to the thumb $\alpha 5$ helix, the β -ball, and to the palm of an adjacent subunit (Salinas et al., 2014; Schroeder et al., 2014). This is very close to the PcTx1 binding site, and it is surprising that PcTx1 and Mambalgin-1 appear not to interact with each other on ASIC1a (Diochot et al., 2012). Mambalgin-1 shifts the activation curve strongly to more acid values, thereby preventing channel opening.

d. Mit-toxin. MitTx of the Texas coral snake is formed by noncovalent interaction between the two peptides MitTx α , a Kunitz type peptide, and MitTx β , a phospholipase A_2 -like protein (Bohlen et al., 2011). This complex resembles the β -bungarotoxins, in which a Kunitz type peptide and a phospholipase A_2 domain are covalently linked by a disulfide bond (Doley and Kini, 2009). MitTx induces a slow activation of ASIC1a, ASIC1b, and ASIC3 with EC_{50} values of 9, 23, and 830 nM, respectively. It only poorly activates ASIC2a, but potentiates ASIC2a currents with an EC_{50} of 75 nM by inducing a strong alkaline shift of the activation curve. The mechanism of action of MitTx is currently not known. However, the most recent ASIC structure was obtained from a cASIC1-MitTx complex, revealing the

structure and binding site of MitTx (Baconguis et al., 2014). Three molecules of MitTx bind to one channel trimer (Fig. 9B). Seen from the top, the toxin heterodimers protrude from the edges of the cASIC1 trimer. The toxin heterodimer is held together by extensive interactions between the two subunits. It interacts mainly with the wrist, palm, and thumb domains of cASIC1 but does not reach into the acidic pocket as does PcTx1 (Fig. 9). The arrangement and possible effect of MitTx is compared with that of a church key bottle opener, with MitTx β forming the upper part, interacting with residues of the two thumb α helices, and MitTx α reaching down to the membrane plane, interacting with residues of the two palm-thumb loops. MitTx α inserts its residue Phe14 (pink in Fig. 9B) between the bottom of the palm domains of two adjacent subunits where it interacts with the $\beta 1$ - $\beta 2$ linker, and Lys16 (dark blue) is placed in the wrist between the β turn and the upper end of the TM1. These interactions may open the palm and change the pore conformation to open the channel pore.

B. Epithelial Na^+ Channel

1. Direct Epithelial Na^+ Channel Antagonists.

a. Properties. Amiloride is the prototypical ENaC blocker. Developed by Cragoe in the early 1960s, it has been widely used as an adjunctive therapy with loop diuretics or thiazides to minimize hypokalemia (Cragoe et al., 1967). Amiloride is a pyrazinoylguanidine bearing amino groups in positions 3 and 5 and a chloro group at position 6 of the pyrazine ring (Fig. 8). Amiloride was found to be a potent reversible inhibitor of the epithelial Na^+ selective conductance in tight epithelia such as the toad urinary bladder, with an IC_{50} of 0.1–0.3 μ M (Lindemann and Van Driessche, 1977). Pharmacodynamic experiments on tight amphibian epithelial models provided the first clues on the mechanism of block of the epithelial Na^+ channels by amiloride. An important observation was that amiloride inhibition depended on the transmembrane voltage that, if negative, would tend to drive the positively charged amiloride in the membrane channel, and that amiloride interacts competitively with permeant cations (Palmer and Andersen, 1989; Schild et al., 1997). These observations were interpreted as amiloride binding within the transmembrane part of the channel pore, thereby inhibiting the flux of permeant ions. Patch-clamp experiments on renal CCD cells provided evidence that amiloride directly interacts with a highly selective Na^+ channel at the apical membrane, resulting in a concentration-dependent decrease in the open-channel probability, by promoting a nonconducting state of the channel (Palmer and Frindt, 1986).

A large number of amiloride analogs were synthesized (Kleyman and Cragoe, 1988). Among these analogs, the most specific inhibitors of ENaC, such as benzamil, bear hydrophobic substituents on the terminal nitrogen atom of the guanidinium moiety (Fig. 8) (Kleyman and Cragoe, 1988). The positively charged guanidinium is needed for

ENaC block (Palmer, 1984). Amiloride and its analogs were found to be rather poorly specific and inhibit other Na^+ transporters, such as Na^+/H^+ or $\text{Na}^+/\text{Ca}^{2+}$ exchangers. A higher specificity of amiloride analogs for the Na^+/H^+ exchanger is conferred by addition of hydrophobic substituents on the 5-amino group of the pyrazine ring (Kleyman and Cragoe, 1988).

