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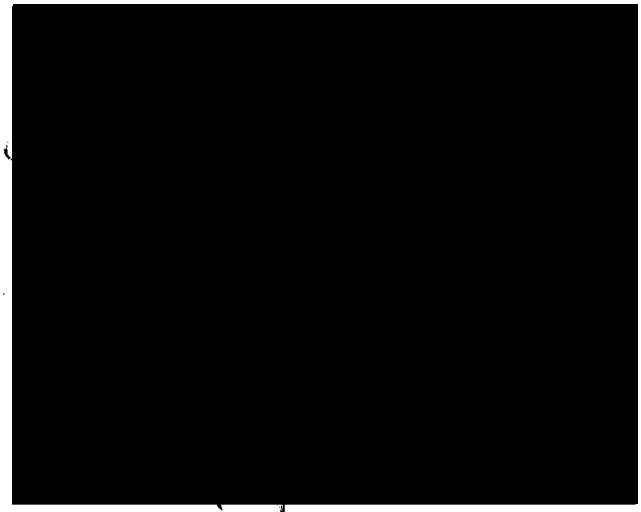

ACTIVATION AND DESENSITIZATION OF ACID-SENSING ION CHANNELS

by

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List of Abbreviations used in this work:

ASIC—Acid-sensing Ion Channel

ENaC—Epithelial Sodium Channel

DEG—Degenerin

FaNaCh—FMRFamide Gated Sodium Channel

MTS—Methanethiosulfonate

TM—Transmembrane Domain

NMDA—N-methyl-D-Aspartate

CNS—Central Nervous System

DRG—Dorsal Root Ganglion

FMRFamide—The peptide Phe-Met-Arg-Phe-amide

RFRP—RF-amide Related Protein

NPFF—Neuropeptide FF

NPSF—Neuropeptide SF

VR1, TRPV1—Vanilloid Receptor

HCN—Hyperpolarization Activated and Cyclic Nucleotide Gated Channel

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Abstract:

This thesis is centered on acid-sensing ion channels (ASICs) and their properties of activation and desensitization. These channels are acid gated channels that are expressed throughout the nervous system. Although their physiological role is unclear, it is thought that they may be sensors of nociceptive signals brought on by changes in extracellular pH.

What these channels are doing in the central nervous system is less clear. Recently, it has been proposed that they may play a pathological role during such ischemic conditions as stroke and seizure. Under ischemic conditions in the CNS, several ionic changes occur which may activate or enhance activation of ASICs: 1) anaerobic respiration causes the extracellular pH to decrease to 6.3, from a resting level of pH 7.4, 2) extracellular calcium can fall as low as 0.1 mM 3) extracellular zinc can increase to as high as 300 μM. In this thesis, I look at how these conditions affect activation of ASIC1a and ASIC1a/2a. I find that all of these conditions can activate or enhance ASICs in the CNS. In addition, these ASICs also exhibit a voltage dependent block by magnesium, which would be relieved by depolarizations induced during ischemia. Entry of sodium or calcium through ASICs during ischemia could then act to cause or enhance neuronal death through both necrotic and apoptotic pathways.

ASICs exhibit desensitization under prolonged acidification. The time course of this can vary depending on the subtype from milliseconds to tens of seconds. I performed some structure function experiments to try and uncover both the mechanism of desensitization as well as identify what creates the distinct kinetics seen in different subtypes. I found a conserved region (T75-S82 in ASIC3) outside the first

transmembrane domain that influences desensitization. Mutations in this region led to changes in desensitization kinetics and the pH dependence of desensitization. I also found that some variable residues, particularly residue 82, can create some of the differences seen in the kinetics of different subtypes. By using substituted cysteine accessibility experiments, I also showed that there is a conformational change that accompanies desensitization. This change buries a highly conserved glutamate at position 79 in ASIC3. This provides new evidence that there is a conformational change outside TM1 during desensitization. This region appears to be critical for regulating desensitization of ASICs and is likely involved in a large conformational change that leads to the desensitized state.

Chapter 1

Introduction

Chapter 1

The work in this thesis focuses on acid-sensing ion channels (ASICs). These ligand gated ion channels are activated by a decrease in extracellular pH. ASICs are expressed in sensory neurons where there is strong evidence suggesting that these channels are the transduction molecule for certain types of pain. They are also expressed in the central nervous system (CNS) where their physiological role is unclear. Although their physiological role in the CNS is uncertain, there is evidence that these ion channels may play a detrimental role during pathological processes such as stroke and seizure. This chapter will elaborate on the properties of these ion channels and their possible roles in physiological and pathological conditions.

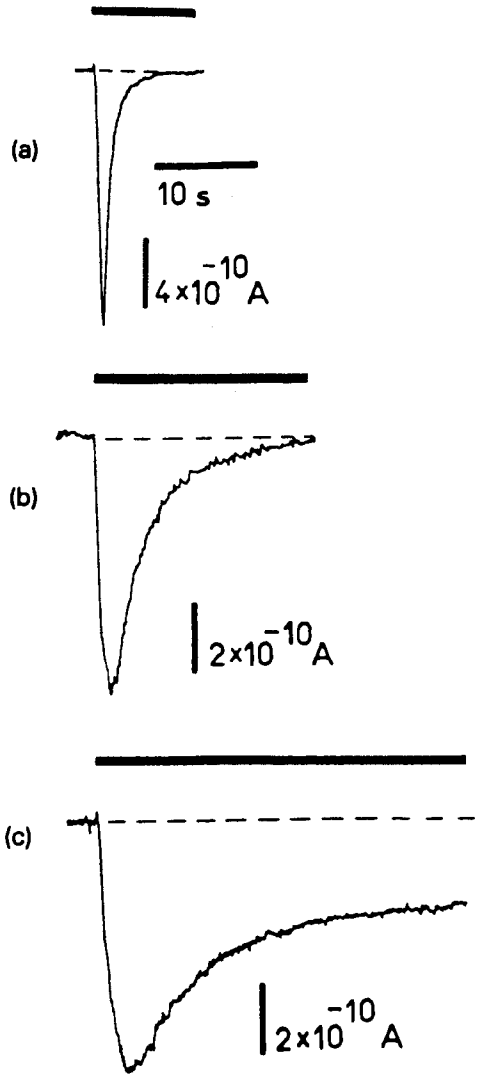
A Brief History

Krishtal and Pidoplichko discovered acid-sensing ion channels in 1980 using voltage clamp recordings from rat sensory neurons (Krishtal and Pidoplichko 1980). This and a series of subsequent papers (Krishtal and Pidoplichko 1981; Krishtal and Pidoplichko 1981; Korkushko and Krishtal 1984) from the same group established the essential features of these molecules: 1) the channels open when pH drops below 7.0; 2) they pass Na^+ over K^+ about as well as voltage-gated Na^+ channels do; 3) amiloride blocks them at rather high concentrations (tens of micromolar); 4) distinct subtypes of the channel evident in different sensory neurons differ in their pH sensitivity and rates of activation and desensitization (Fig. 1).

Krishtal argued that these acid-evoked currents were caused by unique ion channels, whereas another group thought they might be acid-modified Ca^{++} channels

Figure 1

Different desensitization kinetics of acid evoked currents observed in trigeminal ganglia cells. Currents recorded in response to pH stimulus of 6.2. These different kinetics suggested different channels or subunits existed. From Krishtal and Pidoplichko (1981).


(a)
10 s
4x10^-10 A
(b)
2x10^-10 A
(c)
2x10^-10 A

(Morad 1988). The debate was loud but the audience was small and the subject fell from view until 1997. That was when Lazdunski and colleagues cloned an acid-sensing, amiloride-sensitive, Na^+-selective channel and coined the acronym, ASIC (acid-sensing ion channel) (Waldmann, Champigny et al. 1997). The sequence showed that ASICs were a subfamily of mammalian epithelial Na^+ channels (ENaCs) and their relatives in *C. elegans*, degenerins. These channels are all Na^+ selective and amiloride sensitive but differ in their gating mechanisms.

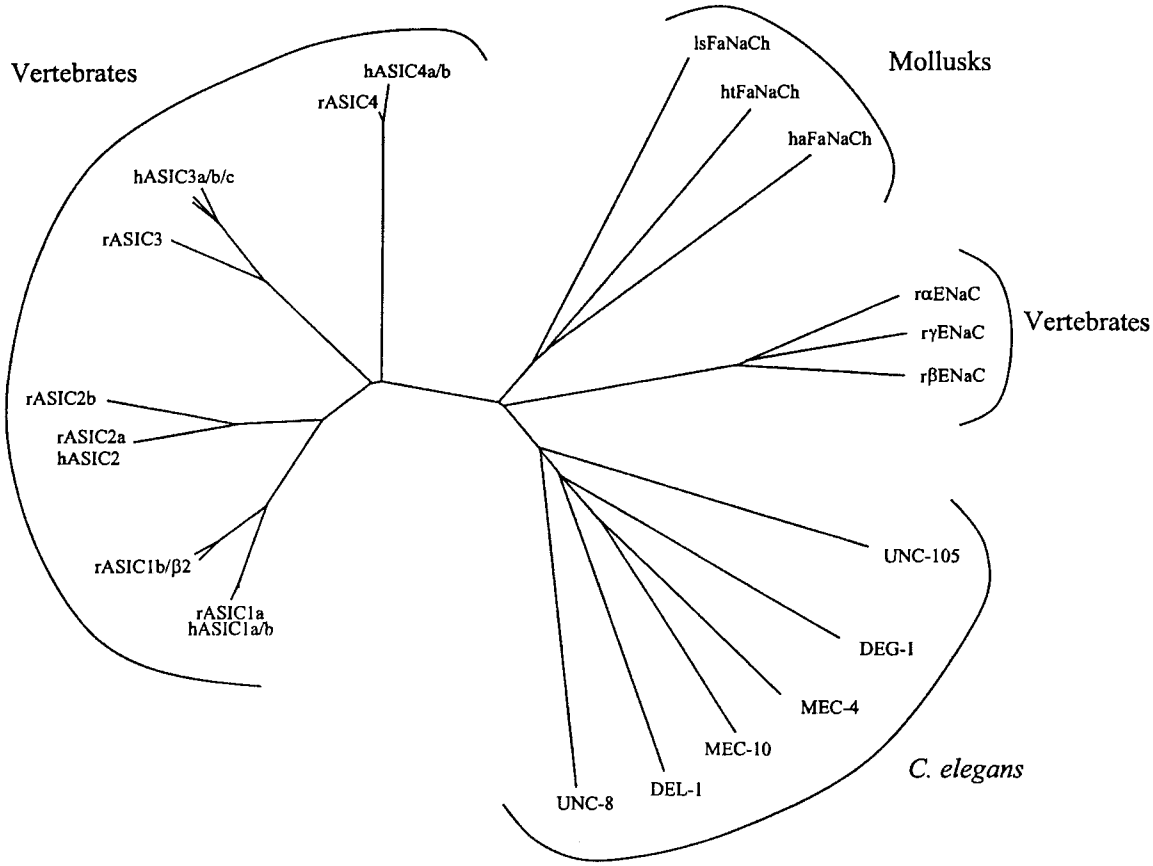
From the first, ASICs were proposed to be transducers for acid-evoked pain (Krishtal and Pidoplichko 1981). They also have been implicated in mechanosensation, taste transduction, and learning and memory. A variety of reviews discuss their various properties and proposed functions (de la Rosa, Canessa et al. 2000; Kellenberger and Schild 2002; Krishtal 2003; Wemmie, Price et al. 2006).

ASICs and the DEG/ENaC Superfamily

ASICs belong to the superfamily of epithelial sodium channels (ENaCs) and degenerins (DEGs) (Fig. 2). All channels in the DEG/ENaC family are sodium selective, voltage insensitive, and blocked by amiloride (albeit at very different concentrations). Some are constitutively open, protons gate some, a peptide (FMRFamide) gates one (Lingueglia, Champigny et al. 1995), and some are mechanosensitive. The ASICs are defined by homology to each other rather than by their acid sensitivity. ASIC1 and ASIC3 are readily opened by small pH changes, but ASIC2 needs extreme pH (5.0) to open and ASIC4 may not be acid sensitive at all. The variability in acid sensitivity suggests that ASICs may prove to have functions that are unrelated to their name.

Figure 2

Phylogenetic tree of DEG/ENaC family members. For ASICs and ENaCs, r denotes rat protein, h denotes human. For FaNaChs, ha is *Helix aspersa*, ht is *Helix trivolvis*, and ls is *Lymnaea stagnalis*. All DEGs are from *Caenorhabditis elegans*. Protein sequences aligned using ClustalW.


Vertebrates
hASIC4a/b
rASIC4
hASIC3a/b/c
rASIC3
rASIC2b
rASIC2a
hASIC2
rASIC1b/β2
rASIC1a
hASIC1a/b
lsFaNaCh
htFaNaCh
haFaNaCh
Mollusks
rαENaC
rγENaC
rβENaC
Vertebrates
UNC-105
DEG-1
MEC-4
MEC-10
DEL-1
UNC-8
C. elegans

ENaCs are among the most critical ion channels to mammalian biology. Expressed in epithelial cells, they control sodium reabsorption for fluid homeostasis. For example, by controlling sodium flux across kidney epithelia, they control the amount of blood in the body (Benos and Stanton 1999). Amiloride can block ENaCs at tens of nanomolar, making it clinically useful for control of blood pressure. Concentrations at least a thousand-fold greater are needed to block ASICs.

The dominant "degenerin" mutations cause the neurons that express this protein in *C. elegans* to swell and lyse (Chalfie and Wolinsky 1990; Hall, Gu et al. 1997; Garcia-Anoveros, Garcia et al. 1998), due to a constitutive activity of the ion channel (Hong and Driscoll 1994). Most of the DEGs are expressed in mechanosensory cells, leading to "mechanosensory abnormal" (MEC) or uncoordinated (UNC) phenotypes in the mutants and raising the possibility that these channels are mechanosensors.

The first ASIC cloned was ASIC2a, originally called MDEG (mammalian degenerin) and BNC1 (Brain Na^+ Channel 1) (Price, Snyder et al. 1996; Waldmann, Champigny et al. 1996). It has homology to DEGs (20-29% identity) and is constitutively active with a degenerin mutation. Neither group that cloned it connected it with Krishtal's acid-gated sodium channels; indeed, ASIC2a requires quite extreme acid (~pH 5) to open.

ASIC1a was cloned by two groups: Corey's, who named it BNaC2 (Brain Na^+ Channel 2) (Garcia-Anoveros, Derfler et al. 1997), and Lazdunski's, who realized its acid sensitivity and named it ASIC (Waldmann, Champigny et al. 1997). ASIC1a in rats is a 526 amino acid protein that is expressed throughout the peripheral and central nervous system. An extracellular pH drop below 6.9 activates a transient amiloride sensitive

sodium current, with a half maximal activating pH ($pH_{0.5}$) near 6.5 (table 1) (Waldmann, Champigny et al. 1997; Alvarez de la Rosa, Zhang et al. 2002; Babini, Paukert et al. 2002). ASIC1a has a significant Ca^{++} permeability, and is blocked by higher levels of Ca^{++}. This channel has similar kinetics, expression pattern, pharmacology, and selectivity to some of the currents described by Krishtal and Pidoplichko.

The Lazdunski group then cloned and characterized rat ASIC3 (Waldmann, Bassilana et al. 1997). It was originally called DRASIC (dorsal root ASIC) because its mRNA was found only in dorsal root ganglion sensory neurons in rat. ASIC3 is more sensitive to acid than ASIC1a with a $pH_{0.5}$ of 6.7 and a steeper activation curve (Sutherland, Benson et al. 2001). ASIC3 desensitizes much faster than ASIC1a at pH 6. Calcium ions carry current through ASIC3 in the absence of Na^{+} (Sutherland, Benson et al. 2001), but there is no reported Ca^{++} permeability in the presence of Na^{+}.

Lazdunski's group then showed that ASIC2a is also a proton gated ion channel (Lingueglia, de Weille et al. 1997). ASIC2a is much less sensitive to protons than either ASIC3 or ASIC1a, with a $pH_{0.5}$ of 4.35 (Champigny, Voilley et al. 1998). They also cloned a splice variant of ASIC2 with a different amino terminus, ASIC2b (MDEG2). ASIC2b is inactive as a homomer but modulates the kinetics, selectivity and sensitivity of both ASIC2a and ASIC3 when coexpressed (Lingueglia, de Weille et al. 1997). ASIC2a and ASIC2b are both expressed throughout the central and peripheral nervous systems (Lingueglia, de Weille et al. 1997; Alvarez de la Rosa, Zhang et al. 2002).

Other labs have since cloned further genes and splice variants in this family. ASIC1b (ASIC-β) and ASIC-β2 are splice variants of ASIC1 (Chen, England et al. 1998; Bassler, Ngo-Anh et al. 2001; Ugawa, Ueda et al. 2001). ASIC1b lacks the Ca^{++}

Table 1

Summary of properties of ASIC homomers and heteromers

* = Sodium selective initial current, nonselective sustained current

S = Sensory Neurons

C = Central Nervous System

Channel	Other Names	P_{Na}/P_K	P_{Na}/P_{Ca}	$pH_{0.5}$	Location	Modulating Peptides
ASIC1a	ASIC, ASICα, BNaC2	7.8-13	2.5-18.5	5.5	C/S	FMRF-RFRP1/2
ASIC1b	ASICβ	14	high	5.9	S	
ASIC2a	BNC1, BNaC1, MDEG1	10	20	4.35	C/S	
ASIC2b	MDEG2	Inactive	-	-	C/S	
ASIC3	DRASIC	13.5	high	6.5-6.7	S	NPFF-FMRF-NPSF-RFRP1/2
ASIC4	SPASIC	Inactive	-	-	C	
ASIC1a/2a		7.2	36	4.8		
ASIC2a/2b		*		3.9		
ASIC2a/3		>1 for both	high	6.5		NPFF-FMRF
ASIC3/2b		*		6.5-6.7		

permeability of ASIC1a, has a decreased sensitivity to protons ($pH_{0.5}$=5.84) (Babini, Paukert et al. 2002), and is restricted to sensory neurons. ASIC-β2 does not form functional homomers, but does associate with ASIC1b to lower its pH sensitivity.

ASIC4 was the last member of the ASIC family to be cloned (Akopian, Chen et al. 2000; Grunder, Geissler et al. 2000). It is expressed in the CNS and has neither been shown to form a functional ion channel nor to modulate the acid sensitivity of other ASICs. This channel has limited expression in the CNS, being highly expressed in the pituitary. Currently, there is no proposed role for this channel, and nothing has been shown to activate it.

The human ASICs are different from the rat ASICs described above. They show different expression patterns, isoforms, and pharmacology. Human ASIC3 for example has three isoforms, and is in the CNS, sensory neurons, and internal organs like lung and kidney (Babinski, Le et al. 1999).

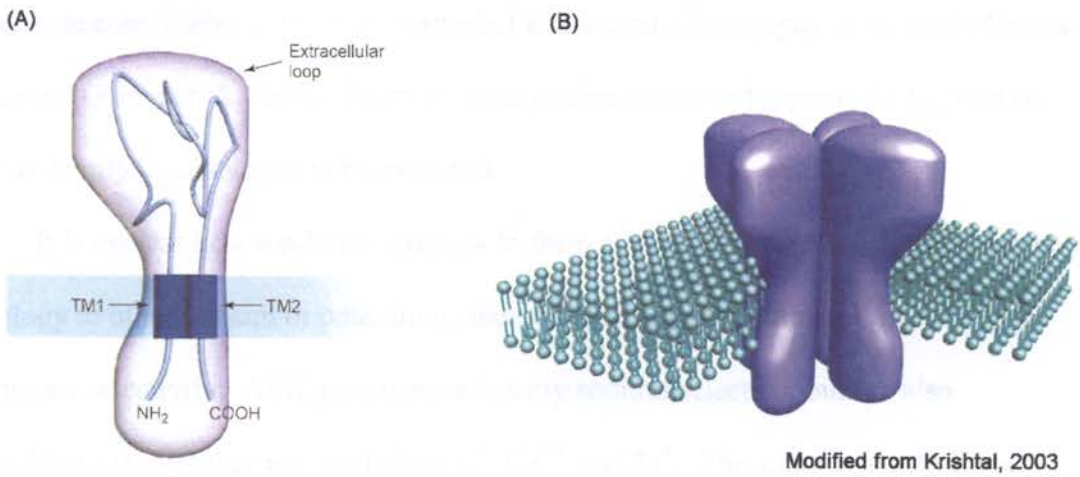
Amino Acid Structure

ASICs have two predicted transmembrane domains in their structure, with most of the protein being in the extracellular loop. A possible arrangement of subunits is shown in figure 3. This shows four subunits coming together to form a functional channel. The stoichiometry of these channels is controversial however, as will be discussed further.

There are two conserved cysteine-rich domains (CRDs) in the extracellular loop that are common to all DEG/ENaC family members. These CRDs in ENaCs play a role in proper expression and activation of the channel (Firsov, Robert-Nicoud et al. 1999).

Figure 3

Schematic for possible ASIC assembly. (A) shows the topology of one subunit of a channel with most of the protein making up a large extracellular loop. (B) A possible arrangement of a functional channel with four subunits assembling and TM2 forming a large part of the pore. (Modified from Krishtal 2003)



Modified from Krishtal, 2003

Since this family has such a large extracellular loop, disulfide linkages within these CRDs are likely critical for correct folding and stabilization of the channel.

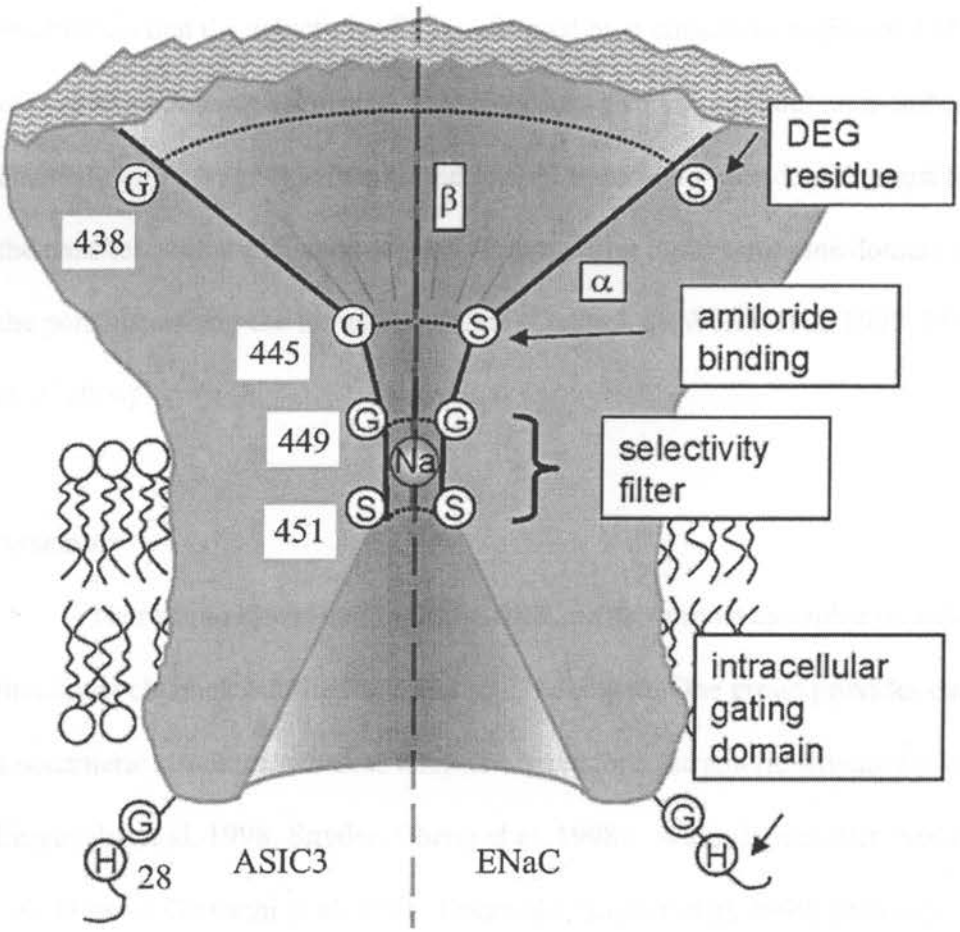
A number of putative gating regions have been conserved among family members. There is an HG motif on the intracellular side of all family members (Fig. 4). These two amino acids have been found to affect the gating of ENaCs: when mutated, the open probability drops dramatically (Grunder, Jaeger et al. 1999). In contrast, the "degenerin" site which is conserved as either a glycine or a serine seems to act as a negative modulator of gating. Mutations of this amino acid to bulky hydrophobic amino acids cause constitutive activity of channels (Waldmann, Champigny et al. 1996; Garcia-Anoveros, Garcia et al. 1998). There are many other conserved regions throughout this channel family that have yet to be explored.

It is unclear how a subunit arranges to form a functional channel. They share no homology to other sodium or potassium channels that have a re-entrant pore loop that determines selectivity. ASICs are predominantly sodium selective, but are also permeable to many other ions including K^+, Ca^{++}, and Li^+. This contrasts with ENaCs however, which are almost purely sodium selective. The only other permeant ion in ENaCs is lithium, which has a smaller ionic radius than Na^+, suggesting that ENaCs have a very narrow pore that excludes anything larger than sodium.

Evidence from ENaCs is inconclusive as to how the selectivity filter is arranged in the pore. Some speculate it may form a pore loop similar to potassium channels, whereas others argue it has a simpler shape as illustrated in figure 4. The best evidence arguing against the pore loop model comes from the proposed location of the amiloride binding site. If the channel were to form a pore loop, then the amiloride binding site

Figure 4

Model of the pore of ASIC/ENaCs. ASIC3 amino acids are shown on the left, with the analogous residues of αENaC on the right. Modified from Kellenberger et al., 2002.


DEG
residue
β
α
438
445
449
451
amiloride
binding
selectivity
filter
Na
intracellular
gating
domain
28
ASIC3
ENaC

would be internal to the selectivity filter. This would not fit with the observed data on amiloride block of the channel (Kellenberger, Gautschi et al. 2003).

The second transmembrane domain (TM2) is another region of the channel that has been conserved throughout the evolution of this family. As the conservation suggests, this region is very important to the channel structure and function. It is well established that the selectivity filter is formed by a conserved region of TM2 (Sheng, Li et al. 2000). A short sequence (G-X-S) is conserved throughout all members of the ion channel family (Fig. 4). Clearly, the second transmembrane domain must line the pore of the channel. Other evidence suggests that the first transmembrane domain may also line the pore, especially the intracellular side (Coscoy, de Weille et al. 1999; Pfister, Gautschi et al. 2006).

Assembly

There is no doubt that multiple ASIC proteins must assemble in order to form a functional channel, but the stoichiometry is debated. One group provides data arguing for a nonameric structure, whereas another argues for a tetrameric structure (Coscoy, Lingueglia et al. 1998; Snyder, Cheng et al. 1998). A similar conflict exists in the ENaC field (Firsov, Gautschi et al. 1998; Eskandari, Snyder et al. 1999; Staruschenko, Adams et al. 2005). It seems likely that ASICs assemble with the same stoichiometry as ENaCs given their conserved topology. It is unclear at this point which stoichiometry is correct, as seemingly similar experiments have yielded contrasting results.

Unlike ENaCs, whose functional channels must contain different channel subtypes (Canessa, Schild et al. 1994), most ASICs can form homomeric ion channels.

ASIC1a, 1b, 2a and 3 can all form functional homomeric channels that are activated by low pH. ASIC2b can form heteromers with other ASICs to modify their activity, but is inactive by itself. It is unclear if it is inactive because it does not assemble, is not trafficked to the membrane, or is simply not activated by low pH. ASIC4 has neither been shown to form a functional ion channel nor to modulate other subunits.

Heteromeric assembly

ASIC1a and ASIC2a share common patterns of expression in the central nervous system, suggesting they may co-localize to form heteromers. Heteromeric channels with unique properties have been seen in oocytes, cell lines and neurons (Bassilana, Champigny et al. 1997; Baron, Waldmann et al. 2002; Hesselager, Timmermann et al. 2004). The heteromer is less selective than either homomer, has a decreased permeability to Ca^{++}, and intermediate proton sensitivity.

ASIC2b is electrically silent as a homomer, but can change the properties of ASIC2a and ASIC3 when coexpressed. ASIC2a loses sensitivity to protons and creates a sustained non-selective current when ASIC2b is present (Lingueglia, de Weille et al. 1997; Ugawa, Yamamoto et al. 2003). Coexpression of ASIC2b with ASIC3 does not change the pH sensitivity of ASIC3; however, the normal sodium selective sustained current seen with ASIC3 at low pH becomes non-selective (Lingueglia, de Weille et al. 1997). ASIC2b has no significant effect when coexpressed with ASIC1a.

ASIC2a and ASIC3 are coexpressed in many DRG neurons. When expressed together in oocytes, they form a functional heteromeric channel with novel properties (Babinski, Catarsi et al. 2000). There is a large increase in low pH evoked sustained current that is sodium selective like the sustained current seen in ASIC3 homomers.

Additionally, ASIC2a/3 heteromers exhibit a prominent sustained current within physiological pH ranges (Yagi, Wenk et al. 2006). Unlike a similar sustained current in ASIC3 homomers, the heteromers have a larger current that persists as the pH continues to drop.

The ASIC currents seen in mouse DRG are different than those evoked from cloned channels suggesting the presence of heteromers in vivo. Benson reproduced the currents evoked from medium to large DRG cells by expressing three subunits together: ASIC1a, 2a, and 3 (Benson, Xie et al. 2002). It is not clear whether or not the three subunits all combine to form one channel, or if native currents are due to a mix of heteromers composed of two subunits. In individual knockouts of ASIC1, ASIC2a, or ASIC3, DRG ASIC currents matched heteromeric currents of the remaining two subunits. ASIC3 seems necessary for the fast desensitization kinetics seen in DRG, since the ASIC3 knockouts had slow kinetics. ASIC1 in mouse seems significantly different than the rat clone. Rat ASIC1 is substantially less sensitive to pH than rat ASIC3, whereas the two mouse clones both activate at and just below pH 7.

Pharmacology

Like all members of the DEG/ENaC family, ASICs are blocked by amiloride. The amiloride block is voltage dependent (Adams, Snyder et al. 1999) with an IC_{50} ranging from 10-100 μM depending on the subunit composition of the channel. ENaCs have a much higher affinity for amiloride, typically on the order of 100 nM (McNicholas and Canessa 1997). At high enough concentrations, amiloride can block L and T-type Ca^{++} channels (Tang, Presser et al. 1988; Garcia, King et al. 1990), Na^{+}/Ca^{++} exchangers,

Na^+/H^+ exchangers, Na^+ pumps, Ca^{++} pumps (Murata, Harada et al. 1995) and other channels. The low affinity of amiloride for ASICs, coupled with its promiscuity as a blocker makes it a weak pharmacological tool. The mechanosensing ion channel blocker Gd^{3+} also blocks ASIC2a/3 heteromers (Babinski, Catarsi et al. 2000), but this is even less selective than amiloride.

Residues contributing to amiloride binding are well-established in ENaCs; amiloride is thought to bind to two sites, one in the extracellular domain, and one near the pore (Fig. 4)(Ismailov, Kieber-Emmons et al. 1997; McNicholas and Canessa 1997; Schild, Schneeberger et al. 1997). Insight into the reason why ASICs are so much less sensitive to amiloride than ENaCs arises from a study that mutated the residues in ASICs that are analogous to the ENaC amiloride binding site near the pore. The mutation eliminated block and unmasked a second action of amiloride: enhancement of the ASIC current (Adams, Snyder et al. 1999). The interpretation is that ASICs have two amiloride binding sites, one that activates and one, like in ENaCs, that blocks. ASICs also lack a binding site in the extracellular domain that is conserved in ENaCs which may explain the lower affinity for amiloride.

There have been two reports of peptide toxins that act as ASIC specific blockers: Psalmotoxin 1 (PcTx1) which comes from the venom of a South American tarantula (Escoubas, De Weille et al. 2000; Escoubas, Bernard et al. 2003), and APETx2 from a sea anemone (Diochot, Baron et al. 2004). PcTx1 is specific for ASIC1a homomers and blocks with an IC_{50} of 0.9 nM. This potent toxin has proven useful to distinguish ASIC1a homomers from heteromers in the CNS (Baron, Waldmann et al. 2002). APETx2 is

selective for ASIC3 containing channels (except ASIC2a/3 heteromers) with an affinity ranging from 63 nM to 2μM.

The block of ASIC1a by PcTx1 has been extensively characterized. It is not a voltage dependent blocker like amiloride, and acts at a different site (Salinas, Rash et al. 2006). It has a higher affinity for the desensitized state of ASIC1a and prevents opening by trapping channels in the desensitized state (Chen, Kalbacher et al. 2006).

New small molecule inhibitors of ASICs are also being developed by the pharmaceutical industry in the hopes of developing new analgesics. One such blocker is A-317567 developed by Abbott Laboratories (Dube, Lehto et al. 2005). Unfortunately, this inhibitor lacks the selectivity and the high affinity of the natural peptide inhibitors. This small molecule does however show effectiveness against inflammatory pain models.

Divalent ions heavily regulate ASIC activity. The divalent ion Zn^{++} potentiates current in ASIC2a containing channels (Baron, Schaefer et al. 2001). Zn^{++} does not enhance ASIC3 or ASIC1a homomers, but does potentiate heteromers formed with ASIC2a. The potentiation is greater at higher pH values, with a greater than seven fold enhancement at pH 6.0 for ASIC2a with an EC_{50} of 120 μM. The presence of Zn^{++} (300 μM) caused a leftward shift of the activation curve of ASIC1a/2a heteromers ($pH_{0.5}$ from 5.5 to 6.0). Two histidine residues are essential for this potentiation; when either one is mutated to alanine the effect disappears.

Zinc has the opposite effect on ASIC1a homomeric channels. It acts as an inhibitor of these channels by binding at a high affinity site formed by a lysine in the extracellular domain (Chu, Wemmie et al. 2004). This lysine is not present on other ASIC subunits, explaining its specificity for ASIC1a.

ASIC currents are very sensitive to Ca^{++} (Krishtal and Pidoplichko 1981). Ca^{++} decreases both single channel conductance and open probability (Korkushco, Krishtal et al. 1983; de Weille and Bassilana 2001). Dropping extracellular Ca^{++} increases the current through ASICs and shifts its activation curve to more basic pHs (Immke and McCleskey 2001). The steady state desensitization curves of ASICs are also shifted by Ca^{++}, with higher levels of Ca^{++} preventing desensitization (Babini, Paukert et al. 2002). Ca^{++} has a central role in gating the channel as will be discussed further.

FMRFamide (Phe-Met-Arg-Phe-amide) gates one DEG/ENaC channel called, FMRFamide-gated sodium channel (FaNaCh), to which ASICs have significant homology (38% between *H. Aspersa* FaNaCh and rat ASIC3). This led to the question of whether or not FMRFamide or other peptides can modulate ASICs. Injection of RFamide peptides have been shown to cause algogenic behavior in mice (Yudin, Tamarova et al. 2004). Mammals do not have FMRFamide, but they have related peptides such as neuropeptide FF (NPFF). NPFF is involved in pain modulation via both opioid dependent and independent pathways (Roumy and Zajac 1998). Although RFamide peptides do not gate ASICs, they do slow the rate of desensitization, and introduce a sustained current (Askwith, Cheng et al. 2000). Interestingly, the peptides need to be applied prior to proton activation to have an effect. NPFF heavily modulates ASIC3 and ASIC2a/3 heteromers in oocytes, but has no effect on ASIC1a or 2a (table 1). FMRFamide modulates all the above channels except homomeric ASIC2a. Unlike the rat channels, neither NPFF nor FMRFamide modulates human ASIC3 (Catarsi, Babinski et al. 2001). NPSF, another mammalian RFamide peptide, also potentiates ASIC3 and DRG acid evoked currents (Deval, Baron et al. 2003). Experiments with other RFamide

related peptides show slowed desensitization and increased peak amplitude in DRG and heterologously expressed ASIC1 and ASIC3 (Xie, Price et al. 2003). The ability of RFamide peptides to induce sustained currents in some ASICs raises the idea that they can increase the sensitivity of ASICs in sustained pain states.

Often changes in extracellular pH are accompanied by intracellular pH. For example, during ischemia in the brain, the extracellular pH change is driven by the intracellular pH changes caused by lactic acidosis (Nedergaard, Kraig et al. 1991). This accompanying intracellular pH change has an effect on ASIC currents activated by the extracellular pH change (Wang, Chu et al. 2006). As the internal pH drops, ASIC currents are inhibited. Conversely, ASIC currents are enhanced by an alkalinization of the inside of the cell. The mechanism behind this modulation of ASICs has yet to be elucidated.

Activation

ENaCs are not gated by ligands or voltage, but are constitutively active. They are regulated either through controlling surface expression, or by modifying their open probability. ENaCs are also blocked by Ca^{++} (Berdiev, Latorre et al. 2001), which can be removed with mechanical forces to open the channel (Ismailov, Berdiev et al. 1997). ASICs evolved from ENaCs, so it is curious how they evolved ligand activation.

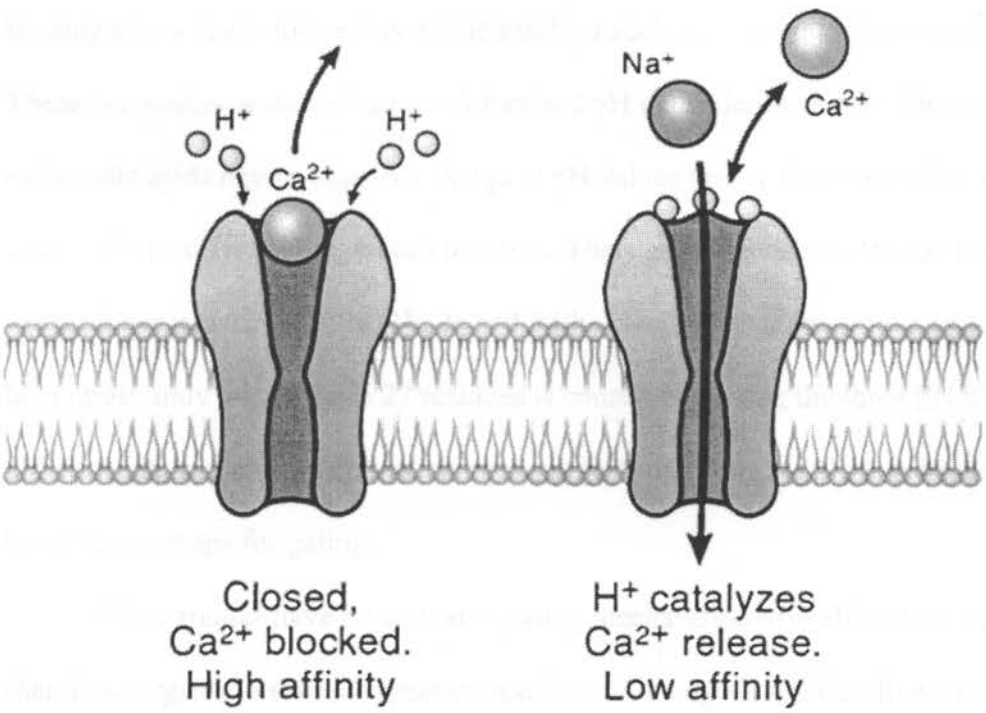
Previous work in the lab has led to an idea for ASIC gating that has been controversial. ASIC gating is highly sensitive to calcium concentration in the extracellular medium (Krishtal and Pidoplichko 1981; Korkushco, Krishtal et al. 1983; de Weille and Bassilana 2001; Babini, Paukert et al. 2002; Immke and McCleskey 2003).

This raises the possibility that the proton binding site on ASICs is a titrateable calcium binding site, more or less like calcium chelators such as EGTA (Fig. 5). They tested this hypothesis on ASIC3 and, consistent with it, found that: a) Ca^{++} and H^{+} compete at the gating site; b) eliminating extracellular Ca^{++} opens the channel without any change in extracellular pH (Immke and McCleskey 2003). A mathematical model, which is qualitatively the same as the math for EGTA, could qualitatively predict channel opening by decreased Ca^{++}, and its H^{+} dependence, from data describing opening by increased H^{+} and its Ca^{++} dependence. Like a Ca^{++} chelator, the affinity of the channel for Ca^{++} diminishes as pH drops. It has an apparent affinity of around 150 nM at pH 9, 10 μM at pH 7.4, and 100 μM at pH 7.0. Thus, protons essentially catalyze release of Ca^{++} from this binding site and they suggested that this release is the event that opens the channel. At physiological Ca^{++} (mM) concentrations, negligibly few channels will be free of Ca^{++} at pH 7.4 whereas nearly 10% will be free at pH 7.0, more or less the fraction of channels open at this pH. Multiple protons must bind for effective Ca^{++} release, conveniently explaining the very steep proton activation curve of ASIC3.

The mathematical model that fit all their gating data also quantitatively fit Ca^{++} block data on single channels. This led them to suggest that the Ca^{++} ion involved in gating simply blocks the pore—in other words, that the channel does not open due to a proton-induced conformation change but, rather, due to a relief of Ca^{++} block. In contrast, desensitization clearly appears to be a slow, H^{+}-driven conformation change. The model seems appealing and explained a variety of data on ASIC3, but it needs to be critically examined on other ASICs. At present, one study in the literature seems supportive.

Figure 5

Schematic showing proposed gating mechanism for ASIC3. At rest, ASIC3 has a high affinity for Ca^{++}, which blocks the channel. When H^{+} bind they decrease the affinity for Ca^{++}. When Ca^{++} is released, the channel is relieved from block and opens. From Immke and McCleskey (2003).


H+
H+
Ca2+
Na+
Ca2+
Closed,
Ca2+ blocked.
High affinity
H+ catalyzes
Ca2+ release.
Low affinity

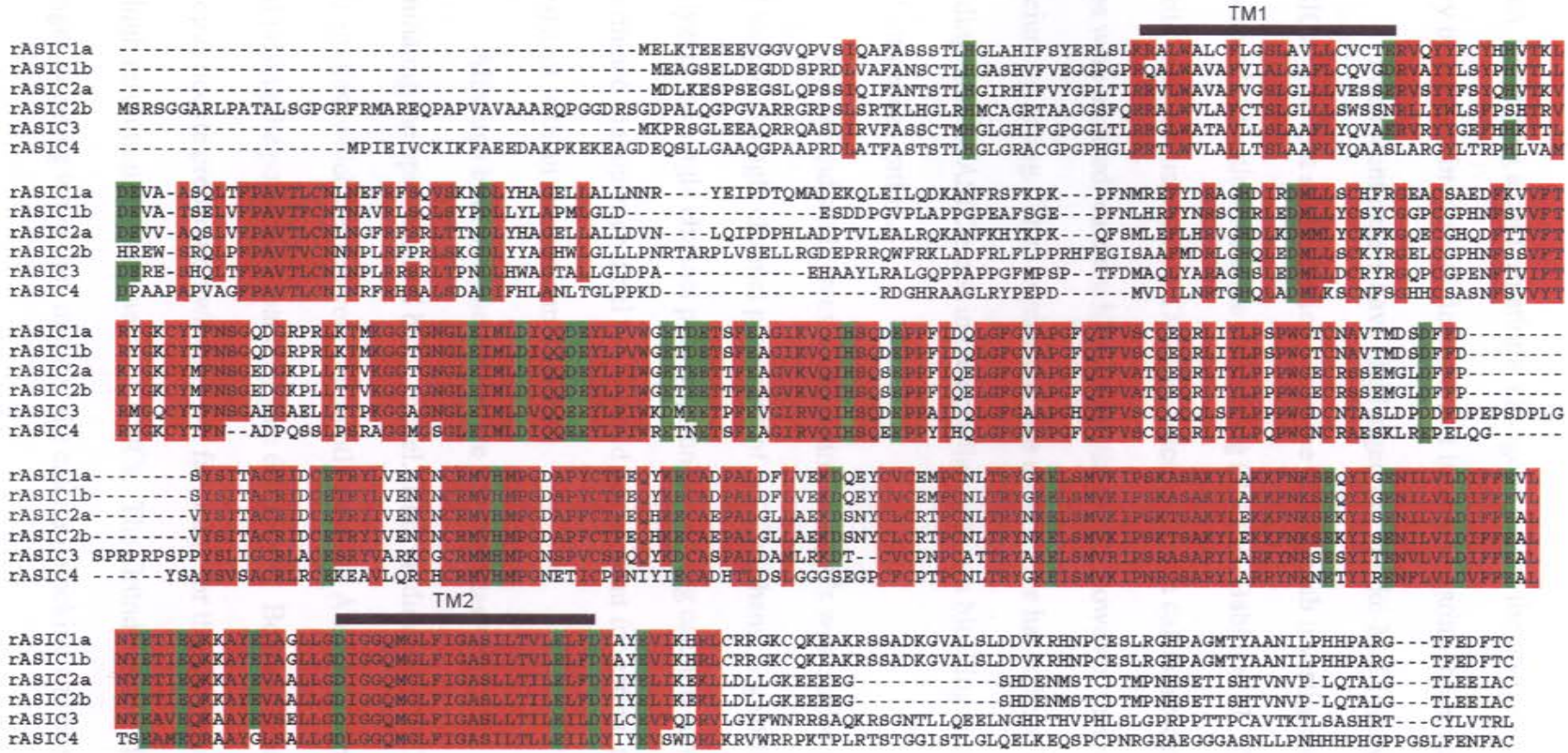
Askwith et al. found that decreasing temperature slows ASIC desensitization with little effect on activation rate (Askwith, Benson et al. 2001). Because conformational changes are affected by temperature far more than channel block, this supports the idea that channel activation is not a conformation change.

Supporting the EGTA model for gating is the striking number of acidic residues conserved in ASICs (Fig. 6). If ASICs are gated by a calcium block, then the calcium binding site is likely formed by acidic residues such as glutamate (E) or aspartate (D). These two amino acids will bind calcium in a pH dependent manner. Their terminal carboxylic acids have a negative charge at pH values higher than their pKa which can bind to the positively charged calcium ion. There are 27 conserved acidic residues on the extracellular loop of ASIC1a, 1b, 2a and 3 (the channels that are proton gated) (Fig. 6). In contrast, only one of these 27 residues is conserved among the three ENaCs. This difference in conserved negative charges suggests that they are playing a functional role in ASICs, perhaps for gating.

Other studies have investigated gating mechanisms of ASICs using mutations or chemical reagents to reveal important residues. A study on ASIC2a found that when Gly430 is mutated to a bulkier amino acid, the proton sensitivity can increase more than two orders of magnitude (Champigny, Voilley et al. 1998). Besides increased proton sensitivity, the mutant channel also fails to desensitize. Gly430 is the same residue that causes the degenerin phenotype when mutated (Waldmann, Champigny et al. 1996). This residue in ASIC2a is also implicated in gating due to its MTS reactivity in open but not closed states (Adams, Snyder et al. 1998). It is only accessible when the channel is

Figure 6

Alignment of the six major rat ASICs. The two black bars indicate the two putative transmembrane domains. Amino acids highlighted in green are conserved acidic residues or histidines. All other conserved residues are highlighted in red. Sequences aligned using ClustalW (http://align.genome.jp/).


TM1
TM2

open, suggesting a conformational change exposing the residue. Clearly, this residue is very important for channel function, but its role in gating the channel is uncertain.

Two recent papers have challenged the Immke-McCleskey gating model for ASICs. The first study, carried out in the Gründer lab investigated the claim that calcium block and activation were the same thing (Paukert, Babini et al. 2004). They found some acidic residues just outside TM2 that acted to form a calcium blocking site. After these sites were mutated, calcium block was eliminated; however, there was still an effect of calcium on gating. They argued that this meant there has to be at least two calcium binding sites on ASICs: one in the pore that acts as a blocking site and another elsewhere that influences gating.

The second study to examine gating in ASICs was carried out in the Canessa lab and looked at single channel properties of ASICs (Zhang, Sigworth et al. 2006). By analyzing the single channel properties under varying calcium concentrations they determined that open channel block could not account for gating, and that gating was consistent with an allosteric mechanism.

Although both of these studies are very persuasive studies, neither is conclusive in eliminating the open channel block model. Both studies use ASIC1 (rat or toadfish) in their studies, which behave very differently from rat ASIC3 that Immke and McCleskey used raising the possibility that they gate differently. Both of these studies also used a xenopus oocyte preparation which is not favorable for the fast solution exchanges needed for high resolution measurements of ASICs. The Gründer study also did note some changes in gating when they mutated the calcium blocking site from ASIC1a. There is

definitely still controversy over the mechanism of activation of ASICs and more experiments are needed to define how ASICs activate.

Desensitization

All acid-sensing ion channels exhibit desensitization upon prolonged acid stimulation. Each subunit desensitizes with different time courses and pH sensitivities. With ASIC1a and ASIC2a, the speed of desensitization increases as the pH decreases. The desensitization kinetics of ASIC3 are relatively unaffected over the pH range of its activation curve. ASIC3 does have unique desensitization properties at very low pH values. Below pH 5, it has a biphasic current, with a rapidly desensitizing component, followed by the generation of a sustained phase. It is unclear whether or not this plays any physiological relevance given the extremely low pH required for activation of this sustained current. There is another sustained component of ASIC3 current that seems more physiologically relevant (Yagi, Wenk et al. 2006). This persistent current is generated between pH 7.2 and 6.8, which is within the range of proton concentration created during ischemic muscle contraction. A sustained current occurs in this range where there is activation, yet desensitization is incomplete. Channels can go back and forth between desensitized, activated, and closed states creating a relatively small current that lacks the appearance of desensitization.