The identification of the primary structure of ENaC made experiments possible that provided a more precise picture of the ENaC block by amiloride. Site-directed mutagenesis coupled to functional expression of ENaC mutants identified specific residues in the TM2 that when mutated considerably decreased the affinity of the channel for amiloride. In the center of TM2, a Ser-Gly-Gly ring in the α , β , and γ subunits (corresponding to cASIC1-G439; Fig. 6B) was found to contain the most critical structural determinants of the high amiloride affinity of ENaC (Schild et al., 1997). Substitution of α S583 with residues such as Ala, Gly, or Cys increased the IC_{50} for amiloride by 6- to 25-fold depending on the type of mutation. Similar substitutions in the β or γ subunits increased the IC_{50} by more than 1000-fold and 100- to 1000-fold, respectively. Analysis of the kinetics of ENaC block by amiloride revealed that the decrease of the amiloride affinity in ENaC mutants by 1–3 orders of magnitude was essentially due to an increase in the dissociation rate constant of amiloride from its receptor site (Kellenberger et al., 2003). In other words, mutations of the G/S ring in the pore of ENaC destabilize the binding interaction of amiloride with its binding site. These experiments identified the amiloride binding site in the channel pore of ENaC and confirmed observations from early experiments that amiloride acts on ENaC as a pore blocker (Palmer and Andersen, 1989). The mutations in the ENaC pore that affect the amiloride potency decreased to a similar extent the affinity of other ENaC channel blockers such as triamterene or the amiloride analog benzamil, indicating a common pore blocking mechanism (Kellenberger et al., 2003).

Currently no allosteric inhibitors other than the pore blockers such as amiloride and its derivatives are known to block ENaC activity. However, functional domains important for ENaC activity and its modulation could represent potential target sites for allosteric antagonists. An interesting candidate among them is the N-terminal intracellular domain with highly conserved Cys residues that lead to efficient inhibition of ENaC when modified by sulfhydryl reagents or oxidizing agents (Kellenberger et al., 2005).

b. Renal effects. The renal effects of amiloride are directly related to ENaC inhibition. Amiloride reaches its target in the lumen of the CCD by filtration and secretion in the proximal tubule by an organic cation transporter. The ENaC inhibition results in an increase in urinary Na^+ excretion and a decrease in K^+ secretion. The effect on K^+ secretion is due to the decrease in the

apical Na^+ conductance, which hyperpolarizes the apical membrane and decreases the driving force for K^+ secretion from the tubular cell into the lumen of the CCD. Interestingly, K^+ secretion in the distal nephron depends under normal K^+ diet entirely on ENaC-mediated Na^+ absorption. In contrast, a part of the K^+ secretion is independent of ENaC activity under high K^+ diet (Frindt and Palmer, 2009a).

Because of the limited contribution of ENaC to the overall reabsorption of the filtered Na^+ along the nephron, its effect on Na^+ excretion is smaller than that of loop diuretics or thiazides. Because the K^+ secretion coupled to ENaC-mediated Na^+ absorption is the main secretory pathway for K^+ secretion under standard K^+ diet, the effect of amiloride on K^+ secretion is larger than its effect on Na^+ absorption.

In a randomized, crossover trial involving young healthy volunteers under a standardized diet, amiloride was not different from placebo regarding a short-term 24-hour blood pressure or a Na^+ or K^+ excretion, consistent with the presence of compensatory mechanisms to maintain Na^+ and K^+ homeostasis in healthy subjects exposed to amiloride (Matthesen et al., 2013).

c. Pulmonary effects. As mentioned previously, ENaC is expressed in airway epithelial cells and is crucial for the maintenance of an appropriate ASL level and mucociliary clearance. These processes represent the primary innate defense mechanism against respiratory tract infections. Because cystic fibrosis (CF) pathogenesis involves defective mucus clearance, amiloride was tested as an inhalation therapy for rehydrating ASL, enhancing mucus clearance in the respiratory tract of CF patients. Amiloride failed as an inhalation therapy (Graham et al., 1993; Grasemann et al., 2007). Among the possible reasons that were evoked to explain this failure were first a restricted drug delivery associated with conventional inhalation therapy due to the limited solubility of amiloride, second a limited potency and a rapid dissociation from the receptor, and third a rapid absorption by the airway epithelia. The amiloride derivative benzamil is a more potent inhibitor of ENaC but was not more efficient than amiloride in the treatment of CF. These observations led to an optimization strategy of the amiloride structure-activity relationship. Interestingly, amiloride shows a high structural tolerance for substitutions, allowing the generation of a variety of ENaC blockers (Hirsh et al., 2006). For instance, modifications of the distal guanidinium group to introduce a permanent positive charge reproduced features that resemble quaternary amines with a high potency for ENaC inhibition. This permanent positive charge introduces an increased polarity of the blocking molecule that results in an increased solubility and a restricted permeability across cell membranes. Other amiloride derivatives were obtained by long-chain substitutions at the guanidine nitrogen. One of the compounds, 552-02, was shown to be more potent

and more selective than amiloride, less epithelium-permeant, and able to increase mucus clearance in vivo and ASL volume in CF bronchial epithelial cells (Hirsh et al., 2008; Schoenberger and Althaus, 2013).