Although the mechanism behind activation is controversial, there is general agreement that desensitization involves a conformational change. The Q10 of desensitization(4.4) is much greater than that of activation (Askwith, Benson et al. 2001). This strongly suggests that desensitization involves a conformational change.

An area outside the first transmembrane has been identified by Canessa as being involved in desensitization (Coric, Zhang et al. 2003). By comparing ASIC1a from rats and toadfish, they identified a sequence that accounts for the desensitization differences in the two species. A recent study also points to an area near the first transmembrane domain as being involved in desensitization (Pfister, Gautschi et al. 2006). Unlike the previous sequence, this one is inside the cell. They observed an intracellular residue that altered its accessibility to MTS reagents depending on the state of the channel. Both these studies suggest that the TM1 area is involved in desensitization, which appears to involve a conformation change.

Different ASIC subunits have very different desensitization kinetics and sensitivities. ASIC3 desensitizes relatively quickly at pH 6.0 (~400 ms), and its speed is relatively unaffected by pH over its range of activation. ASIC1a desensitizes more than 5 times slower than ASIC3 at pH 6.0 (~2s), and its desensitization kinetics are highly pH dependent with very fast desensitization at lower pH values (~100 ms at pH 4.5). ASIC2a behaves similarly to ASIC1a with slow kinetics that increase as the pH drops. It is unknown what causes these differences between different subunits.

Proposed Sensory Functions

The ability to sense pH makes ASICs prime candidates for transducing acid-evoked pain and taste sensations. Their relation to degenerins makes them appealing as possible mechansensing ion channels. Expression patterns and channel properties may be consistent with these ideas, but definitive tests in whole animals are lacking.

Pain/Nociception:

Krishtal and Pidoplichko found that 74% of DRG neurons expressing an acid-sensitive current were smaller that 26 μm (Krishtal and Pidoplichko 1981), demonstrating expression in neurons that make Aδ and C axons that carry the bulk of nociceptive information (Waddell and Lawson 1990). Immunocytochemistry shows ASIC1 co-expresses with vanilloid receptors (VR1), Substance P and CGRP, all markers for nociceptors (Olson, Riedl et al. 1998). The distribution is consistent with ASICs playing a role in pain, but does not mean this need be their only function.

Experiments on humans have shown that amiloride diminishes the pain associated with injection of a pH 6.0 solution (Ugawa, Ueda et al. 2002). The VR1 antagonist capsazepine has no effect on the intensity of pain perceived at pH 6.0, but does reduce it at pH 5.0, although to a lesser extent than amiloride. This provides evidence for the role of ASICs in transducing pain from acidic stimuli. It suggests that VR1 plays little or no part in sensing acid within the physiological range.

Inflammation causes transcript levels of ASICs to increase (Voilley, de Weille et al. 2001) through transcriptional control that involves serotoninergic and nerve growth factor pathways (Mamet, Baron et al. 2002). Nonsteroid anti-inflammatory drugs (NSAIDs) such as aspirin and ibuprofen suppress this increase. In addition to suppressing the increase in transcript levels, NSAIDs also directly inhibit the currents produced by ASICs in both DRG and ASIC3 or ASIC1a expressing COS cells. The sensitivity of ASIC expression to persistent pain conditions is further circumstantial evidence for a role in pain.

Benson et al. fluorescently tagged sensory neurons that innervate heart muscle and found that they all expressed ASIC3-like current at exceedingly high levels (Benson, Eckert et al. 1999; Sutherland, Benson et al. 2001). As a sensory system, heart is unusual in that the only conscious sensation that arises from it is pain (angina) and the only trigger for this is ischemia, when the heart gets insufficient oxygen for its metabolic demand. Thus, the expression is consistent with ASIC3 being a sensor for ischemic pain by detecting lactic acid. Moreover, ASIC3 is clearly sensitive enough to detect the fairly small (1/2 pH unit) changes in extracellular acidity that occurs in ischemic muscle. ASIC3 also responds better to lactic acid than to other forms of acid (Immke and McCleskey 2001) making it better for detecting the lactic acidosis that accompanies ischemia than the carbonic acidosis typical of most metabolic acidoses. Together these observations make a circumstantial case that ASIC3 is a sensor for angina and related ischemic pain. The idea is difficult to test in transgenic mice because there is no rodent model of ischemic pain.

The clearest demonstration for a role of an ASIC in pain has come from Sluka, who created a persistent pain paradigm triggered by muscle acidity in mice. Two muscle injections of acid some time apart creates a long-lived hypersensitivity to touch of nearby skin (Sluka, Kalra et al. 2001). This very reproducible syndrome simply does not occur in mice that lack ASIC3 (Sluka, Price et al. 2003). The condition may be analogous to some forms of temperomandibular joint syndrome, in which muscle or joint problems cause a chronic pain throughout the face. Looking at the afferents of this muscle, Connor et al. found that more than 40% of the afferents that project to the trigeminal ganglia express

ASIC3 like current (Connor, Naves et al. 2005) which is consistent with the idea that ASIC3 may be a sensor for muscle pain.

Along these same lines, data from rat DRG show high levels of ASIC3 expression in neurons that innervate muscle (Molliver, Immke et al. 2005). These sensory neurons were labeled by DiI injections into a leg muscle. Specifically, small neurons showed ASIC3 expression, further suggesting that ASIC3 may be responsible for nociception. In contrast, small diameter sensory neurons that innervate skin are less likely to express ASIC3. This may be why there are no strong phenotypes in skin pain assays of knockouts.

More traditional assays provide mixed messages as to ASICs role in pain. Two labs have made ASIC3 knockout mice that differ in their behavior. With i.p. injections of 0.6% acetic acid, the Zimmer lab knockout shows an increase (not decrease) in writhing over wild type (Chen, Zimmer et al. 2002). The Welsh lab knockout exhibits no change in paw licking after acid injections, and a decrease in mechanical hyperalgesia after intramuscular acid injection (Price, McIlwrath et al. 2001). With regards to noxious heat, the Zimmer knockout is more sensitive to temperatures above 50°C in a hot-plate test (Chen, Zimmer et al. 2002); the Welsh knockout shows a decreased response of heat sensitive C-fibers to noxious heat, and normal paw withdrawal to radiant heat (Price, McIlwrath et al. 2001). The Zimmer lab knockout only differs from the wild type in tests at high intensity, suggesting a role for ASIC3 in high intensity pain in different modalities. The Welsh lab knockout demonstrates a role for ASIC3 in acid and carageenan evoked hyperalgesia. In both cases, neither knockout shows a severe phenotype indicating that there are other players in all of these types of pain. It seems fair

to summarize data on knockout mice to indicate that they give subtle effects in pain assays and different labs report different results. A crucial concern is that these experiments cannot address the hypothesis that ASICs are sensors for ischemic pain such as angina because there is no mouse model for such pain.

An in vitro model for ischemic skeletal muscle has been recently developed (Wenk and McCleskey 2006). In this model, sensory neurons that innervate the plantar muscles of a mouse or rat foot can be recorded from while the muscle is stimulated. By removing oxygen and nutrient flow, ischemic responses can be studied. This model has found sensory neurons that seem to respond specifically to ischemic muscle contractions. By using pharmacology and genetically altered mice, this model may help demonstrate whether ASICs play a role in sensing ischemia.

Mechanosensation:

ASICs are considered as possible mechanosensing channels because of their homology to degenerins, which are expressed on mechanosensing *C. elegans* neurons (O'Hagan, Chalfie et al. 2005). ASIC2a and ASIC3 are expressed on mechanosensory terminals in rat skin (Garcia-Anoveros, Samad et al. 2001; Price, McIlwrath et al. 2001). ASIC2a knockouts show decreased sensitivity of rapidly adapting mechanoreceptors (Price, Lewin et al. 2000). ASIC3 knockouts have altered mechanosensation, however in this case the rapidly adapting mechanoreceptors become more sensitive (Price, McIlwrath et al. 2001). Neither knockout has dramatically altered mechanosensation like the degenerin mutants. However, the degenerin mutation causes loss of the entire mechanosensing cell, not just a single molecule. Current research suggests that ASICs

may play a mechanosensory role in the gut, rather than cutaneously (Drew, Rohrer et al. 2004; Page, Brierley et al. 2004; Roza, Puel et al. 2004; Jones, Xu et al. 2005).

How might mechanosensitivity arise? Is acid sensitivity relevant? Most models of mechanosensation surmise that the detecting channel is tethered to other proteins in the extracellular and intracellular media. The DEG proteins are thought to form ion channels in a multi-subunit complex necessary for transducing mechanical forces. Many proteins associate with DEGs to form this complex, but very few interacting proteins have been found that associate with ASICs. One protein that associates with ASIC3 is CIPP (channel-interacting PDZ domain protein). CIPP interacts with the carboxy terminus of ASIC3 using one of its four PDZ domains. When expressed with ASIC3 in COS cells, CIPP increases the current five fold, and shifts the activation curve towards more basic values (Anzai, Deval et al. 2002). CIPP may act as a scaffold protein to link ASIC3 to other intracellular proteins, since it has four PDZ domains.

In conclusion, there is no direct evidence showing that ASICs can act as mechanosensing ion channels. There is some weak circumstantial evidence as well as some vague knockout results. The best evidence for ASICs being mechanosensors is their homology to DEGs. At this point, it is purely speculation and cannot be taken very seriously without stronger data.

Taste:

Foods are perceived as sour due to an acid receptor in taste cells. Currents generated by acid in taste cells generally have a low amiloride and proton sensitivity. Recently it has been shown that both ASIC2a and 2b are present in a population of rat

taste cells, and that the heteromer is insensitive to amiloride at low pH (Ugawa, Minami et al. 1998; Lin, Ogura et al. 2002; Ugawa, Yamamoto et al. 2003). ASIC2a/2b heteromers have similar proton sensitivity to that seen for sour taste. Mouse knockouts of ASIC2 have shown no effects on sour taste sensing ability (Richter, Dvoryanchikov et al. 2004). However, ASIC2 is not expressed in mouse taste buds as it is in rats, so these two species may have different sensory transduction molecules for sour taste.

Two other proton-activated channels have also been implicated in sour taste transduction: VR1 and HCN channels. They can both be activated by acid and are expressed in taste cells. It is unclear to what extent these channels participate in sour taste transduction, or if all are involved.

Vision:

Interestingly, ASICs are expressed in the retina (Brockway, Zhou et al. 2002; Lilley, LeTissier et al. 2004). They are expressed in retinal ganglion cells. Knockout studies suggest that they are important for normal retinal function (Ettaiche, Guy et al. 2004; Ettaiche, Deval et al. 2006). Although it is unlikely they play any part in the sensory transduction, they may play synaptic roles in the transmission of these signals.

CNS ASICs

Immunolocalization studies show that ASIC1a is expressed in areas of high synaptic concentration such as cortex, olfactory bulb, hippocampus, amygdala, and cerebellum (de la Rosa, Krueger et al. 2003; Wemmie, Askwith et al. 2003). This bolsters the hypothesis that ASICs have a synaptic function. ASICs may be activated in

the CNS due to synaptic release. Vesicles become acidified when they are loaded with their neurotransmitter. When vesicles empty their transmitter, they also unload protons, thereby rapidly acidifying the synaptic cleft (Krishtal, Osipchuk et al. 1987; Palmer, Hull et al. 2003). This could be a stimulus that activates ASICs, but nobody has shown ASIC activation by any physiological stimulus.

ASICs in the CNS may enhance learning and memory formation. The Welsh group knocked out ASIC1 in mice and found deficits in LTP and learning in the knockouts (Wemmie, Chen et al. 2002; Wemmie, Askwith et al. 2003). The mutant mice have decreased facilitation of EPSPs during high frequency stimulation, deficits in spatial memory, impaired LTP, and impaired eyeblink and fear conditioning. Even with all these defects, the mice appear normal, and are capable of learning. There is always the stipulation with knockouts that the phenotype may be a result of a non-related gene that is affected by the knockout. Given the relatively mild phenotype of the knockout, this is definitely something to consider.

No role for ASICs in normal synaptic transmission has been demonstrated. Blockade of ASICs with amiloride or desensitization with low pH causes no apparent effect on synaptic transmission (de la Rosa, Krueger et al. 2003). Furthermore ASIC1 was not found to be enriched in postsynaptic density (PSD) fractions of cell homogenates from whole brain (de la Rosa, Krueger et al. 2003). Another study did find ASIC1 in dendritic spines in hippocampus (Zha, Wemmie et al. 2006). They also showed that down regulation of ASIC1 caused a reduction in the number of spines. ASIC2a has also been shown to be enriched in synaptic fractions from cerebellum (Jovov, Tousson et al. 2003). This further supports the idea that ASICs may play some synaptic role.

Although ASICs are widely distributed throughout the CNS, it is very unclear what they are doing there. Knockouts have shed little light on this subject. It is likely that they may have some sort of synaptic role, but that has yet to be demonstrated. Better pharmacology may aid in the understanding of the function of ASICs in the CNS. There is always the possibility that ASICs are gated by additional ligands. The fact that ASIC2b and ASIC4 are both insensitive to acid indicates that this may be a very real possibility.

CNS Ischemia (Stroke and Seizure)

Since ASICs are proton-gated, they may have deleterious side effects in the CNS during pathological conditions like stroke and seizure. During the ischemic conditions of a stroke or seizure, the pH can drop as low as 6.2 due to lactic acidosis (Nedergaard, Kraig et al. 1991), increasing extracellular lactate and proton concentrations. In addition to the pH change, the extracellular Ca^{++} concentration can drop from 1.2 mM to 0.1 mM (Kristian, Gido et al. 1998), due to the translocation of Ca^{++} into cells. Synaptic release occurring during these pathologies can also release large amounts of Zn^{++} into the extracellular medium. All of these ionic conditions: increased H^{+}, Zn^{++}, lactate, and decreased Ca^{++}, are known potentiators of ASIC currents.

If ASICs are activated strongly in the CNS, they could conceivably lead to neuronal death, much as constitutively active degenerins kill cells expressing them (Chalfie and Wolinsky 1990). Hyperactive ASICs are capable of loading a cell with sodium, creating an osmotic stress, thereby killing the cell. ASIC1a has a significant Ca^{++} permeability, which could let excess Ca^{++} into the cell, thereby killing it via Ca^{++}

activated apoptotic pathways. For either of these to occur, substantial activation would be necessary. The transient nature of ASIC currents may protect against such damage, but sustained ASIC currents may occur.

Allen and Atwell subjected cerebellar Purkinje cells to many different conditions occurring during CNS ischemia (Allen and Attwell 2002). The ASICs in the cerebellar slices are activated when pH falls below 6.8 with a $pH_{0.5}$ of 6.4. Many ischemic factors enhanced both the maximal and sustained ASIC current in these cells. Arachidonic acid, cell swelling, and lactate all potentiate the acid-gated current, often inducing a sustained component.

In a rat model of global ischemia, Johnson et al. found that ASIC2a is upregulated in surviving cells (Johnson, Jin et al. 2001). Since the ASIC1a/2a heteromers are less sensitive to acid than ASIC1a, the increase in ASIC2a may act to suppress activity of ASIC1a homomers, by increasing the proportion of ASIC1a in heteromers. Using a pilocarpine model of epilepsy, both ASIC1a and 2b are downregulated (Biagini, Babinski et al. 2001). This may be a neuroprotective mechanism to prevent neuronal death.

Roger Simon's group has shown a more direct role of ASICs in ischemic cell death (Xiong, Zhu et al. 2004). By using knockouts of ASIC1 and some pharmacology, they demonstrated that blockade or knockout of ASIC1 reduces ischemic cell death in mice. A further study showed that calcium influx through NMDA receptors enhances ASIC activation during ischemia (Gao, Duan et al. 2005). This is accomplished through phosphorylation of the channel on two sites by Ca^{++}/calmodulin-dependent protein kinase II (CamKII).

One other feature of ASICs that will be discussed in Chapter 3, is its sensitivity to Mg^{++}. Magnesium has shown promise in preventing ischemic cell death (Muir 2002). Much of this affect has been attributed to NMDA receptors, which are blocked by Mg^{++} in a voltage dependent fashion (Mayer, Westbrook et al. 1984). In Chapter 3, I will show that ASIC1a and the heteromer, ASIC1a/2a, have qualitatively similar properties.

A type of glutamate receptor, the NMDA receptor, has been heavily implicated in neuronal death occurring during ischemia. There are a number of reasons that this channel might mediate cell death. First, there is ample ligand around during ischemia. As neurons depolarize during ischemia, glutamate is released from cells into the extracellular space. NMDA receptors have a high affinity for glutamate, and do not exhibit the fast desensitization of AMPA receptors. Second, NMDA receptors have high calcium permeability. Excess calcium entry into cells can create both osmotic and enzymatic stresses to cells. Calcium is an activator of apoptotic pathways that are responsible for programmed cell death. One other feature of NMDA receptors is their voltage dependent Mg^{++} block (Mayer, Westbrook et al. 1984; Nowak, Bregestovski et al. 1984). This block normally restricts current through the channel, except when the cell is significantly depolarized to remove the block. During ischemia cellular depolarization removes this block, allowing influx of Ca^{++} and Na^{+}. These two stressors could then promote cell death through necrotic and apoptotic pathways.

Although NMDA receptors have many of the right properties to be activated during CNS ischemia, clinical trials have shown little promise. One problem with NMDA receptors is their pH dependency. Unlike ASICs, these receptors are inhibited by low pH, meaning that lactic acidosis is actually neuroprotective against NMDA receptor

mediated death. It is likely that these receptors contribute to ischemic cell death, but there is no single target that causes the cell death. Indeed, Gao et al. have shown that Ca^{++} entry through NMDA enhances ASICs (Gao, Duan et al. 2005). The idea that one specific ion channel or protein is responsible for cell death during ischemia is likely an oversimplification. Indeed there are numerous theories explaining ischemic cell death; most have shown much promise in animal models, yet few have born out any clinically effective treatments.

Other pH gated channels

ASICs may be the most sensitive, but they are not the only channels activated by protons. The vanilloid receptor (VR1 or TRPV1), a member of the transient receptor potential (TRP) family of ion channels, is opened by many types of stimuli, including heat, protons, and capsaicin (Tominaga, Caterina et al. 1998). Protons activate VR1 receptors by lowering their temperature threshold. The pH needs to drop below 5.9 at room temperature to activate the channel. The current through VR1 receptors is easily distinguished from ASICs since it does not desensitize, and is a non-selective cation channel. VR1 is thought to transduce noxious heat and capsaicin sensitivity in vivo (Caterina, Leffler et al. 2000).

Hyperpolarization activated and cyclic nucleotide gated channels (HCNs) are also activated by protons. These cation channels are normally opened by hyperpolarization, however both cyclic nucleotides and extracellular protons shift the activation curve to more positive voltages (Chen, Wang et al. 2001; Stevens, Seifert et al. 2001). At very low pH values, this shifts to above the resting potential of the cell, thereby activating the

cell. As sensory transduction channels, HCN1 and HCN4 are thought to participate in sour taste transduction.

There is also a family of potassium channels that show proton-gating. The TASKs (TWIK-related Acid-Sensitive K^+ channels) are members of the tandem pore potassium channel family. Instead of being activated by low pH, TASK1 and TASK3 are inactivated by acid. The IC_{50} for protons of TASK1 and TASK3 are pH 7.3 and 6.3 respectively, putting them within the physiological range of pH changes, especially TASK1 (Lesage 2003). Low pH excites cells expressing these channels by eliminating a resting potassium conductance.

A member of the cys-loop family of ligand gated channels has been shown to be activated by protons (Bocquet, Prado de Carvalho et al. 2007). This prokaryotic relative of nicotinic acetylcholine receptors activates at pH 6. Proton gradients are very important for this photosynthetic organism's metabolism, and this channel is thought to help sense and adapt to them.

Most ion channels show some sort of pH dependence to their function. For instance, voltage gated sodium channels are inhibited by low pH through a proton block in the pore (Woodhull 1973). NMDA receptors are also inhibited by low pH, with very little current remaining at pH 6.6 (Tang, Dichter et al. 1990). Protons can bind with varying affinity to seven of the twenty standard amino acids, so it is no surprise that pH effects can be quite common. The physiological relevance of many of these effects is questionable, as pH is generally very tightly regulated.

pH gated or modulated ion channels are widespread throughout biology. The ability to sense pH is crucial to the homeostasis of the organism. The evolution of all of

these pH sensors attests to the fact that sensing pH is relatively simple. A binding site for protons can be formed by any one of at least 7 amino acids with varying pKas. On the other hand sensing a more complex molecule involves a more complex binding site. This is perhaps why the diversity of proton gated channels is so high.

Questions of this Thesis

Since ASICs have been implicated in ischemic cell death in the CNS, I tested what the changes in ionic condition would do to the activity of ASICs. Looking at the two predominant subunits in the CNS, ASIC1a, and ASIC2a, I examined the influence of Mg^{++}, Zn^{++}, pH and Ca^{++} on activation. I will show that ASICs are greatly affected by the decrease in $[Ca^{++}]$ that occurs during ischemia in the CNS. Additionally, changes in Zn^{++} concentration will have varying effects on the activity of different CNS ASICs. Mg^{++} appears to block ASICs in voltage-dependent manner, implying that ASICs may act as a coincidence detector of acidosis and depolarization. These ion channels are very well suited to respond to the conditions that occur during ischemia, making them likely mediators of neuronal cell death.

All ASICs undergo desensitization in response to prolonged acidic stimuli. The mechanism behind this is not well understood. I will show a region extracellular to the

first transmembrane domain that appears to be involved in this process. Amino acids within this region help determine both the kinetics and pH sensitivity of desensitization. This would therefore greatly regulate the activity of these channels. I also show that there is a conformation change that occurs within this region following desensitization. There are many highly conserved residues within this sequence suggesting that they play an important role in acid sensitivity of this channel family.

Chapter 2

Methods

Chapter 2

Cell Culture

All experiments in this thesis were performed using transfected cell lines. The major cell type used in these experiments was CHO-K1 cells. These cells are an immortalized cell line derived from Chinese hamster (*Cricetulus griseus*) ovary cells (Puck 1957). These cells were chosen due to their low background currents. In control experiments they exhibited <10 pA of inward current in response to a pH 5.0 stimulus. Comparatively, HEK-293 and COS-7 cells displayed high background levels of acid-sensitive currents. COS-7 cells were initially used for experiments shown in Chapter 3, until their background currents (~250 pA) were judged to be too large. Consequently, all experiments were repeated in CHO cells, which displayed identical results to those obtained with COS cells. All data presented in this thesis are from CHO cells.

CHO cells can grow relatively quickly in culture. They are maintained in a culture media containing 90% Ham's F-12 media (Invitrogen), and 10% fetal bovine serum. The cells are maintained in a 37°C incubator with 5% CO_2. Every 2-3 days the cells were split with trypsin/EDTA (0.5g/l trypsin, 0.2g/l EDTA, Fisher #BW17-161E) to keep them from becoming confluent. This was typically done when the cells were 40-70% confluent. The media was aspirated from the cells, at which point the trypsin/EDTA (3 ml) was added. After 3-5 minutes at 37° the cells would detach from the dish. Media (2-7 ml) was then added to quench the trypsin. A dilution of the cells, typically 10-20% was then made in a new dish for propagation. CHO cells were maintained in culture for approximately 15 passages before being discarded.

To obtain new cells, a cryovial containing CHO cells frozen in freezing media (5% DMSO in 95% culture media) was quickly thawed in a 37°C water bath. They were then immediately placed in a 10 cm dish of warm culture media. This was placed in a 37°C incubator and allowed to sit for 3-5 hours so the cells could adhere to the dish. After this time, the cells were rinsed with warm media. This removes dead cells and DMSO from the media.

To freeze cells for stocks, I took a dish that was 50-70% confluent. The cells were detached with trypsin/EDTA at 37° as previous. After being quenched with media, the cells were then moved to a 15 ml centrifuge tube. They were then centrifuged at 1000 RPM on a Sorvall RT6000B with a H1000B rotor for 3 minutes. The liquid was then aspirated off the cell pellet, after which the cells were resuspended in 1 ml of freezing media consisting of 95% culture media, and 5% DMSO. The cells were then placed in a cryovial and placed in a Nalgene Cryo 1°C Freezing Container filled with isopropanol. This container allows slow cooling of the cells to help prevent crystal formation. The container is then placed in a -80°C freezer. After reaching -80°C in the container the cells are then removed and either left in the -80°C freezer or stored in liquid nitrogen.

Transfection Protocol

We obtained cDNA clones of rat ASICs courtesy of M. Lazdunski. Clones were either in PCI or JPA vectors.

We also used either CD4 in pcDNA3 (Invitrogen) or pCMV-DsRed-Express (Clontech) to identify transfected cells. For CD4 I used 0.5 μg of CD4 cDNA in transfections. To identify CD4 positive cells, I put CD4 antibody coated beads (Dynal)

on the cells. The beads stick to CD4 positive cells enabling us to identify transfected cells. When DsRed was used as the marker, 5-10 μg of DNA was added. Transfected cells were identified under epifluorescence with a rhodamine filter set.

Cells were transiently transfected with ASICs by electroporation. A 10 cm plate of 40-70% confluent CHO cells was used. First, the culture media was aspirated from the cells, followed by addition of 3 ml of trypsin/EDTA. The cells were then placed in a 37°C incubator for 3-5 minutes or until the cells detached from the bottom of the dish. The trypsin was then quenched by the addition of 2 ml of complete media. The cells were then transferred to a 15 ml conical tube for centrifugation at 1000 RPM for 3 minutes (Sorvall RT6000B with H1000B rotor at room temperature). After centrifugation, the supernatant was aspirated from the tube leaving a pellet of cells. The cells were resuspended in 0.5-1 ml of HBS (140 NaCl, 25 HEPES, 2 Na_2CO_3, pH 7.4 with NaOH). 100-200 μl of cell suspension (approximately $4x10^6$ cells/ml) was placed in a 0.4 cm gap cuvette (Invitrogen #P460-50) along with cDNA for channels and markers. The quantity of DNA added varied based on desired expression and channel type. For ASIC3, 0.5 μg gave reasonable current expression. Higher amounts were used for ASIC1a and ASIC2a, on the order of 5 μg. For the transfection markers, 0.5 μg of CD4 cDNA and 5 μg of DsRed were used. Once the DNA was added, the cells were allowed to sit for 1 minute prior to electroporation. The electroporator (Bio-Rad Gene Pulser II) was set at 380V, and 75 μF. The electric pulse lasted on the order of one second. The resulting time constant was 6-12 ms depending on the volume of cell suspension. Initially, the cells were left on ice for 2-5 minutes prior to plating; however, I found later that rapid plating with no ice gave much greater channel expression. The cells were then

plated onto cover slips (9 mm square, Bellco Glass) in one or two 35 mm dishes with complete F-12 media. The uncoated cover slips were rinsed with ethanol and dried prior to plating. The dishes of cells were kept at 37°C/5% CO_2. Experiments were performed 18-48 hours after electroporation. After 48 hours, expression became more variable and cell confluency becomes an issue.

Electrophysiology and Recording

Cells were visualized on a Nikon inverted microscope with a 10X Phase objective and 10X eyepieces. The DsRed was visualized by epiflourescence using a mercury arc lamp (Osram HBO103W/2) and a rhodamine filter set (Nikon DM580, excitation below 546 nm, emission above 590 nm). DsRed is maximally excited at 556 nm, and emits maximally at 586 nm.

Whole cell patch recordings were made with an Axopatch 200 equipped with a CV201 headstage (Axon Instruments). All experiments were performed in voltage clamp configuration. Data was digitized with either a TL-1 and pClamp 6, or a Digidata 1322A and pClamp 8 (all Axon Instruments). Acquisition frequency varied between 400Hz and 10kHz. The lowpass filter on the amplifier was set between 1 and 5 kHz. Series resistance of less than 10 MΩ (usually less than 5 MΩ) was compensated by 70-80%. The pipette was controlled with a Newport motion controller (Model 860-C2) with Newport 860A motorizers on the X, Y and Z axes.

Patch pipettes were pulled with a Sutter P-87 puller. The glass used was either Garner 7052 glass, 1.10 mm ID x 1.5 mm OD for the ASIC1a ischemia study, or A-M systems (#603000) and Sutter (BF150-86-10) borosilicate glass for the ASIC3 studies

(both are 0.86 mm ID x 1.5 mm OD with filament). Typical resistance of patch pipettes was 2-4 MΩ.

Solution exchanges were made with a homemade computer controlled system. The system consisted of up to 10 solenoid valves controlled with an Isolatch Valve Driver II (General Valve Corporation). Syringes containing test solutions were placed above the valves and were driven by gravity. As the valve was opened by a 5V pulse from the software via the digital outputs on the Digidata/TL-1, solution would flow out of one of the 10 μl pipettes that was manually placed in front of the cell. This 5V pulse would simultaneously close another valve that controlled the bath solution. The bath solution flowed out of a pipette that was positioned at 90° to the test solutions, still facing the cell. This arrangement yielded reasonably fast exchange times on the order of 5-10 ms in control experiments. An array of 8 pipettes could be positioned with test solutions, and two for bath solutions allowing full activation curves to be performed on each cell. Each pipette was manually aligned with a manipulator, and selected with a switch box.

One big problem with solution exchange is created by cell adhesion to the cover slip. This creates inefficient solution exchange for at least two reasons: first it is difficult to exchange the solution underneath the cell, and second the oncoming solution hits the bottom of the dish which disrupts laminar flow and allows mixing. For channels that do not undergo fast desensitization these issues are inconsequential, however they can create artifacts when studying quickly desensitizing channels such as ASICs. For this reason, cells were often lifted off the bottom of the dish to improve solution exchange when quick desensitization was a problem.

Reagents/Solutions

The bath solution for the experiments in chapter 3 consisted of 130 mM NaCl, 5 mM KCl, 20 mM HEPES (2-[4-(2-Hydroxyethyl)-1-piperazine]ethanesulfonic acid), 10 mM Glucose, and variable calcium concentrations (External 1, Table 2). The pH in all external solutions was adjusted with N-methyl-D-glucamine so as not to change sodium concentration. Test solutions were the same, except MES (4-Morpholinoethanesulfonic acid) was used instead of HEPES for pH 6.7 and lower.

For the experiments in chapters 4 and 5, the external solution was 150 mM NaCl, 5 mM KCl, 10 mM pH buffer, and 1 mM $CaCl_2$ (unless otherwise noted) (External 2, Table 2). The pH buffer used depended on the pH of the bath or test solution: MES for solutions $\leq$ pH 6.7, MOPS for pH 6.5-7.9, HEPES for pH 6.8-8.2, and TAPS for $\geq$ pH 7.7. NMG was used to pH all solutions.

For the internal solutions in chapter 3, I used 100 mM KCl, 5 mM $MgCl_2$, 10 mM EGTA (Ethyleneglycol Bis(aminoethylether) Tetraacetic Acid), 40 mM HEPES, 2 mM Na_2ATP (Adenosine-5'-Triphosphate Disodium Salt), 0.3 mM Na_3GTP (Guanosine-5'-Triphosphate Trisodium Salt), adjusted to pH 7.4 with KOH (Internal 1, Table 2). The internal solution used in chapters 4 and 5 was 120 mM KCl, 25 mM HEPES, 5 mM EGTA, 5 mM NaCl, adjusted to pH 7.3 with KOH (Internal 2, Table 2).

The methanethiosulfonate reagents were obtained from Toronto Research Chemicals. Unless noted, they were used at 1 mM dissolved in the test solution. MTSBn (Benzyl methanethiosulfonate) was dissolved in DMSO prior to being added to the solution. MTS reagents are notably unstable (Karlin and Akabas 1998; Vemana, Pandey et al. 2004). To ensure their activity I made fresh solutions daily, that were used for no

Table II

Solutions used in patch clamp experiments. External 1 and Internal 1 were used in Chapter 3 experiments, whereas External 2 and Internal 2 were used in Chapters 4 and 5.

	Na^+	K^+	Cl^-	Ca^{++}	Mg^{++}	HEPES*	EGTA	Na_2ATP	Na_3GTP	Glucose	pH
External 1	130	5	137	1.2	-----	20	-----	-----	-----	10	7.4 w/NMG
External 2	150	5	157	1	-----	10	-----	-----	-----	-----	8.0 w/NMG
Internal 1	-----	100	110	-----	5	40	10	2	0.3	-----	7.4 w/KOH
Internal 2	5	120	125	-----	-----	25	5	-----	-----	-----	7.3 w/KOH

more than 4 hours. When not being applied to cells, they were kept on ice. Alkaline pHs also accelerate the breakdown of MTS reagents. Many of my experiments involved addition of MTS reagents at pH 8.0. I always got very robust reactions at these pHs indicating that the MTS compounds were still active, although I may have underestimated rates of modification at these pHs due to breakdown of the reagents.

Mutagenesis

Mutations were introduced into the rat ASIC3 cDNA clone by PCR as described (Weiner, Costa et al. 1994) using Pfu DNA polymerase. Mutant constructs were fully sequenced to ensure accuracy of mutagenesis and to confirm the absence of unintended mutations.

Analysis

Offline analysis was performed with Clampfit 8 (Axon Instruments) and Origin 6 and 7 (OriginLab).

The voltage dependence of a blocking ion depends on whether or not it binds within the electric field of the membrane. This was analyzed with the Woodhull equation (Woodhull 1973):

$$K(V) = K(0)e^{\frac{zF\delta V}{RT}}$$

where K(V) is the dissociation constant of the blocking ion at voltage V, and K(0) is the dissociation constant at 0 volts. The constants z, F, R, and T are the valence, Faraday's constant, the gas constant, and absolute temperature, respectively. δ represents the fraction of the membrane's electric field through which the blocking ion binds. If it is 0,

then the blocking site is outside the membrane's electric field. If δ is 1, then the blocker must cross the electric field completely to bind to its blocking site.

Activation and steady state desensitization curves were fit with Hill equations. This equation describes the binding of a ligand to a binding site. The readout of binding for my experiments was current. The equation as used was:

$I/I_{MAX} = I_{MAX}*[H]^n/([H_{0.5}]^n+[H]^n)$

I/I_{MAX} is the normalized current, [H] is the proton concentration, $[H_{0.5}]$ is the half maximal activation or desensitization proton concentration, and n is the Hill coefficient. The Hill coefficient is a variable that describes cooperativity of binding.

Current decay kinetics were fit with a single exponential: $I=I_0 + Ae^{-t/\tau}$, where τ is the time constant of desensitization. Where noted, currents were fit with a double exponential: $I=I_0 + A_1e^{-t/\tau 1} + A_2e^{-t/\tau 2}$. Fits were made with ClampFit 8, (Axon Instruments).

Alignment of sequences was performed using clustalW (http://align.genome.jp/). This was done using the protein sequences accessed from the National Library of Medicine.

Statistics were performed using either Microsoft Office Excel 2003 (Microsoft), or Origin 7.0 (OriginLab). Errors reported are standard error. To determine significance, a two-tailed students t-test was used.

Chapter 3

Activity of acid-sensing ion channels under conditions that accompany stroke

Chapter 3

Introduction:

Acid-sensing ion channels (ASICs) are proton gated sodium channels that are expressed in the central nervous system (CNS) and in sensory neurons (Krishtal 2003). ASIC1a and its heteromers with ASIC2 are the predominant ASICs in central neurons (Wemmie, Chen et al. 2002). Of the two, ASIC1a is more sensitive and has higher calcium permeability (Waldmann, Champigny et al. 1997; Yermolaieva, Leonard et al. 2004).

NMDA receptors have long been implicated in ischemic cell death (Simon, Swan et al. 1984; Lipton 1999). During ischemia, glutamate is released which activates NMDA receptors causing influx of sodium and calcium. Influx of these two ions can be excitotoxic, resulting in cell death if prolonged. NMDA receptors are blocked by extracellular Mg^{++} in a voltage-dependent manner (Mayer, Westbrook et al. 1984; Nowak, Bregestovski et al. 1984). Since this block is voltage-dependent, it is alleviated by depolarizing the cell. This makes NMDA receptors coincidence detectors for glutamate and depolarization. It is thought that this block would be relieved by depolarization induced by ischemia.

For many of these same reasons, ASICs have been implicated in neuronal death during ischemia. The activation of ASICs would cause cell death by loading cells with sodium and calcium, thereby causing both osmotic and enzymatic stress. There is evidence showing that ASIC activity contributes to neuronal death caused by ischemia. A recent study found that both ASIC1a knockout mice, and mice exposed to an ASIC1a

blocker showed significantly less damage to stroke (Xiong, Zhu et al. 2004; Benveniste and Dingledine 2005).

Ionic concentrations in the extracellular space can change greatly during ischemia. As oxygen is depleted, neurons switch to anaerobic respiration which results in the buildup of lactic acid. This causes a reduction in the pH both intracellularly, and extracellularly. The extracellular pH can change from resting level of 7.4, down to 6.1 (Siemkowicz and Hansen 1981; von Hanwehr, Smith et al. 1986; Katsura, Asplund et al. 1992). As the cell depletes its ATP stores, it is unable to maintain other ionic gradients such as calcium and potassium. The changes in extracellular calcium concentration that occur during ischemia can be dramatic, falling more than ten fold from 1.2 mM to as low as 0.1 mM (Siemkowicz and Hansen 1981; Li, Kristian et al. 1995; Kristian and Siesjo 1997).

As the potassium concentrations increase extracellularly up to 80 mM, the cell's membrane potential depolarizes (Siemkowicz and Hansen 1981). Studies have shown average resting potentials changing from –65 mV to –20 mV (Xu and Pulsinelli 1994). These depolarizations cause activation of voltage gated channels which will further create imbalances in the concentrations of ions both intracellularly and extracellularly.

An additional ion that undergoes a change in its extracellular concentration is zinc. Normally, extracellular zinc concentrations are in the low nanomolar range, but during heavy synaptic activity, like during ischemia, it can be released from synaptic vesicles and increase up to 300 μM (Assaf and Chung 1984). Extracellular zinc has the potential to be neurotoxic (Weiss, Sensi et al. 2000).

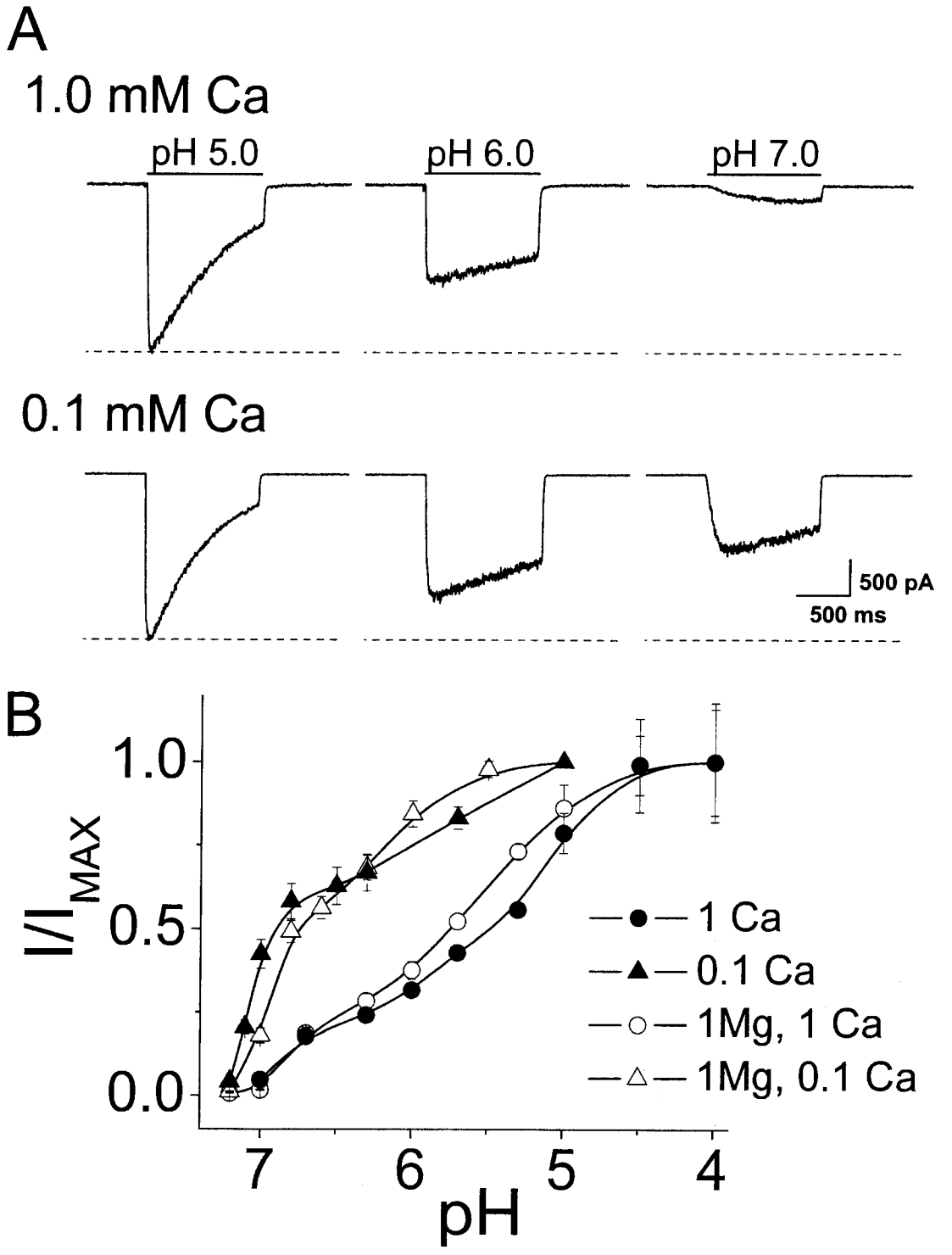
These changes in extracellular ions are likely what results in the activation of ASICs during ischemia. First, pH activates ASICs, and does so within the ranges present during ischemia (Waldmann, Champigny et al. 1997). In addition to the pH changes, we know that calcium concentrations are crucial to ASIC activation, with lower calcium concentrations leading to greater activation (Babini, Paukert et al. 2002; Immke and McCleskey 2003). Studies also point to various effects of zinc on ASICs (Baron, Waldmann et al. 2002; Chu, Wemmie et al. 2004). Here, I find that all of these conditions will favor the activation of ASICs, thereby increasing the likelihood of cell death during ischemia. Unexpectedly, I also found a voltage dependent block of ASICs by Mg^{++}, which is qualitatively similar to the NMDA receptor and would be alleviated by ischemic depolarization.

Effects of ischemic calcium concentrations on ASIC1a

During stroke, extracellular calcium concentrations can decrease from 1.2 mM to 0.1 mM (Siemkowicz and Hansen 1981). Since ASICs are affected by extracellular calcium, I tested the activation of ASIC1a under the calcium conditions occurring during ischemia. I transfected CHO cells with rat ASIC1a, and looked at pH evoked currents using whole cell patch clamp. To elicit ASIC currents, solution was changed rapidly from a bath solution at pH 8.0 with 3 mM Ca^{++} to a test solution with variable pH and Ca^{++} for one second (Fig. 7A). I used 3 mM Ca^{++} in the bath solution in order to speed recovery from desensitization and inhibit rundown. When 1.0 mM calcium was present in the low pH solutions, the activation curve for ASIC1a was shallow, with a $pH_{0.5}$ near 5.5 (Fig. 7B solid circles).

Fig. 7

pH and calcium dependence of ASIC1a. (A) pH evoked currents from ASIC1a transfected CHO cells in either 1.0 mM (top) or 0.1 mM (bottom) extracellular Ca^{++}. Bath solution contains 3 mM Ca^{++}, at pH 8.0. (B) pH activation curves for ASIC1a in either 1 mM Ca^{++} (solid circles), 0.1 mM Ca^{++} (solid triangles), 1 mM Ca^{++} + 1 mM Mg^{++} (hollow circles), or 0.1 mM Ca^{++} + 1 mM Mg^{++} (hollow triangles). 1 mM Ca^{++} curves are normalized to pH 5.7, low Ca^{++} curves are normalized to pH 5.0. +/- SEM. n=6-13. Curves are drawn with B-splines connecting the points, and are not mathematical fits.


A
1.0 mM Ca
pH 5.0
pH 6.0
pH 7.0
0.1 mM Ca
500 pA
500 ms
B
I/I_MAX
1.0
0.5
0.0
7
6
5
4
pH
1 Ca
0.1 Ca
1Mg, 1 Ca
1Mg, 0.1 Ca

To mimic ischemia, I lowered the calcium concentration to 0.1 mM, which resulted in two major changes in the activation curve: it was shifted to the left, and was considerably steeper (Fig. 7B solid triangles). These two effects changed the $pH_{0.5}$ to 6.9, making the channel far more sensitive to pH in lower calcium. Raw current traces demonstrate this best at pH 7.0 (Fig. 7A). In 1.0 mM Ca^{++}, there is only a small current in response to pH 7, whereas in 0.1 mM Ca^{++} the current is much larger.

Comparing a pH 6.3 (ischemic pH) stimulus in the presence of either 1.2 mM (physiological), or 0.1 mM (pathological) Ca^{++}, showed significant enhancement of the current by low calcium (Fig. 8A top panel). The currents were on average 4 times larger (4.03±0.2, n=9) in this lower calcium concentration at pH 6.3. This suggests that the low calcium concentrations that can occur during ischemia will result in a very large increase in the activation of ASIC1a by decreased pH.

ASIC1a/2a heteromers are also expressed throughout the CNS (Baron, Waldmann et al. 2002), and are also enhanced when extracellular calcium is decreased (Fig. 8A bottom panel). Like ASIC1a, these channels were enhanced when extracellular calcium was lowered to 0.1 mM (an average of three fold at pH 6.3 (2.98±0.12, n=11)). Although the heteromeric channels are less sensitive than ASIC1a, they were substantially activated by these pathological levels of protons.

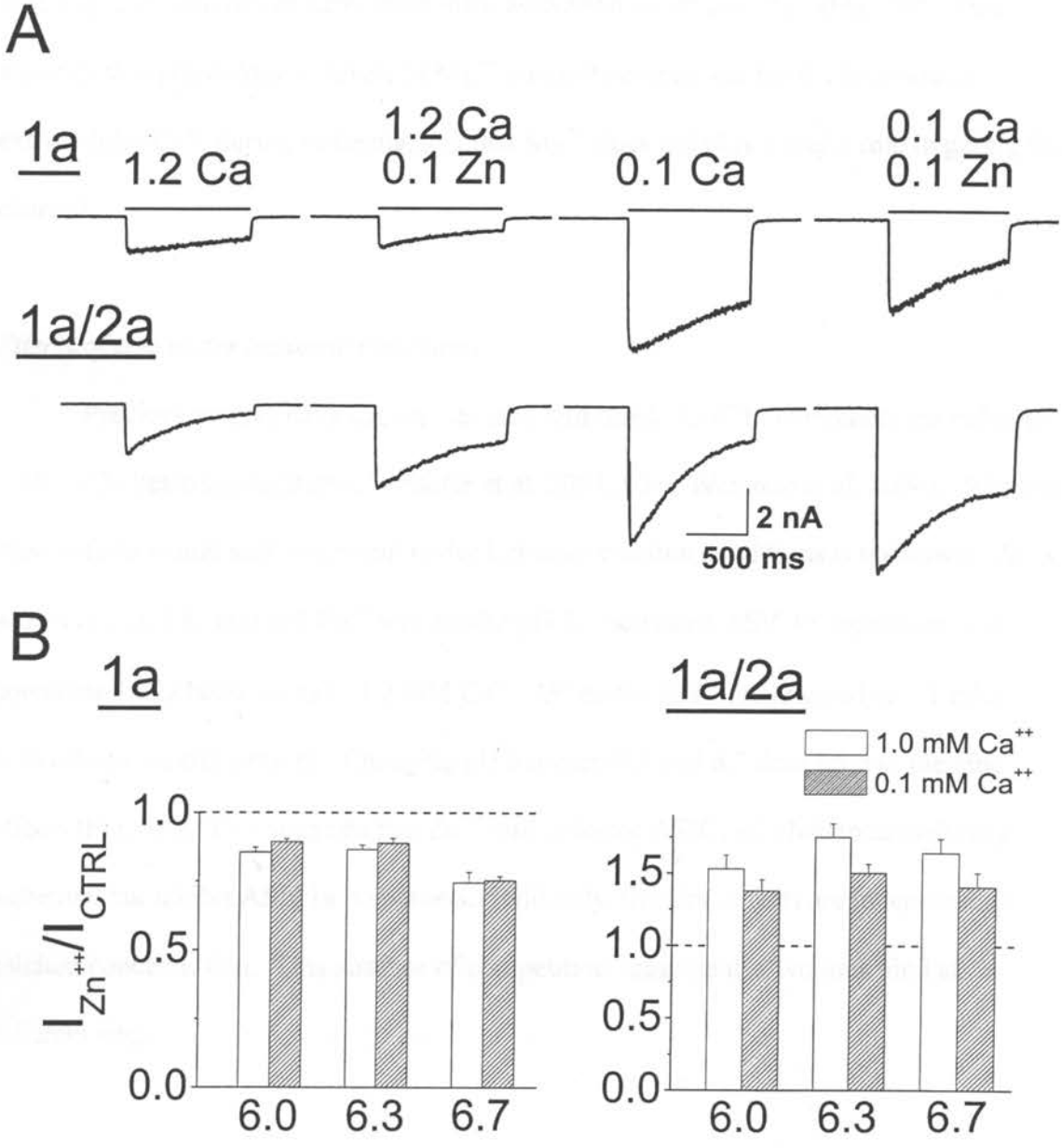
Unlike the dramatic change in extracellular calcium concentrations, magnesium concentrations do not decrease to the same degree during ischemia (Lee, Wu et al. 2002). The effect of Mg^{++} on ASICs is not well described, and since extracellular Mg^{++} may compensate for lower calcium during ischemia, I measured activation curves in the presence of Mg^{++}. When I supplemented 1 mM Ca^{++} with 1 mM Mg^{++}, I saw little

Fig. 8

Effects of Zn and Ca on homomeric and heteromeric ASIC1a. (A) ASIC1a (top) or ASIC1a/2a (bottom) transfected CHO cells exposed to a pH 6.3 stimulus in the presence of varying [Ca^{++}] and [Zn^{++}] (in mM). (B) Summarized data of 100 μM Zn^{++} effects on ASIC1a (left) or ASIC1a/2a (right). Data plotted as relative current in the presence of Zn^{++} compared to current in the absence of Zn^{++}. Hollow bars are in the presence of 1.0 mM Ca^{++}; shaded bars are in 0.1 mM. n=3-12.