2. Indirect Antagonists. Other ENaC antagonists indirectly modulate ENaC activity at the cell surface. The best-known examples are the antagonists of the mineralocorticoid receptor such as spironolactone or eplerenone. The effects on urinary Na^+ or K^+ excretion are very similar to those of amiloride, but these drugs do not require access to the tubule lumen. Endothelin and aldosterone have opposing effects on ENaC-mediated Na^+ absorption in the distal nephron and the collecting duct. Endothelin antagonists are expected to relieve ENaC from inhibition by endothelin, but the physiologic/pharmacological relevance of this phenomenon is unknown.

3. Agonists. As already pointed out, the liquid homeostasis in the adult lung depends on the passive flow of cations via ENaC and cyclic nucleotide-gated ion channels (Berthiaume and Matthay, 2007). A proof of principle for the development of ENaC agonists is provided by the covalent modification of Cys residues introduced at specific sites in the external vestibule of the channel pore, e.g., at the DEG site (section V.C). The compound S3969 was recently reported to reversibly stimulate the human ENaC in heterologous cell expression systems (Lu et al., 2008). This compound acts on ENaC by increasing the channel open probability with an apparent affinity (EC_{50}) of $1 \mu\text{M}$. In addition, a cyclic peptide was shown to activate ENaC by increasing its open probability in heterologous expression systems (Shabbir et al., 2013).

The antinatriuretic effect of vasopressin mediated by the V2R has been attributed to a stimulation of ENaC-dependent Na^+ absorption in the ASDN. In normal healthy volunteers under a high Na^+ diet to suppress aldosterone secretion, the potent V2R agonist desmopressin decreased Na^+ excretion. A 7-day treatment with amiloride prevented the vasopressin-induced natriuresis, consistent with an ENaC-mediated Na^+ absorption (Bankir et al., 2005).

C. Bile Acid-Sensitive Ion Channel

Many of the chemical compounds that inhibit ASICs have also been applied to the related BASIC. Amiloride inhibits the constitutive mouse BASIC current and the bile-activated rat BASIC current with IC_{50} values of 7 and $9 \mu\text{M}$, respectively (Wiemuth and Grunder, 2010; Wiemuth et al., 2012). The diarylamidines diminazene and nafamostat, previously shown to inhibit ASICs, also block BASIC currents with IC_{50} values of 2–6 μM in a voltage-dependent way, suggesting a pore block (Wiemuth and Grunder, 2011; Lefevre et al., 2014). Fenamates such as flufenamic acid are NSAIDs that inhibit the cyclooxygenase in their clinical use and have been known to modulate the function of a large number of ion channels

(Wiemuth and Grunder, 2011). Flufenamic acid and to a lesser extent related drugs, such as diclofenac, activate BASIC at millimolar concentrations, thus at concentrations that are not reached in clinical use of these drugs (Wiemuth and Grunder, 2011). It is interesting to note in this context that diclofenac is known to inhibit ASIC3 with an IC_{50} of $\sim 100 \mu\text{M}$ (Voilley et al., 2001).

VII. Conclusions and Perspectives

The cloning of ENaC and ASICs boosted the research on these channels and made possible the investigation of molecular aspects of their function and cellular roles. The physiologic and pathologic roles of both ENaC and ASICs have been addressed extensively in genetic mouse models. More recently, tissue-specific knockout approaches provided more detailed information on the ENaC physiology. Newly discovered ASIC toxins contributed importantly to the understanding of the involvement of ASICs in pain sensation and neurodegeneration after ischemic stroke. There are still many open questions remaining, and further studies are needed to find answers to questions such as the molecular mechanisms of ENaC regulation by aldosterone or of the involvement of ASICs in pain sensation, synaptic plasticity, and the expression of fear. Studies addressing these questions may identify new drug targets for ENaC regulation and will likely provide more precise information on ASIC physiology and confirm ASICs as promising drug targets.

The recent publication of crystal structures of an ASIC channel has stimulated the structure-function investigation of ASICs. Together, newly available structural and functional information allowed elucidating molecular mechanisms of ASIC and ENaC function. By continuing these investigations, it will be possible to describe the molecular mechanisms by which extra- and intracellular factors control ENaC and ASIC activity and to identify specific sites within the channel proteins that can be targeted by novel drugs.

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