Fig. 8

Effects of Zn and Ca on homomeric and heteromeric ASIC1a. (A) ASIC1a (top) or ASIC1a/2a (bottom) transfected CHO cells exposed to a pH 6.3 stimulus in the presence of varying [Ca^{++}] and [Zn^{++}] (in mM). (B) Summarized data of 100 μM Zn^{++} effects on ASIC1a (left) or ASIC1a/2a (right). Data plotted as relative current in the presence of Zn^{++} compared to current in the absence of Zn^{++}. Hollow bars are in the presence of 1.0 mM Ca^{++}; shaded bars are in 0.1 mM. n=3-12.


A
1a
1.2 Ca
1.2 Ca
0.1 Zn
0.1 Ca
0.1 Ca
0.1 Zn
1a/2a
2 nA
500 ms
B
1a
1a/2a
1.0 mM Ca++
0.1 mM Ca++
I Zn++/I CTRL
1.0
0.5
0.0
6.0
6.3
6.7
1.5
1.0
0.5
0.0
6.0
6.3
6.7

change from activation in the absence of Mg^{++} (Fig. 7B). When Ca^{++} was replaced with 1 mM Mg^{++}, the activation curve resembled activation under low Ca^{++} (Fig. 7B). This suggests that physiological levels of Mg^{++} will not compensate for the decrease in extracellular Ca^{++} during ischemia, and that Mg^{++} does not play a major role in gating this channel.

Effects of zinc under ischemic conditions

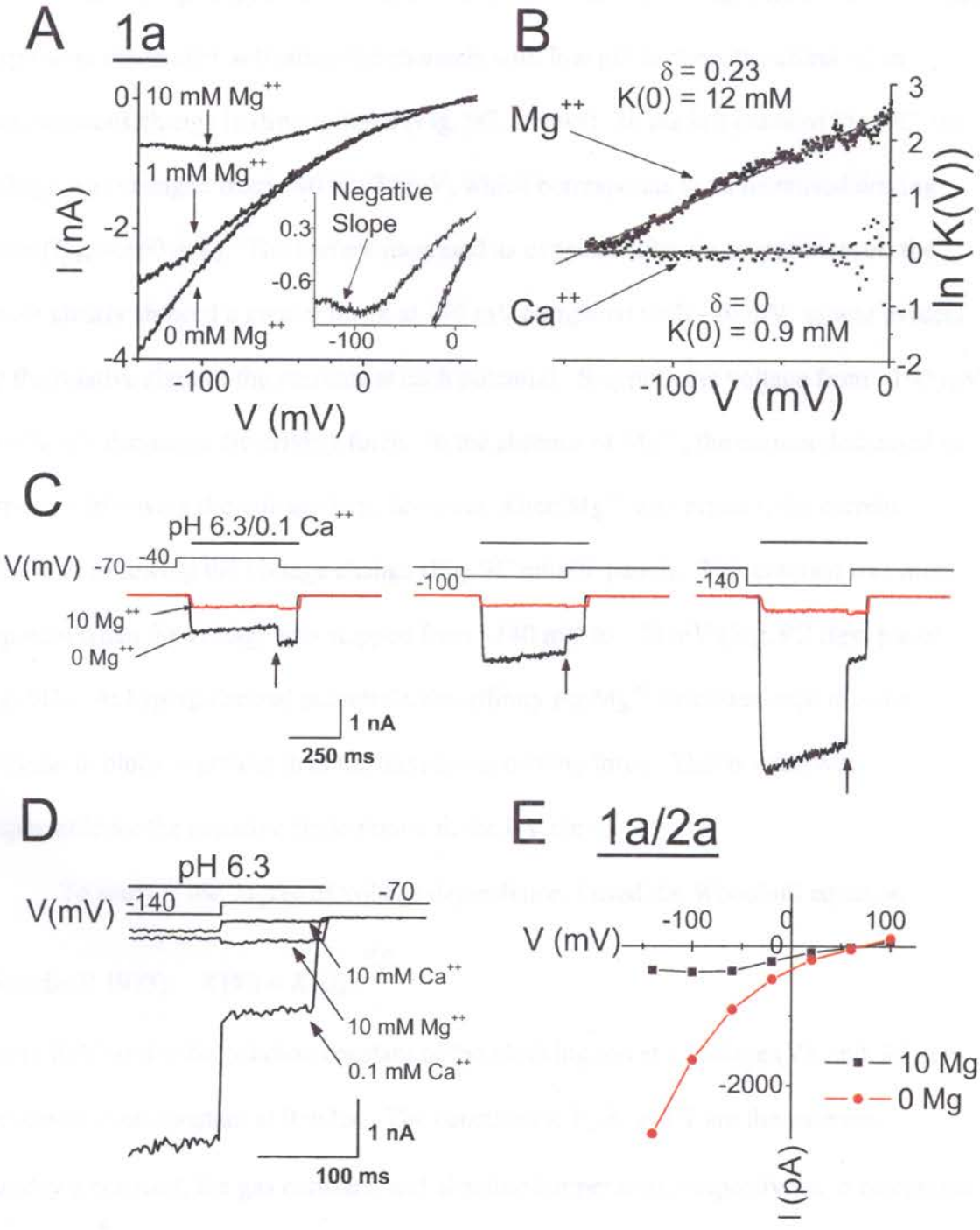
Previous studies have shown that zinc will block ASIC1a homomers yet enhance ASIC1a/2a heteromers (Baron, Schaefer et al. 2001; Chu, Wemmie et al. 2004). Whether these effects would still be present under ischemic calcium conditions is unknown. As is shown in Fig. 8A, 100 μM Zn^{++} will inhibit pH 6.3 activated ASIC1a homomers and potentiate 1a/2a heteromers in 1.2 mM Ca^{++}. When the [Ca^{++}] is lowered to 0.1 mM, both effects are still present. Changing pH between 6.3 and 6.7 does little to the zinc effects (Fig. 8B). This suggests that Zn^{++} will enhance ASIC1a/2a heteromers during ischemia, but inhibit ASIC1a homomers. Evidently, the zinc effects are independent of calcium concentration. This absence of competition suggests the two ions bind at different sites.

Mg^{++} is a voltage-dependent blocker of ASICs

Although Mg^{++} did not significantly affect the activation of ASIC1a (Fig. 7B), it was apparent that it blocked ASIC1a in a dose-dependent manner. Using pH 6.3 (0.1 mM Ca^{++}) as the test solution, a voltage ramp from –140 mV to +70 mV showed a

Fig. 9

Voltage-dependent Mg block of ASICs. (A) Currents elicited by a 1 second voltage ramp during a pH 6.3 stimulus of an ASIC1a transfected CHO cell with 0 (bottom trace), 1 (middle), or 10 mM Mg^{++} (top). Inset shows blowup of the 10 mM Mg^{++} trace to demonstrate the negative slope region. (B) Plot of the log of the dissociation constant, K, as a function of voltage. K(V) is calculated from voltage ramps of CHO cells in either 10 mM Mg^{++} or Ca^{++}, and 0.1 mM Ca^{++} without Mg^{++}. (C) Voltage steps during pH stimuli further demonstrate voltage dependence of Mg^{++} block. During the pH change the voltage is returned to –70 mV from –40 (left), –100 (middle) and –140 mV (right) as is indicated by the arrows. (D) Blowup of voltage change showing an increase in current when 10 mM Mg^{++} is present, but not when 10 mM Ca^{++} is present. (E) I-V curve of ASIC1a/2a expressing cell showing similar voltage dependent block by Mg^{++}.


A
1a
10 mM Mg++
1 mM Mg++
0 mM Mg++
Negative
Slope
I (nA)
V (mV)
B
δ = 0.23
K(0) = 12 mM
Mg++
Ca++
δ = 0
K(0) = 0.9 mM
ln (K(V))
V (mV)
C
pH 6.3/0.1 Ca++
V(mV)
10 Mg++
0 Mg++
1 nA
250 ms
D
pH 6.3
V(mV)
10 mM Ca++
10 mM Mg++
0.1 mM Ca++
1 nA
100 ms
E
1a/2a
V (mV)
10 Mg
0 Mg
I (pA)

negative slope region in the current voltage curve when 10 mM Mg^{++} was present, suggesting a voltage dependent block (Fig. 9A).

This voltage dependent block is further illustrated by voltage steps. These voltage steps were made after activating the channels with low pH to show the effect of an instantaneous change in driving force (Fig. 9C, arrows). In the left panel of Fig. 9C, the voltage was changed from –40 to –70 mV, which corresponds to an increased driving force (E_{rev} ~ +60 mV). The current increased as expected after the step; however, the traces clearly showed a greater block at –70 mV compared with –40 mV, as was evident by the relative sizes of the currents at each potential. Stepping the voltage from –100 mV to –70 mV decreases the driving force. In the absence of Mg^{++}, the current decreased as expected following the voltage step; however, when Mg^{++} was present, the current increased following the voltage change (Fig. 9C middle panel). This contrast was more apparent when the voltage was stepped from –140 mV to –70 mV (Fig. 9C right panel, Fig. 9D). At hyperpolarized potentials, the affinity for Mg^{++} increases such that the increase in block is greater than the increase in driving force. This is what was responsible for the negative slope region in the I-V curve.

To analyze the degree of voltage dependence, I used the Woodhull equation (Woodhull 1973): $K(V) = K(0)e^{\frac{zF\delta V}{RT}}$

where K(V) is the dissociation constant of the blocking ion at a voltage (V), and K(0) is the dissociation constant at 0 volts. The constants z, F, R, and T are the valence, Faraday's constant, the gas constant, and absolute temperature, respectively. δ represents the fraction of the membrane's electric field through which the blocking ion binds. Fig. 9B shows how the membrane potential affects the dissociation constant. The slope of

this line is proportional to δ and the intercept is K(0). The points are taken from a set of voltage ramps in the presence, or absence of 10 mM Mg^{++}. I determined a δ value of 0.23 for ASIC1a indicating that the Mg^{++} binding site lies 23% of the way through the membrane's electric field. The affinity of this site for Mg^{++} at 0 mV is 12 mM.

This voltage dependent block was also present in heteromeric channels (Fig. 9E). Stepwise current-Voltage curves showed the same negative slope region when Mg^{++} was present.

Calcium block is not voltage dependent

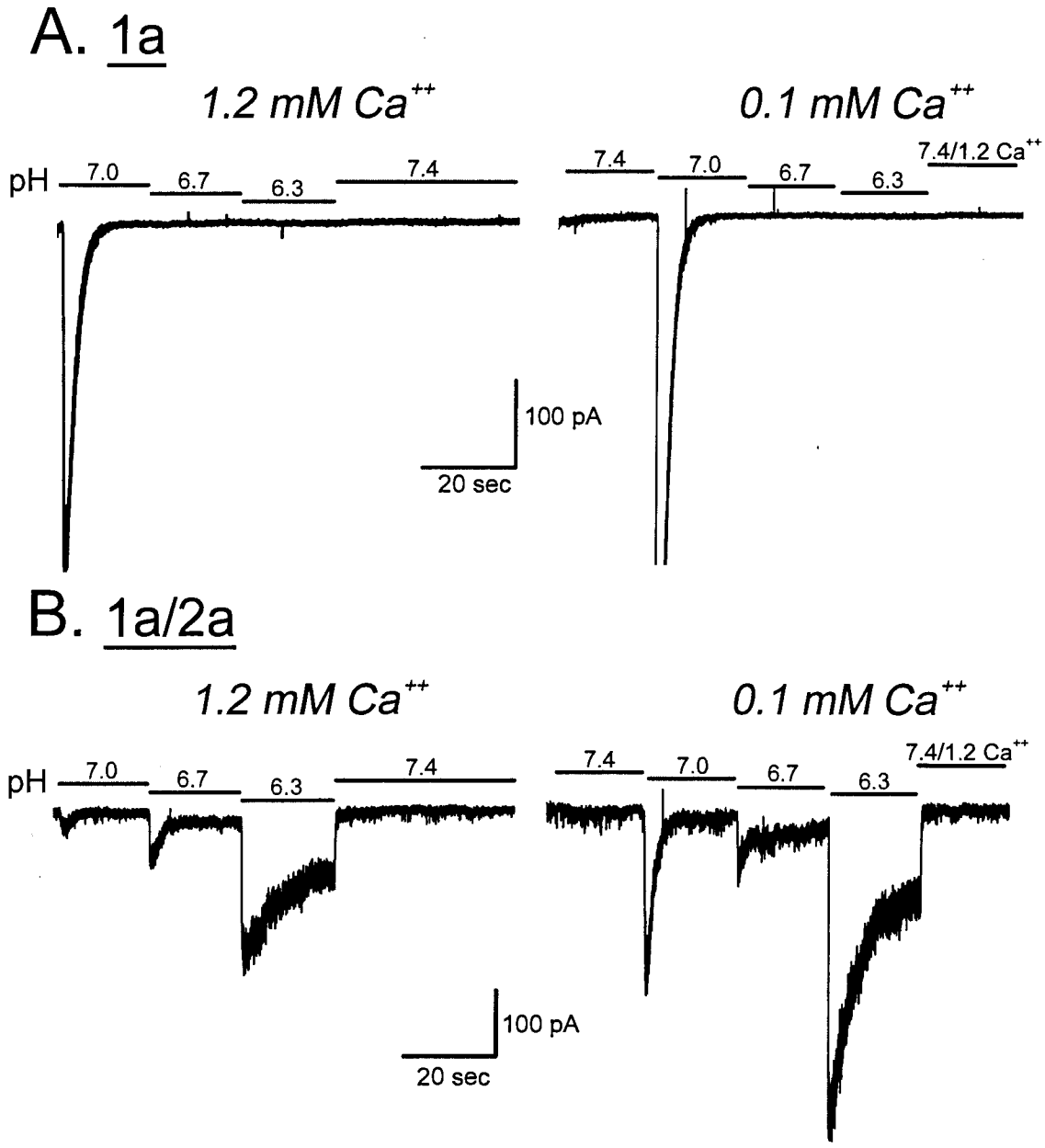
Since Ca^{++} also blocks ASIC1a, I examined the voltage dependence of Ca^{++} block in ASIC1a. Unlike Mg^{++}, Ca^{++} showed no voltage dependence to its block (Fig. 9B, D). Voltage step and ramp protocols with 10 mM Ca^{++} showed no effect of voltage on block, suggesting separate binding sites for Mg^{++} and Ca^{++}.

Sustained currents are seen with heteromeric channels

Transient currents are unlikely to cause significant damage to cells. Both ASIC1a and ASIC1a/2a desensitize in response to prolonged acidification. It is unclear whether or not these channels are capable of producing a sustained current at ischemic pH levels. In order to test this, I crudely simulated a slow pH change to pH 6.3 by making successive steps every 20 seconds as shown in figure 10. Figure 10A shows that ASIC1a homomers were activated by the initial step to pH 7 after which they completely desensitized. Further changes in pH were unable to cause any additional activation. This

Fig. 10

Sustained currents seen in heteromeric, but not homomeric ASICs. Representative currents elicited by consecutively decreasing the pH in either 1.2 (left) or 0.1 (right) mM Ca^{++}. Cells were transfected with either ASIC1a alone (A) or ASIC1a and 2a (B).


A. 1a
1.2 mM Ca++
0.1 mM Ca++
pH
7.0
6.7
6.3
7.4
7.4
7.0
6.7
6.3
7.4/1.2 Ca++
100 pA
20 sec
B. 1a/2a
1.2 mM Ca++
0.1 mM Ca++
pH
7.0
6.7
6.3
7.4
7.4
7.0
6.7
6.3
7.4/1.2 Ca++
100 pA
20 sec

was independent of calcium concentration. This was consistent with a previous study showing complete desensitization of ASIC1a (Yagi, Wenk et al. 2006).

ASIC1a/2a heteromers showed sustained currents (Fig. 10B). In either normal (1.2 mM) or low (0.1 mM) Ca^{++}, desensitization was incomplete and channels continued to be activated as the pH decreased. Additionally, there was a component of the current that was persistent. This is especially evident when the pH is returned to 7.4 after the pH 6.3 stimulus. These results indicate that ASIC2a confers the ability to pass sustained currents on ASIC1a.

Results Summary

I have shown in this chapter that two types of ASICs expressed in the CNS are sensitive to ionic changes occurring during ischemia. Both ASIC1a and ASIC1a/2a heteromers were activated by pH values that can be caused by ischemia. Furthermore, pH evoked currents from ASIC1a and ASIC1a/2a were enhanced when the calcium concentration dropped. Lower extracellular calcium caused a shift in the activation curve as well as increasing the steepness of it. Both of these channels exhibited a voltage dependent block by magnesium, qualitatively similar to NMDA receptors. Unlike NMDA receptors however, the voltage dependence and affinity were fairly weak, and would likely not play a large physiological role. An additional divalent ion, zinc, had contrasting effects on the two channel subtypes: inhibiting ASIC1a, and enhancing ASIC1a/2a heteromers. These effects were independent of extracellular calcium concentrations. The evidence here argues for separate Ca^{++}, Mg^{++}, and Zn^{++} binding sites. Lastly, I could elicit sustained currents through ASIC1a/2a channels, but not

through homomeric ASIC1a. All of this suggests that ionic conditions that are present during ischemia are sufficient to activate ASICs, consistent with the proposal that they cause or enhance neuronal cell death through excitotoxicity.

Chapter 4

A region affecting desensitization in ASICs contributes to differences in kinetics between ASIC1a and ASIC3

Chapter 4

Introduction

Acid-sensing ion channels (ASICs) are members of the DEG/ENaC family of ion channels. They are sodium selective ion channels that are gated by drops in extracellular pH (Krishtal 2003). ASICs desensitize in response to prolonged stimulation by protons. The four functional subunits desensitize with widely different kinetics and sensitivities (Benson, Xie et al. 2002; Hesselager, Timmermann et al. 2004). It is unknown what creates these differences, although an area outside the first transmembrane region (TM1) has been shown to mediate some species specific differences (Coric, Zhang et al. 2003).

The proton sensor for desensitization has remained undiscovered. Likely candidates are acidic residues—glutamates and aspartates—since these amino acids can bind both protons calcium. This is important, since calcium appears to compete with protons for desensitization (Babini, Paukert et al. 2002). Steady state desensitization curves are very steep, suggesting multiple binding sites per subunit (Korkushco, Krishtal et al. 1983). There have been three studies pointing to regions in and around TM1 as being involved in desensitization (Babini, Paukert et al. 2002; Coric, Zhang et al. 2003; Pfister, Gautschi et al. 2006). Each of these studies has found a small region that affects desensitization in some way, but none have found any putative proton binding sites.

Desensitization is critical to the way ASICs function. In sensory neurons, sustained currents through ASICs are thought to drive nociceptive signals from ischemia. These sustained currents occur within the physiological range of pHs that would occur during myocardial ischemia. A study on these currents demonstrates that desensitization regulates both the size, and pH range (Yagi, Wenk et al. 2006). Likely, desensitization

would also be responsible for regulating sustained currents in central neurons during ischemia as discussed in Chapter 3.

Conserved Acidic Residues Near TM1 Affect Desensitization

With the idea that proton binding sites for desensitization would be formed by conserved acidic residues, I mutated glutamates and aspartates to alanines. An alignment of acid-gated rat ASICs shows there are 27 conserved acidic residues in the extracellular domains (Fig. 6). Only one of these charged amino acids is also conserved in epithelial sodium channels (ENaCs), which are proton insensitive. Due to the large number of conserved acidic residues, I began by mutating residues near the two transmembrane domains, as these regions have previously been shown to alter gating in ASICs (Paukert, Babini et al. 2004; Pfister, Gautschi et al. 2006).

We screened ASIC3 mutants for desensitization phenotypes by expressing them in CHO cells, and examined their desensitization kinetics. CHO cells were voltage clamped at -70 mV, in whole cell configuration, and the external medium was rapidly changed from pH 8.0, to pH 6.0 to elicit ASIC currents. Wild-type ASIC3 desensitizes to a pH 6.0 stimulus with kinetics that can be fit by a single exponential with a time constant (τ) near 0.4 seconds (Fig. 12).

Three mutants, all near TM1, immediately distinguished themselves due to their irregular desensitization kinetics (Fig. 12). The one closest to TM1, E63A, was slower to desensitize than wild-type (wt) ASIC3, while mutations at two neighboring residues, D78A and E79A, showed the opposite phenotype, desensitizing very rapidly (0.1 seconds). In contrast, mutants near TM2 (E432A, E435A), or farther from TM1

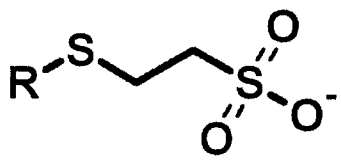
Figure 11

ASIC Alignment and MTS Structures. (A) Alignment of rat ASICs. Highlighted residues correspond to D78 and E79 in ASIC3. (B) MTS Compounds used in experiments. R denotes either the methane sulfinate moiety, or the cysteine to which it attaches.

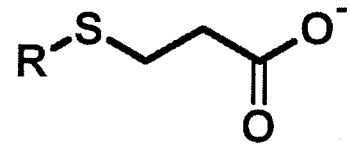
A

```
ASIC3   72HHKTTLDERESHQLT-FPAVTL
ASIC1a    HHVTKLDEVAASQLT-FPAVTL
ASIC1b    PHVTLLDEVATSELV-FPAVTF
ASIC2a    QHVTKVDEVVAQSLV-FPAVTL
ASIC2b    PSHTRVHREWSRQLP-FPAVTV
ASIC4     PHLVAMDPAAPAPVAGFPAVTL
```

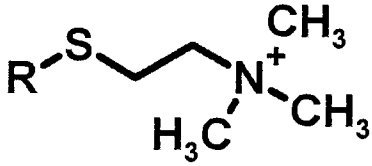
B



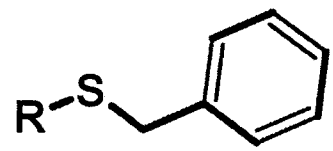
MTSES



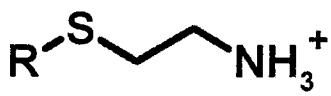
MTSCE



MTSET



MTSBn



MTSEA

(D107A) did not display altered desensitization kinetics (Fig. 12). A double mutant of both D78 and E79 (D78A/E79A) showed even faster desensitization than either alone (Table III).

Both D78 and E79 are very highly conserved in ASICs (Fig. 11). They are also near residues previously identified as affecting desensitization between fish and rat ASIC1. I decided to further explore this region surrounding D78 and E79 by doing an alanine scan. Without exception, alanine mutants from T75-S82 all affected the kinetics of desensitization (Fig. 13). This suggests that D78 and E79 are part of a larger region of ASIC3 that seems to be involved in desensitization. In most cases, only desensitization was affected by the mutation, although in one alanine mutant, L77A, the activation curve was shifted significantly without a change in slope (Table III). The predominance of desensitization phenotypes in the relative absence of effect on activation suggest this region is specialized for a role in desensitization.

Importance of Charge at D78 and E79

There are three acidic residues in the region identified in figure 13. Two of these, D78 and E79, are conserved whereas the third, E81, is only conserved among ASIC3 subunits. These three charges lie very close to each other, and are all possible proton binding sites for desensitization. Since neutralizing these charges leads to faster desensitization, it is possible that titration of these acidic residues by protons is one event that promotes desensitization. I decided to further explore these residues by mutating them to alternate amino acids.

Figure 12

Desensitization phenotypes occur with mutations near TM1. (A) Normalized currents in response to a pH 6.0 stimulus in CHO cells transfected with either mutant, or wt (arrow) ASIC3. (B) Average time constants (τ) of desensitization for mutant channels. (* denotes $p \leq .001$ vs. wt by students t-test). (n=4-27 cells).

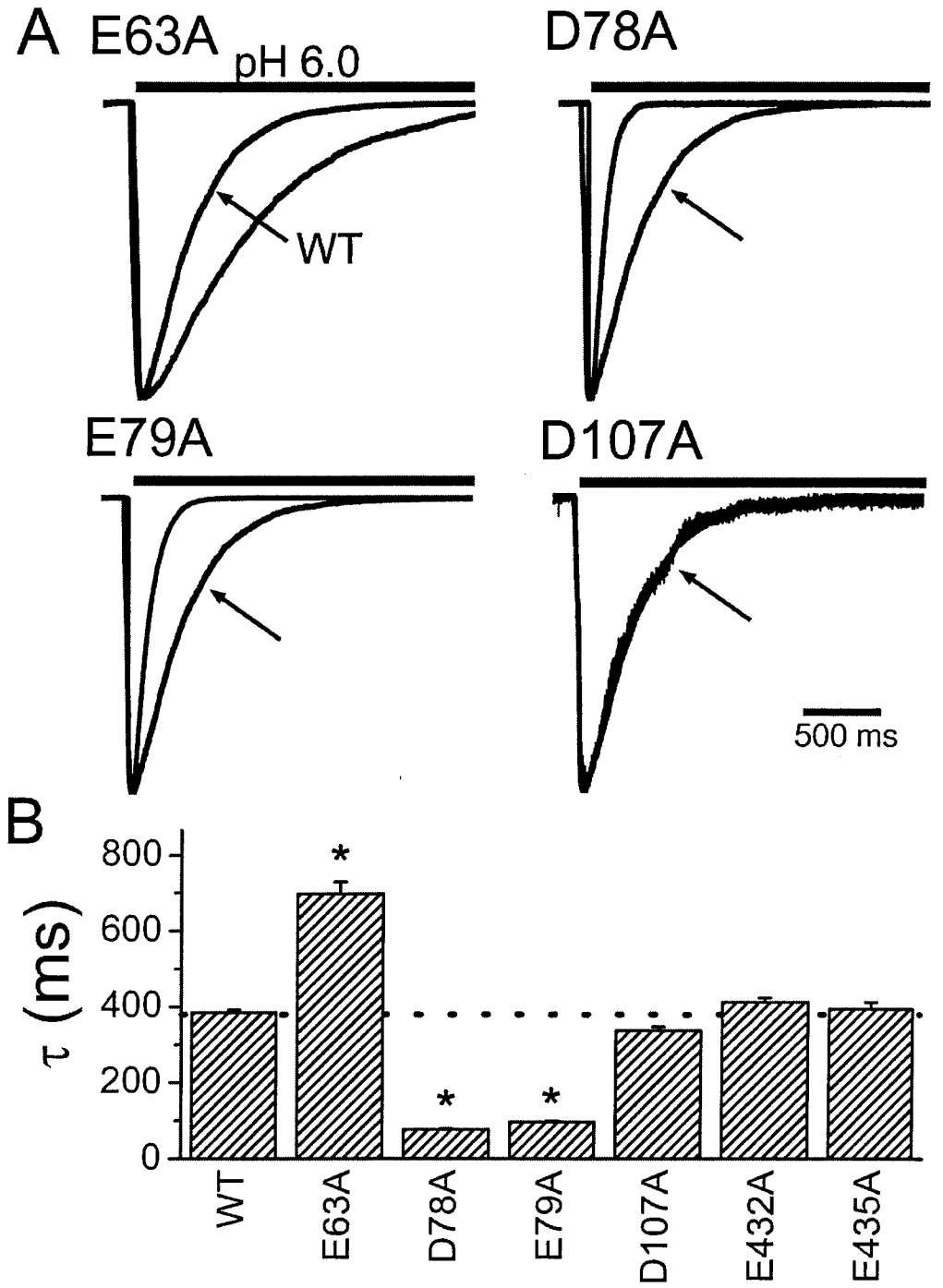

A
E63A
pH 6.0
WT
D78A
E79A
D107A
500 ms
B
τ (ms)
800
600
400
200
0
*
*
*
WT
E63A
D78A
E79A
D107A
E432A
E435A

Figure 13

Alanine scan of region outside TM1. (A) Normalized currents from cells expressing indicated ASIC3 mutant. (B) Average time constants (τ) of desensitization for mutant channels. (* denotes $p \leq .001$ vs. wt). (n=4-27 cells).

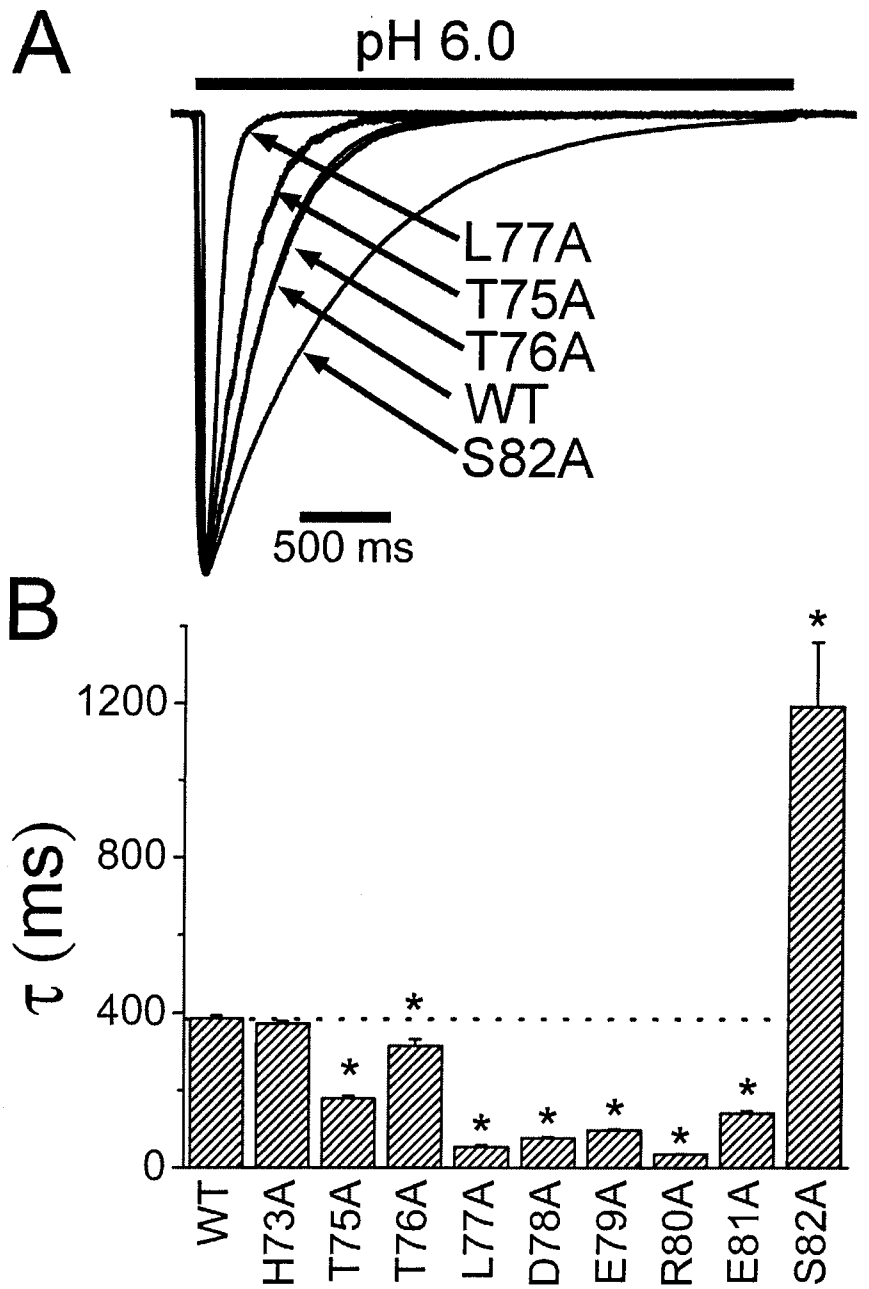

A
pH 6.0
L77A
T75A
T76A
WT
S82A
500 ms
B
τ (ms)
1200
800
400
0
WT
H73A
T75A
T76A
L77A
D78A
E79A
R80A
E81A
S82A

Table III

Properties of ASIC3 mutants. Time constant of desensitization (τ), half maximal pH of Activation ($pH_{0.5}$ Act) and half maximal pH of steady state desensitization ($pH_{0.5}$ Des) of ASIC3 mutants. n = number of cells tested.

	τ (ms)	n	$pH_{0.5}$ Act	n	$pH_{0.5}$ Des	n
WT	385±8	27	6.8	12	7.2	6
E63						
A	698±31	5				
H73						
A	371±6	6				
T75						
A	178±7	4			7.3	4
T76						
A	314±17	6	6.9	4	7.1	6
L77						
A	53±5	14	6.4	4	6.9	5
D78						
A	77±3	17	6.5	14	7.1	8
N	39±2	12				
E	401±16	15	6.9	3		
C	173±6	17			7.2	4
R	22±2	11				
E79						
A	97±3	31	6.7	8	8.0	5
Q	153±6	8			7.5	4
D	36±1	9			7.3	3
C	119±4	16			7.9	9
P	18±1	9			7.8	4
R80						
A	35±1	9			7.3	4
V	NC					
E	41±1	3			7.2	4
C	175±9	5			7.3	5
E81						
A	140±6	14	6.8	8	7.3	5
Q	270±11	5	6.9	4	6.9	3
C	676±72	8	6.9	3		
V	826±95	7	7.2	3	6.9	4
S82						
A	1192±166	15	6.8	3	7.0	5
R99						
A	394±17	3				
R102						
A	371±8	3				
D107						
A	338±10	4				
E432						
A	412±12	11	6.8	3		
E435						
A	395±17	8	6.8	5		
D439						
A	NC					
D78/E79						
A	22±1	13			8.1	4

The residue at position 78 showed a very clear pattern among its mutations: negative charge was critical for normal desensitization kinetics (Fig. 14A). Desensitization kinetics reflected the charge of the amino acid at this position, with negative charges (glutamate) desensitizing much slower than neutral (asparagine), or positive charges (arginine).

E79 and E81 mutants showed less distinct patterns among their mutations (Fig. 14 B, C). With E79, the conservative neutral amino acid glutamine desensitized quickly arguing that perhaps charge is important. On the other hand, replacing glutamate with aspartate led to very quickly desensitizing channels, which argues that the size of this residue is very important. No mutations I performed at this site did anything but speed desensitization. These experiments suggest that both size and charge may be critical to this residue's function. Indeed, the conservation of a glutamate at this site is absolute among functional ASICs.

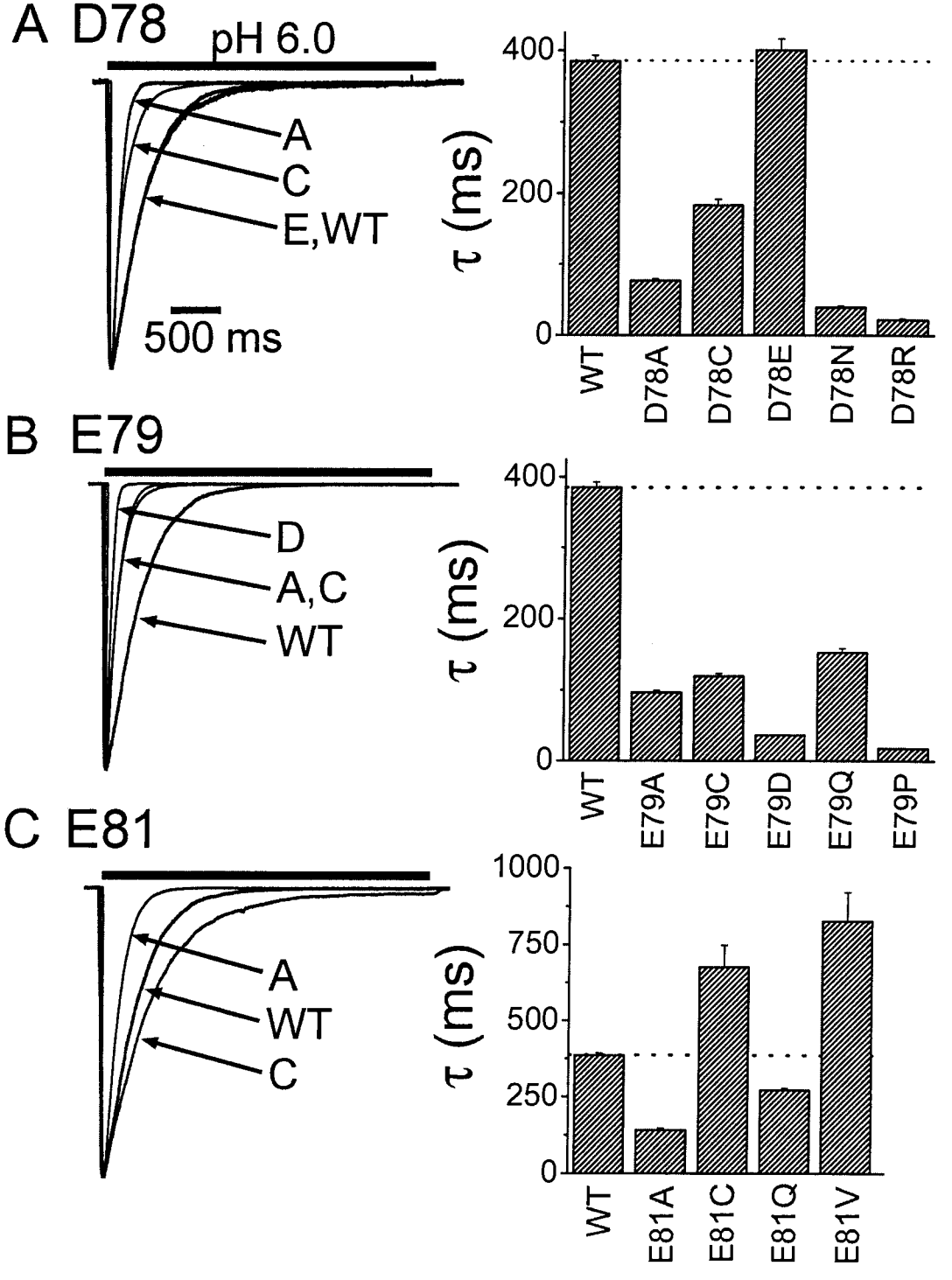
No clear pattern emerged with mutations of E81. Some mutants sped desensitization (A, Q) whereas some greatly slowed desensitization (V, C). Since this residue is not conserved between different subunits, it may play a role in determining desensitization kinetics between subunits, as will be discussed further in this chapter.

MTS Modification of E79C

To further test the accessibility and function of E79, I made a cysteine mutant, E79C, whose terminal sulfhydryl group can react with methanethiosulfonate (MTS) groups (CH_3SO_2SX, where X is a variable functional group) (Akabas, Stauffer et al. 1992; Karlin and Akabas 1998). Fig. 11B shows the various functional groups (-SX) of

Figure 14

Mutagenesis of acidic residues. Normalized currents from D78 (A), E79 (B), or E81 (C) mutants and their corresponding average time constants. (n=5-27 cells).


A D78
pH 6.0
A
C
E,WT
500 ms
τ (ms)
400
200
0
WT
D78A
D78C
D78E
D78N
D78R
B E79
D
A,C
WT
τ (ms)
400
200
0
WT
E79A
E79C
E79D
E79Q
E79P
C E81
A
WT
C
τ (ms)
1000
750
500
250
0
WT
E81A
E81C
E81Q
E81V

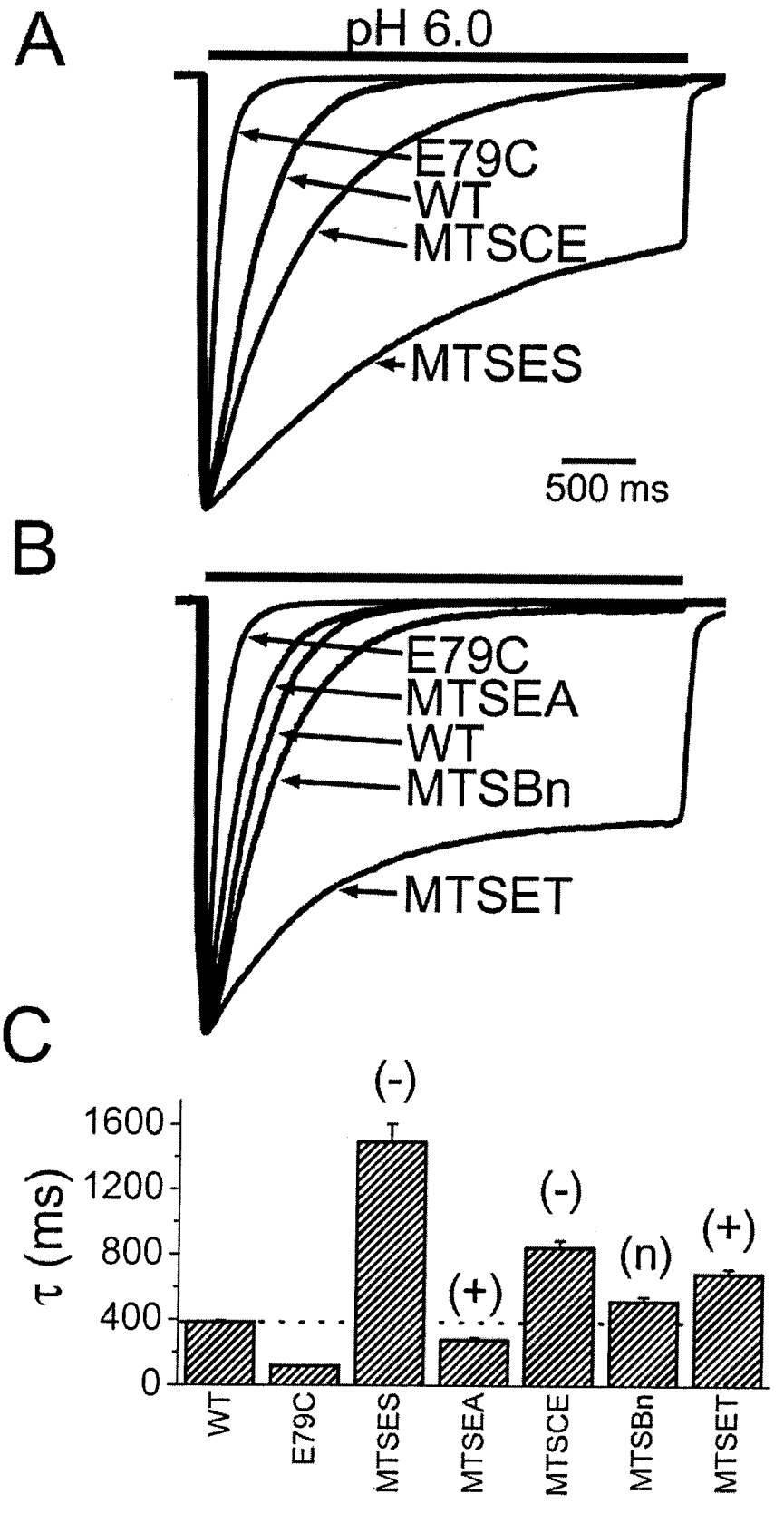
the reagents used in my experiments. None of these reagents had any effect on gating kinetics of wild-type ASIC3, indicating that native cysteines in the channel are either unavailable to react with MTS compounds or do not alter gating upon reaction. This is consistent with previous studies on ASIC2a (Adams, Snyder et al. 1998; Poet, Tauc et al. 2001).

Like E79A, the E79C mutant had much faster desensitization kinetics than wild-type channels (Fig. 14A). Cells transfected with E79C channels were exposed to MTS compounds (1 mM, 5 minutes) in the bath solution at pH 8, and this irreversibly changed the desensitization kinetics (Fig. 15). Desensitization slowed enormously after a permanent charge—either negative (MTSES, Sodium (2-sulfonatoethyl) methanethiosulfonate) or positive (MTSET, [2-(Trimethylammonium)ethyl] methanethiosulfonate bromide)—was attached. A titrateable acidic group (MTSCE, 2-carboxyethyl methanethiosulfonate), which might most closely mimic the native glutamate, slowed desensitization beyond the wild-type channel, but not nearly as much as the permanent charges. A large neutral group (MTSBn, benzyl methanethiosulfonate) and a titrateable positive group (MTSEA, 2-aminoethyl methanethiosulfonate hydrobromide) also slowed desensitization, roughly to the neighborhood of wild-type channels.

Modification of E79C with either MTSES or MTSET created a non-desensitizing component of the current. This component is more pronounced with MTSET, and causes it to appear to have a slower time constant. The current, however, still fits a single exponential with an average time constant of 680 ms, which is significantly less than MTSES modified channels (1500 ms).

Figure 15

Modification of E79C by MTS reagents. Cells expressing E79C mutant channels were exposed to the indicated MTS reagents (1mM) for 5 minutes. Traces shown (A, B) were recorded after modification. (C) Average time constants from cells after exposure to the indicated MTS reagent. Symbols denote charge of the modifying reagent. (n=4-16).


A
pH 6.0
E79C
WT
MTSCE
MTSES
500 ms
B
E79C
MTSEA
WT
MTSBn
MTSET
C
τ (ms)
1600
1200
800
400
0
(-)
(+)
(-)
(n)
(+)
WT
E79C
MTSES
MTSEA
MTSCE
MTSBn
MTSET

Overall, the results of figure 14 and 15 suggest that charge and size of residue 79 are important for the speed of desensitization. They further suggest that any charge on this residue slows desensitization.

D78 and E79 Mutants in ASIC1a

Both D78 and E79 are absolutely conserved in ASIC1a, ASIC1b, ASIC2a, and ASIC3. There are deviations in one, or both of these residues in ASIC2b and ASIC4, neither of which are acid-sensitive (Fig. 11). It follows that if these residues are critical for acid-sensitivity, then I should see similar effects by mutating them in ASIC1a.

Both D78 and E79 were converted to alanine in ASIC1a, and representative desensitization kinetics are shown in Figure 16. As expected, both mutations sped desensitization in ASIC1a (Table IV). Although the D78A mutant had a less robust effect when compared with E79A, both significantly altered desensitization kinetics. These mutants still had increased desensitization kinetics over wild-type channels at pH 5, when desensitization is faster (Fig. 16B). These data show that D78 and E79 play a role in desensitization across the ASIC family, and are not ASIC3 specific.

Effects of Mutating Residue 82

The only two residues in this region of ASIC3 in which mutations slowed desensitization were E81 and S82 (Figs. 13, 14). The interesting thing about these two residues is that neither of these residues is conserved between ASIC3 and ASIC1a, but both are conserved across species in their respective subunits (Fig. 17A). This suggests

Figure 16

Mutations of D78 and E79 have similar effects in ASIC1a. (A) Normalized currents recorded from cells transfected with either WT, or mutant ASIC1a channels. (B) Average time constants of desensitization at either pH 6.0 (hollow) or pH 5.0 (hatched). n=6-8 cells for each measurement. * indicates $p < .05$, ** indicates $p \leq .001$ compared to WT as measured by students t-test.

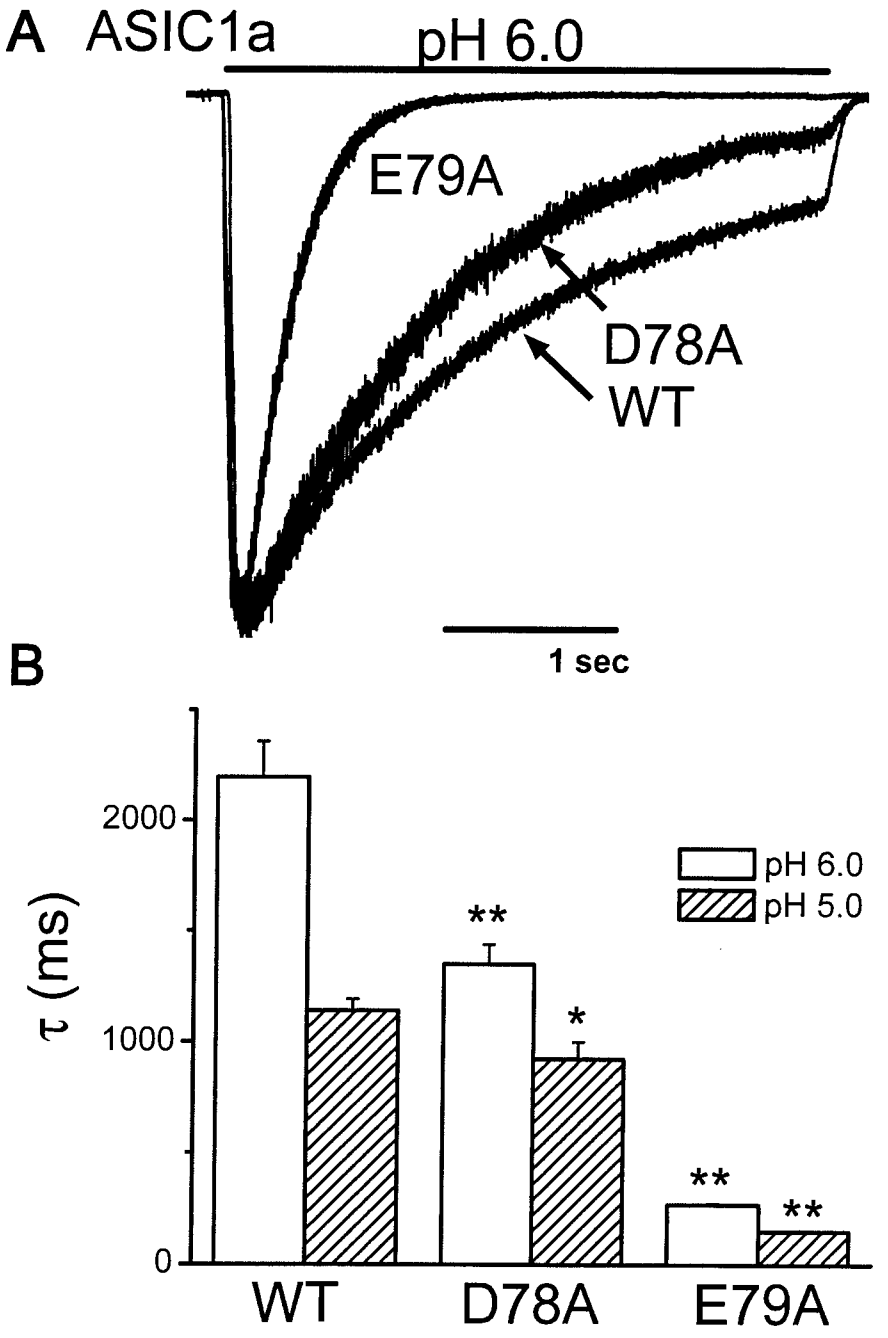

A ASIC1a
pH 6.0
E79A
D78A
WT
1 sec
B
τ (ms)
2000
1000
0
pH 6.0
pH 5.0
**
*
**
**
WT
D78A
E79A

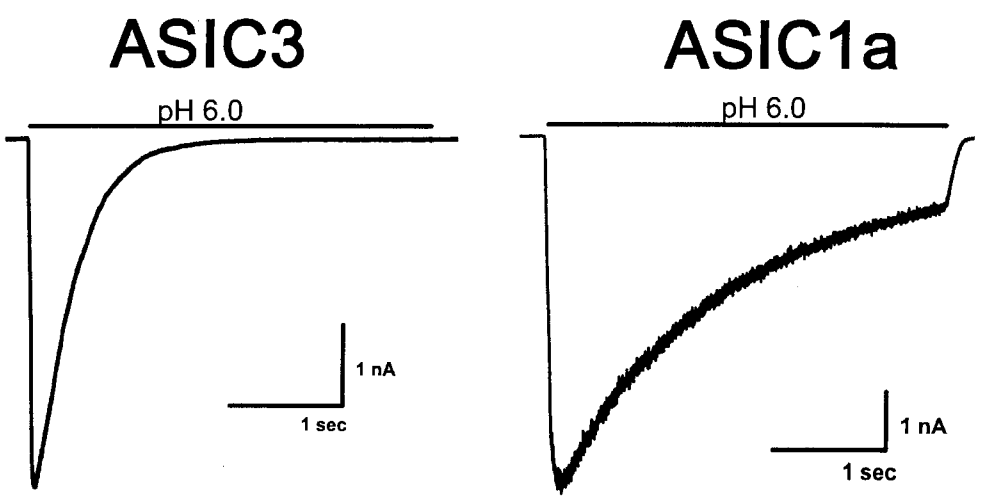
Figure 17

Sequence and desensitization kinetics of ASIC3 and ASIC1a. (A) Alignment of rat, human and mouse ASIC3 and ASIC1a outside TM1. The shaded box surrounds residues 80-83. The numbering at the top corresponds to rat ASIC3 and rat ASIC1a residues. (B) Desensitization of ASIC3 (left) or ASIC1a (right) currents in response to a pH 6 stimulus in transfected CHO cells.

A

```
                  72-73-74-75-76-77-78-79-80-81-82-83-84-85-86-87-88
rASIC3      HHKTTLDERESHQLTFP
mASIC3      HHKTTLDERESHQLTFP
hASIC3a     HHQTALDERESHRLIFP
rASIC1a     HHVTKLDEVAASQLTFP
mASIC1      HHVTKLDEVAASQLTFP
hASIC1      HHVTKLDEVAASQLTFP
```

B



that these residues may contribute to subunit specific differences in desensitization kinetics. Figure 17B shows wild-type currents from ASIC3, and ASIC1a. ASIC1a desensitizes much slower than ASIC3 at pH 6 (Table IV). Their sequences show key changes in the amino acids following residues 78 and 79, which may underlie these kinetic differences (Fig. 17A). I made further mutations within this sequence in an attempt to test whether or not these residues help create the distinct kinetics of each subtype.

Figure 13 suggested that the residue at position 82 might play an important role. In ASIC3, this is a serine, whereas it is an alanine in ASIC1a. The serine to alanine mutation in ASIC3 caused a slowing of desensitization (Fig. 18A, Table IV). Since ASIC1a desensitizes more slowly, and has an alanine at this position, I hypothesized that a serine at position 82 in ASIC1a would speed desensitization. To test this, I made the A82S mutant in ASIC1a, and as Fig. 18B shows, desensitization is dramatically sped up (τ = 50 ms, Table IV)). This argues that this residue might be critical in determining the desensitization kinetics of different subtypes. Both ASIC1a and ASIC2a have an alanine at this position, and both desensitize more slowly than ASIC3.

Four Residues Help Determine Kinetics of Subunit

Two other amino acids in this region of interest are variable among different subunits: residues 80 and 81. In ASIC3, both of these are charged residues, an arginine and a glutamate. ASIC1a has a valine and an alanine at these respective locations. Both of these mutants were made in ASIC3. The R80V mutant did not express and the E81A mutant desensitized more quickly than wt ASIC3 (Figure 14, Table III). In addition to

Figure 18

Residue 82 greatly affects desensitization kinetics. Normalized currents from pH 6.0 stimulus of cells transfected with either WT or mutant ASIC3 (A), and ASIC1a (B). (C) Average time constant of desensitization for WT or mutant channels. (** indicates $p \leq .001$ vs. WT, n = 8-27 cells).

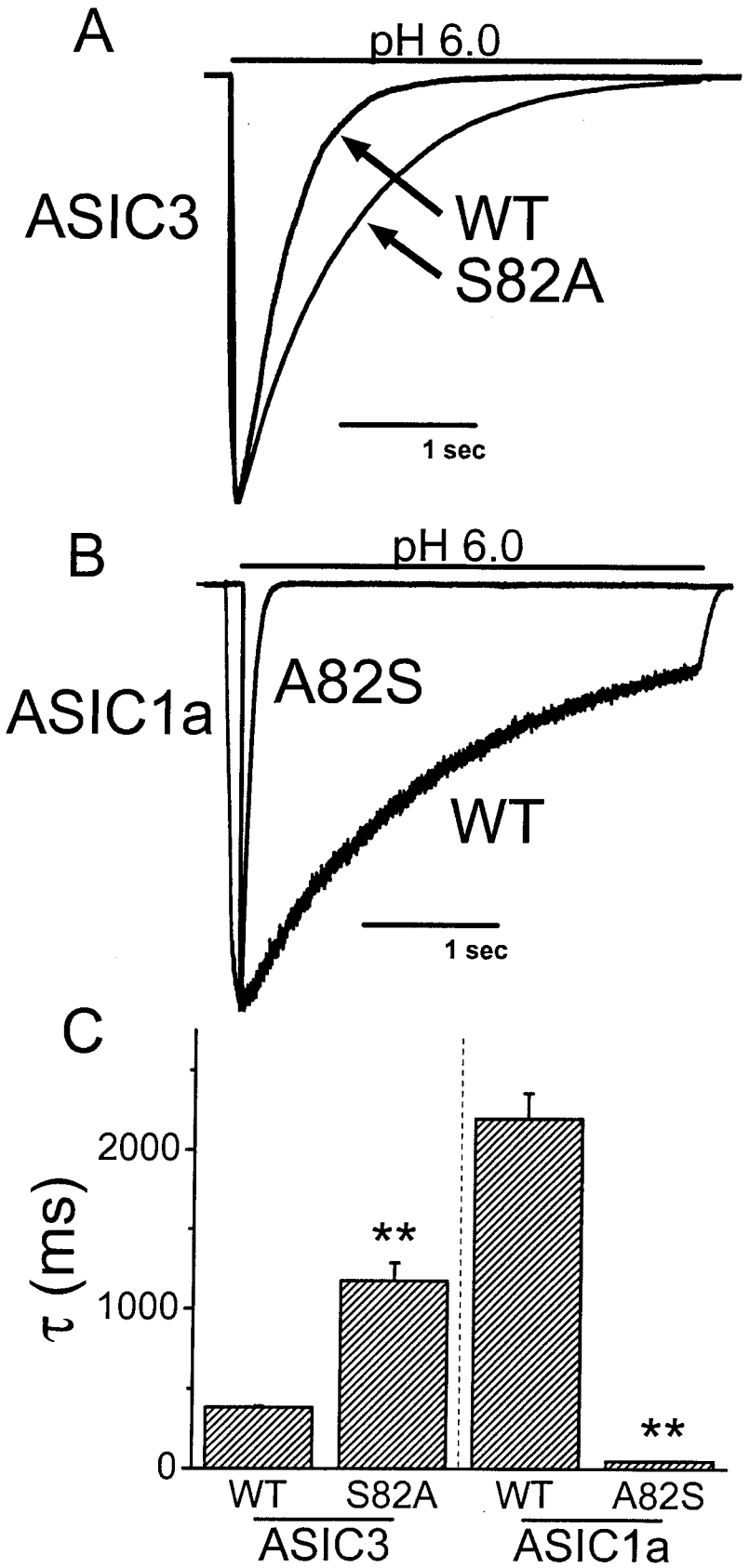

A
pH 6.0
ASIC3
WT
S82A
1 sec
B
pH 6.0
ASIC1a
A82S
WT
1 sec
C
τ (ms)
2000
1000
0
**
**
WT
S82A
WT
A82S
ASIC3
ASIC1a

Table IV

ASIC3 mutants vs. ASIC1a mutants. Time constant of desensitization for mutants made in either ASIC1a, or ASIC3. Quad indicates an exchange of residues 80-83 between ASIC1a and ASIC3.

	ASIC1a	ASIC3
WT	2196±162	385±8
D78A	1349±89	77±3
E79A	268±6	97±3
82S	50±1	(WT)
82A	(WT)	1192±166
Quad	340±23	892±35

these residues, the amino acid at position 83 has been shown to affect desensitization kinetics in ASIC1 (Coric, Zhang et al. 2003). Together these four residues are shaded in Fig. 17. Since all four have been shown to affect desensitization either in ASIC3 (R80, E81), ASIC1a (S83) or both (82), I mutated all four of these residues simultaneously (Fig. 19A). These quadruple mutants essentially swapped four amino acids between ASIC1a and ASIC3. The hypothesis behind this was that these four residues help impart the characteristics of desensitization upon each subunit. If this is true, then swapping these four residues should also convert desensitization kinetics between the two subunits.

The first mutant, ASIC3-Quad, had the ASIC1a sequence inserted into ASIC3. This desensitized more slowly than wt ASIC3 (Fig. 19B), but still more rapidly than ASIC1a. The time constant of desensitization averaged about 900 ms (Fig. 19D, Table IV), which is more than 2 fold slower than wtASIC3.

The ASIC1a-Quad mutant had kinetics that very closely resembled ASIC3, desensitizing with a τ of 340 ms (Fig. 19C, Table IV). In this case, desensitization kinetics were almost indistinguishable between ASIC3 and the mutant ASIC1a-Quad.

Both of these data suggest that this sequence can account for some of the differences seen between these two subunits, but clearly there are other important residues that determine how fast these channels desensitize.

Results Summary

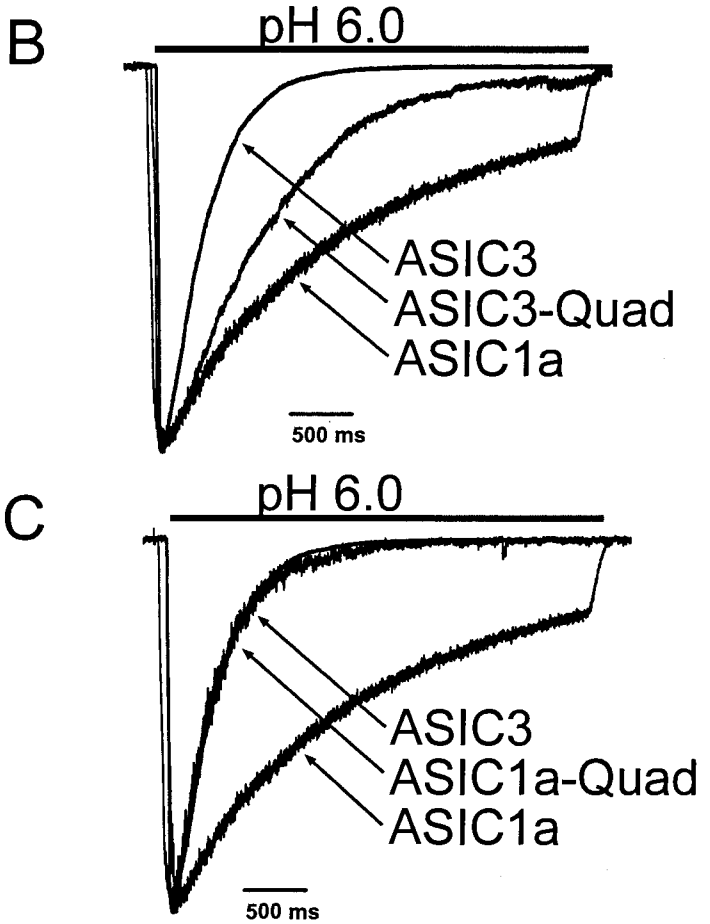
This chapter establishes a region outside TM1 that is involved in ASIC desensitization. There are two highly conserved putative proton binding sites—D78 and E79—within this region that help to regulate desensitization kinetics among both ASIC1a

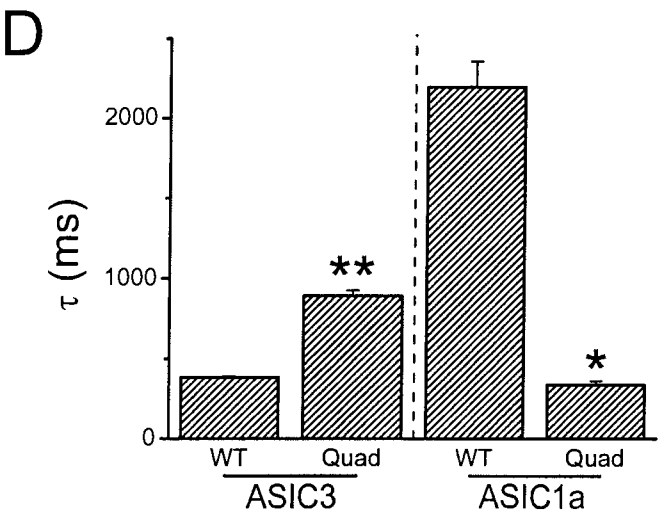
Fig. 19

Exchange of four residues imparts opposite kinetics. (A) Partial sequence of quadruple mutants of ASIC1a or ASIC3. Changed residues are shaded. (B, C) Representative traces from cell expressing quadruple mutant of ASIC3 (B) or ASIC1a (C) and WT channels of each of those subunits. (D) Average time constant of desensitization of the mutants. n = 5-27 cells. ** indicates $p < .001$ compared to WT ASIC1a and ASIC3, * indicates $p < .05$ vs. WT ASIC3, and $p < .001$ vs. WT ASIC1a.

A

```
ASIC3-Quad   72HHKTTLDEVAASQLTFP
ASIC1a-Quad  72HHVTKLDERESHQLTFP
```





and ASIC3. Mutagenesis and cysteine modification establish the importance of charge on these two residues. It seems possible that titration of these two residues helps drive desensitization. Other residues within this sequence that differ between these subunits appear to cause some of the differences in desensitization kinetics, particularly the residue at position 82, where a serine creates fast desensitization, and an alanine causes slower desensitization. Finally, a sequence of four amino acids that differ between ASIC1a and ASIC3, but are highly conserved among ASIC1a and ASIC3 in different species, seems to contribute in large part to the different desensitization kinetics of the two channels.

Chapter 5

A conformational change outside the first transmembrane domain that accompanies desensitization of acid-sensing ion channel (ASIC) 3

Chapter 5

Introduction:

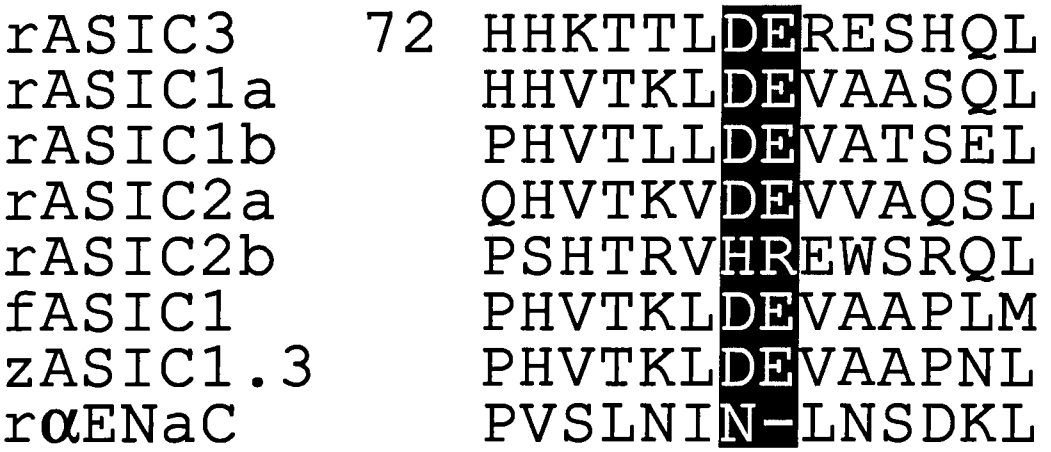
Chapter 4 showed a region outside the first transmembrane domain that was important for desensitization. Many residues within this region altered desensitization kinetics and pH sensitivity, with few effects on activation. Two highly conserved residues are putative proton binding sites: D78 and E79 (Fig. 20). Data suggested that these residues might be titrated as part of desensitization. This chapter aims to test whether or not this region undergoes a conformational change during desensitization. Previous evidence indicates that desensitization involves a conformational change of the channel.

The first study to show evidence of a conformational change during desensitization was done by the Welsh lab (Askwith, Benson et al. 2001). They altered the temperature during pH stimuli of ASIC3 and found that while activation was not affected much, the desensitization rate was greatly affected by the ambient temperature. The relatively high Q10 of desensitization (4.4) suggested that the channel underwent a significant conformational change to desensitize. For comparison, the Q10 of conductance of a sodium channel is 1.3, and many enzyme reactions have Q10 values near 3 (Hille 2001).

Schild and colleagues investigated R43, which is on the intracellular side of the first transmembrane domain of ASIC1a. They found that Cd^{2+} applied to the R43C mutants slows desensitization and concluded that the transmembrane helix undergoes a conformation change during desensitization (Pfister, Gautschi et al. 2006).

Figure 20

Alignment of protein sequence surrounding D78-E79. The DE pair is absent from the epithelial Na channel (rαENaC) and absent from rASIC2b, which is the one ASIC in the list that fails to make acid-gated current when expressed alone.


rASIC3 72 HHKTTLDERESHQL
rASIC1a HHVTKLDEVAASQL
rASIC1b PHVTLLDEVATSEL
rASIC2a QHVTKVDEVVAQSL
rASIC2b PSHTRVHREWSRQL
fASIC1 PHVTKLDEVAAPLM
zASIC1.3 PHVTKLDEVAAPNL
rαENaC PVSLNIN-LNSDKL

In chapter 4, I showed that modification of E79C with MTS reagents greatly slowed desensitization. Mutations that decreased the size or charge of E79 sped desensitization. One interpretation of these two findings is that E79 gets buried during desensitization. Removal of charge might aid in this transition, whereas increasing the size or charge might result in the opposite. In this chapter I will test whether or not E79 or adjacent residues alter their accessibility to MTS reagents during desensitization.

E79 mutants alter steady state desensitization

Mutating the acidic amino acids, D78 and E79, to alanine created channels that desensitized notably faster than wild-type (Fig. 21A) and a double mutant (D78A/E79A) was faster still (Table V). We converted five other acids in the extracellular domain to alanine and none of them caused this effect (Table V). Three of these were just outside the second transmembrane domain; E432A and E435A were no different from wild-type and D439A did not express. D107A, nearer the center of the extracellular domain was no different from wild-type and E63A, within a few amino acids of TM1, prolonged the desensitization rate. D78 and E79 are noteworthy for their high conservation in ASICs, but not in ENaCs or in ASICs that cannot by themselves form acid-gated channels (Fig. 20).

Steady state desensitization measures the fraction of channels in the desensitized state at a given pH. To measure this, the pH of the bath solution was changed prior to a pH 6.0 stimulus (as shown in the protocol on Fig. 21B). The resulting current from the pH 6.0 stimulus was plotted against the pH of the bath solution ("Conditiong pH"). Wild-type channels show a half maximal pH of desensitization at pH 7.2 (Fig. 21B, C). At pH 7.4,

Figure 21

Properties of D78A and E79A. (A) Faster rate of desensitization compared to wild-type (WT) channels. Channels are opened by a step to pH 6.0 from pH 8.0. (B) Currents from the indicated channels evoked by a step to pH 6.0 from a bath solution of the indicated pH. Wild-type and D78A channels are half-desensitized near pH 7.2/7.1, whereas E79A channels are half-desensitized at pH 8. (C) Steady state desensitization curves (open symbols) and activation curves (closed symbols) for the indicated channels. E79A channels have identical activation curves to wild-type, but greatly shifted desensitization. D78A channels are little changed from wild-type for either curve. Desensitization curves plot the normalized peak currents against the conditioning pH, as in (B). Activation curves plot normalized peak current against the test pH (raw data not shown).

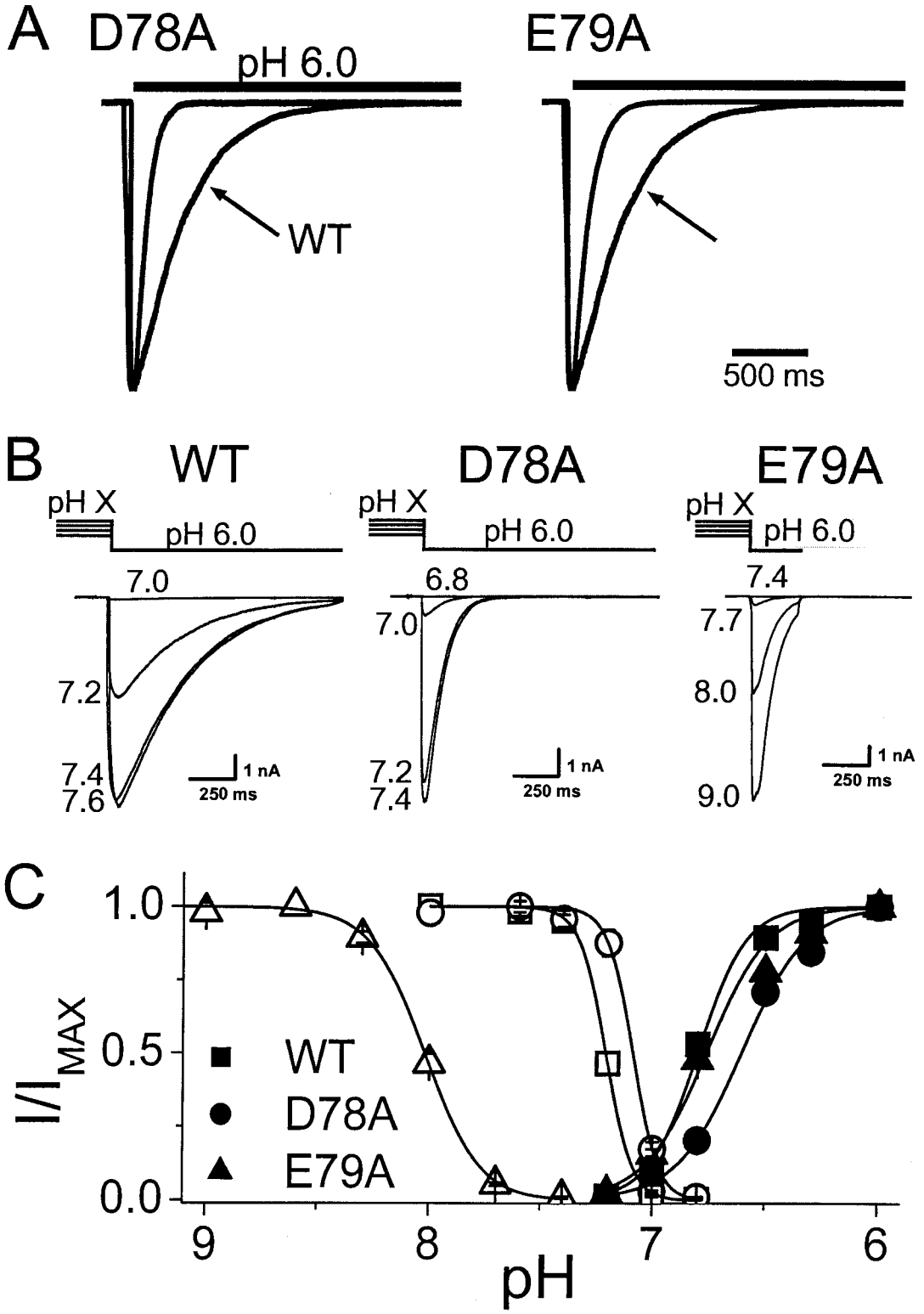

A
D78A
pH 6.0
E79A
WT
500 ms
B
WT
D78A
E79A
pH X
pH 6.0
7.0
7.2
7.4
7.6
1 nA
250 ms
6.8
7.0
7.2
7.4
7.4
7.7
8.0
9.0
C
1.0
0.5
0.0
I/I MAX
WT
D78A
E79A
9
8
7
6
pH

Table V

Desensitization time constants (τ) of ASIC3 mutants. D439A mutants failed to express significant acid-evoked currents.

	τ (ms)	n
WT	385±8	27
E63A	698±31	5
D78A	77±3	17
E79A	97±3	31
D107A	338±10	4
E432A	412±12	11
E435A	395±17	8
D439A	No Expression	
D78A/E79A	22±1	13

nearly all the channels are available to be opened, while at pH 7.0, most channels are desensitized. This steep dependence on pH can be described by a Hill slope of more than 7.

The steady state desensitization curve of E79A was shifted to 10-fold lower proton concentrations than wild-type and the slope of the desensitization curve was halved (7.3 to 3.5 Hill slope) (Fig. 21 B, C). In contrast, the activation curves (closed symbols) of wild-type and E79A channels were indistinguishable.

D78A mutants, although they share the same increased speed of desensitization as E79A, did not exhibit the dramatic shift in steady state kinetics. The steady state desensitization curve of D78A channels was shifted slightly to the right. Although this shift seems negligible ($pH_{0.5}$ = 7.1), it is very noticeable since the Hill slope is so steep. The traces show significant differences at pH 7.0 and 7.2 over wild-type channels. D78A also had a slight rightward shift in its activation curve, which was of similar amplitude to its shift in steady state desensitization.

MTS accessibility of E79C is state dependent

The large shift in steady state desensitization of E79A suggests that the desensitized state is stabilized (has lower potential energy) in the mutant channel compared to wild-type. This could be from a conformational change. To explore this possibility, I made cysteine mutants whose terminal sulfhydryl group can react with methanethiosulfonate (MTS) groups (CH_3SO_2SX, where X is a variable functional group) (Akabas, Stauffer et al. 1992; Karlin and Akabas 1998). I tested a variety of these reagents and none affected gating kinetics of wild-type ASIC3 (not shown). In contrast,

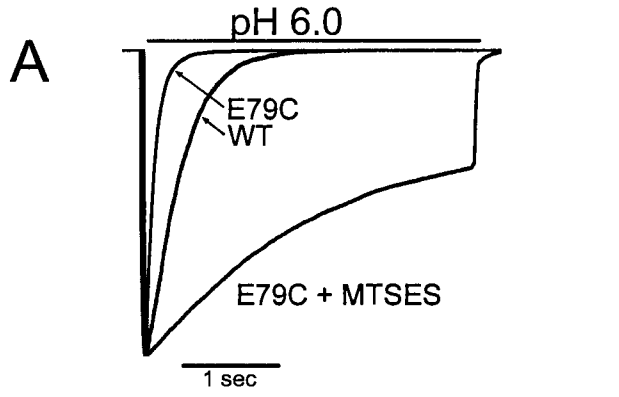
all the MTS reagents slowed desensitization of E79C; the most dramatic change occurred with the negatively charged MTSES (Fig. 22A). The 120 ms desensitization rate of E79C changed to 1500 ms when MTSES (1 mM) was applied at either pH 8.0 or 7.6 for 5 minutes. In contrast, there was virtually no change when MTSES was applied at pH 7.0. At pH 7.4 the current had both a fast and a slow component of desensitization (Fig. 22B). Currents were fit with a sum of two exponentials and the histogram in Fig. 22C shows the percentage of total current fit by a slow time constant when MTSES was applied at the indicated pH. I take the height of the bar as an indicator of MTS reactivity. MTS reactivity dropped off in the range of pH where there is substantial steady state desensitization (inset).

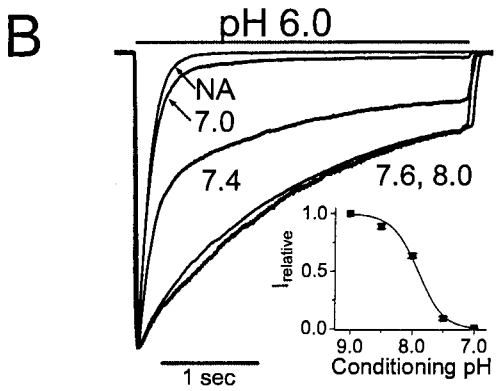
The simplest interpretation of Fig. 22B is that the desensitized channels cannot react with MTSES. However, an alternate possibility is that decreasing pH protonates the thiolate ion on the cysteine, rendering it unreactive to MTS reagents (Roberts, Lewis et al. 1986). I tested this possibility by shifting the desensitization curve to more acidic pH using increased divalent ion concentration in the extracellular medium (Babini, Paukert et al. 2002). Fig. 22C shows that channels reacted readily with MTSES at pH 7.2, 20 mM Ca^{2+}, where desensitization is incomplete (inset), but not at pH 7.2, 1 mM Ca^{2+}, where channels are fully desensitized. I conclude that MTS reactivity is determined by the gating state of the channel, not by pH itself.

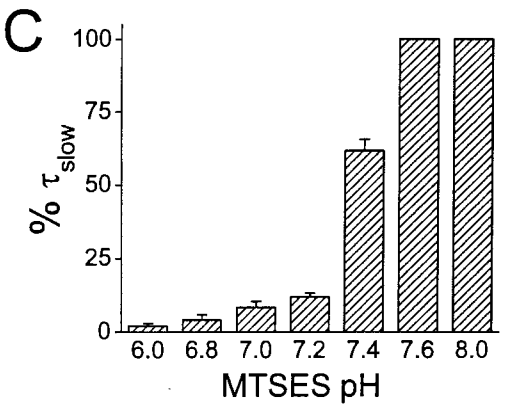
I tried to quantify reaction kinetics of MTSES with residue 79 by varying the concentration and exposure time (Fig. 23). Gradual modification of both desensitization rate and peak amplitude was evident when 200 μM MTSES was applied at pH 8 (Fig. 23A). In contrast to pH 8, no reaction was ever detected at either pH 7 or 6 (Fig. 23B).

Figure 22

E79 accessibility is state dependent. (A) Normalized wild-type currents (WT) and E79C current before (E79C) or after (E79C + MTSES) exposure to 1 mM MTSES for 5 minutes at pH 8.0. (B) E79C currents before (NA) and after exposure to 1 mM MTSES for 5 minutes at the indicated pH. Inset shows steady state desensitization for E79C ($pH_{0.5}$ of 7.91, n=9); at pH 7.0, channels are fully desensitized. Currents were fit with a double exponential with fast (~120 ms) and slow (>600 ms) components. Histograms (C) give the percentage of the current that was fit with a slow time constant against the pH of the MTS incubation solution. (D) MTSES sensitivity depends on desensitization, not pH itself. Cells were exposed to 1 mM MTSES for 5 minutes at pH 8.0 or pH 7.2 in 1 mM Ca^{++}, or pH 7.2 in 20 mM Ca^{++}. The addition of 20 mM Ca^{++} shifts the steady state desensitization curve (hollow circles, inset) so channels are available to open at pH 7.2, thereby rendering them sensitive to MTSES.


A
pH 6.0
E79C
WT
E79C + MTSES
1 sec


B
pH 6.0
NA
7.0
7.4
7.6, 8.0
1.0
0.5
0.0
I relative
9.0
8.0
7.0
Conditioning pH
1 sec


C
100
75
50
25
0
% τ slow
6.0
6.8
7.0
7.2
7.4
7.6
8.0
MTSES pH

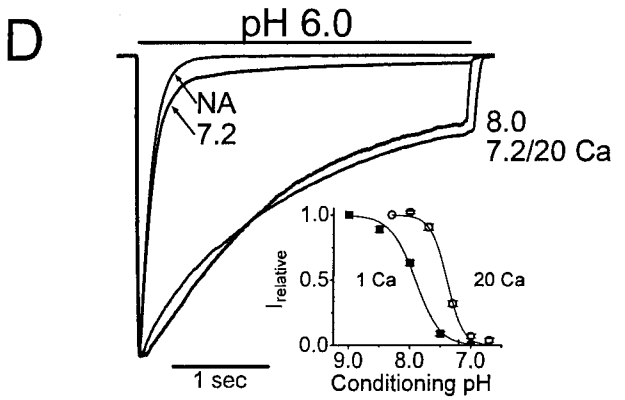
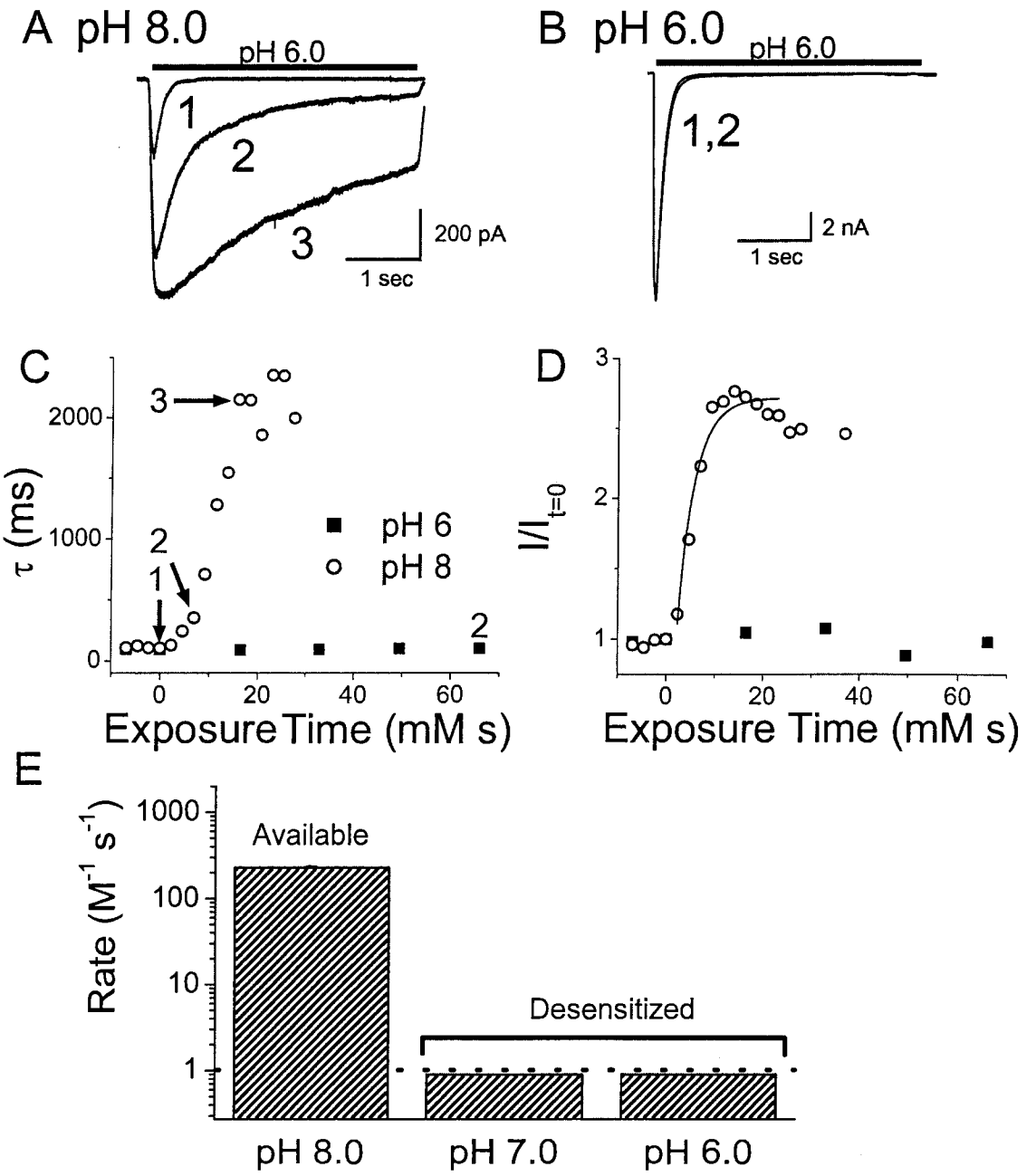

D
pH 6.0
NA
7.2
8.0
7.2/20 Ca
1.0
0.5
0.0
I relative
1 Ca
20 Ca
9.0
8.0
7.0
Conditioning pH
1 sec

Figure 23

MTSES reaction rate at E79C. (A) Currents from E79C channels before application of MTSES (1), and 35 sec (2) or 81 sec (3) after exposure to 200 μM MTSES at pH 8.0. The increase in amplitude at pH 8 was caused by MTSES because it shifts the steady state desensitization curve of E79C to the right (not shown). (B) Before and after 5 minutes in 1 mM MTSES at pH 6; there is no evident change when MTSES is applied to desensitized channels. (C, D) Modification as a function of exposure time (time exposed * concentration MTSES) for E79C. (C) plots the time constant (τ) of desensitization; (D) plots the relative peak current amplitude, which is fit with a single exponential used to obtain the modification rate constant. (E) Modification rate constants of E79C at pH 8, 7, and 6. The rate at pH 8.0—226 $M^{-1}s^{-1}$—could be quantified, whereas rates when channels are desensitized (either pH 6.0 or 7.0) are below 1 M^{-1} s^{-1}.


A pH 8.0
pH 6.0
1
2
3
200 pA
1 sec
B pH 6.0
pH 6.0
1,2
2 nA
1 sec
C
τ (ms)
2000
1000
0
3
2
1
2
pH 6
pH 8
0
20
40
60
Exposure Time (mM s)
D
I/I t=0
3
2
1
0
20
40
60
Exposure Time (mM s)
E
Rate (M-1 s-1)
1000
100
10
1
Available
Desensitized
pH 8.0
pH 7.0
pH 6.0

Exponential fits of the fractional change vs. exposure time (in mM x seconds, Fig. 23C, D) yielded a reaction rate of 226 $M^{-1}sec^{-1}$ at pH 8, and rates below my detection level (<1 $M^{-1}sec^{-1}$) at the two lower pH values (Fig. 23E). Evidently, MTSES can react with residue 79 if the channel is available to open, but cannot react with desensitized channels.

MTSES modification of E79C shifts steady state desensitization

The increase in peak amplitude seen after modification (Fig. 23A, D) can be explained by a shift in the steady state desensitization curve. Figure 24 shows steady state desensitization curves of E79C mutants either before (squares) or after (circles) modification with MTSES. Modification of E79C with MTSES caused a large rightward shift in steady state desensitization, shifting the $pH_{0.5}$ from 7.9 to 7.1. Since modification rates were tested at pH 8, about half the channels were desensitized. As channels were modified, more became available to be opened at this pH which led to the increase in peak current.

Modification of D78C and R80C lacks state dependence

Do residues adjacent to E79 also change accessibility during desensitization? As with E79, the adjacent cysteine mutants, D78C and R80C, exhibited significantly faster desensitization than wild-type channels (Fig. 25); however, they showed clearly different response to MTSES. D78C mutants were not obviously changed by MTSES, whereas R80C was modified by MTSES equally well at pH 8 and pH 6 (Fig. 25A, B). The effect of MTSES on R80C—desensitization was slowed by about 30%—was modest compared to MTS modification of E79C, but it was absolutely consistent and statistically

Figure 24

MTSES shifts steady state desensitization of E79C mutants. (A) Steady state desensitization of E79C currents either before (left) or after (right) modification by MTSES. Numbers indicate pH of bath solution prior to a pH 6.0 stimulus as indicated by the black bar. (B) Steady state desensitization curves of E79C before (squares) and after (circles) modification by MTSES. Curves are average of at least three cells.

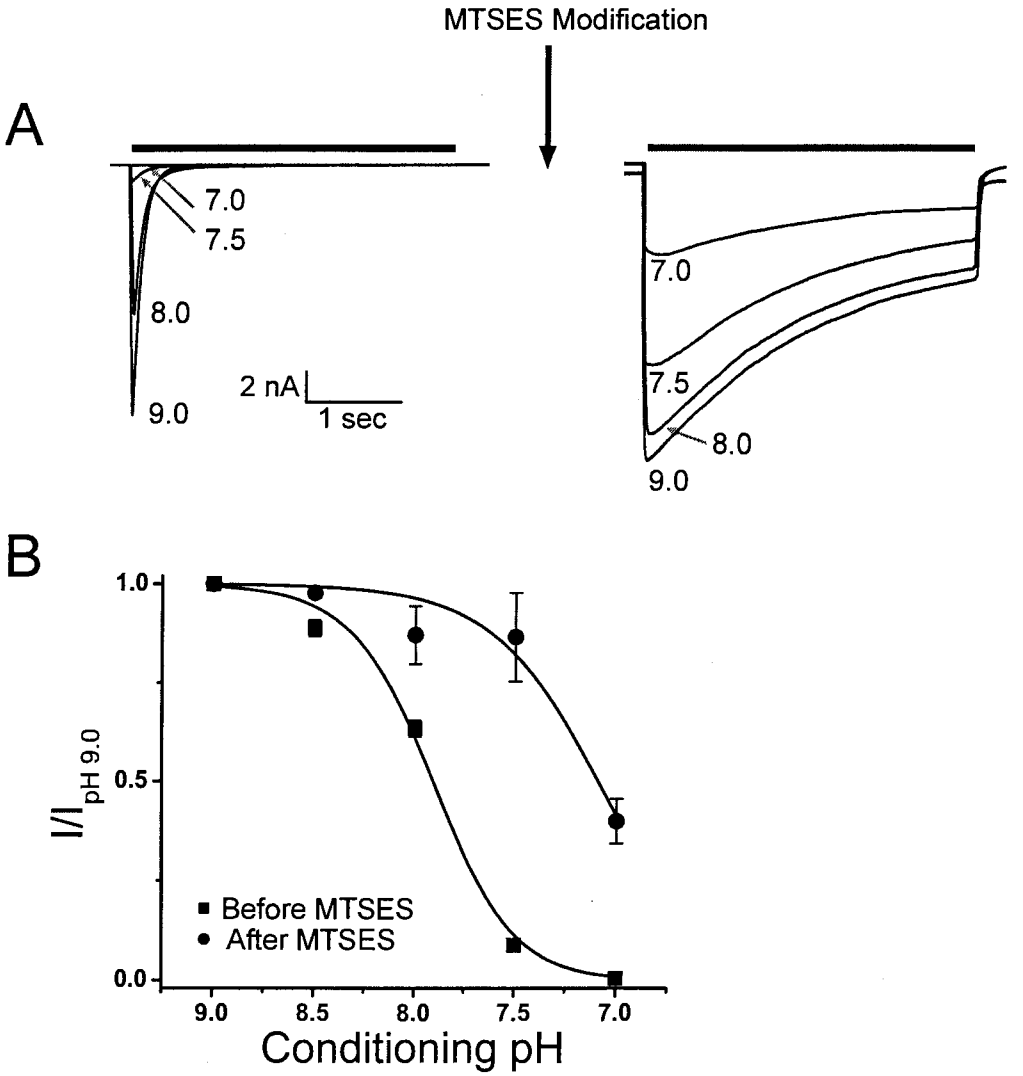
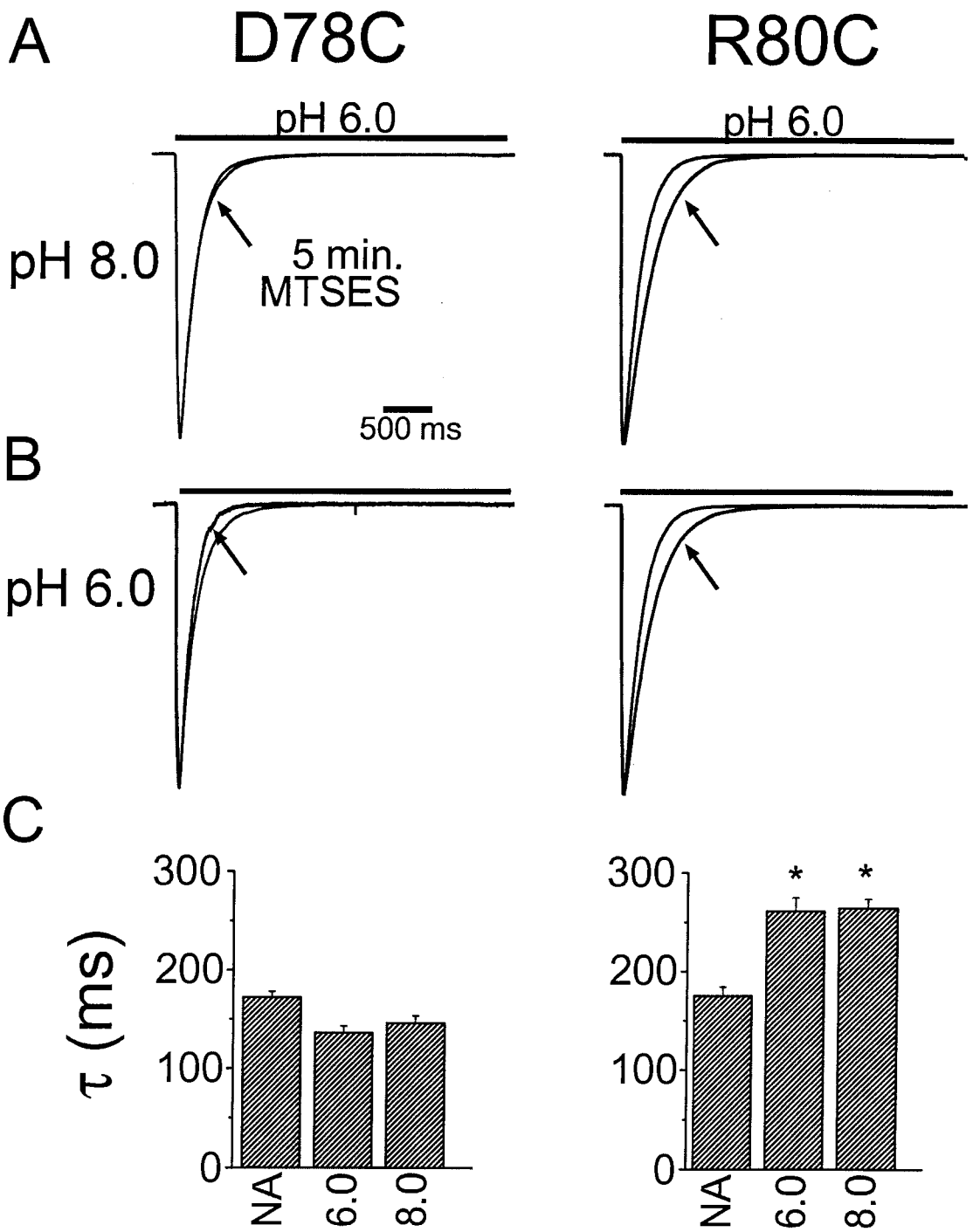

MTSES Modification
A
7.0
7.5
8.0
9.0
2 nA
1 sec
7.0
7.5
8.0
9.0
B
1.0
0.5
0.0
I/I pH 9.0
Before MTSES
After MTSES
9.0
8.5
8.0
7.5
7.0
Conditioning pH

Figure 25

Residues adjacent to E79C differ in their apparent state dependency of modification. (A,B) Representative traces from cells transfected with either D78C (left) or R80C (right) without and with (arrows) exposure to 1 mM MTSES for 5 min at pH 8 (A) or pH 6 (B). (C) Average time constants for desensitization. NA indicates no application of MTSES. A minimum of 5 cells was tested for each condition. D78C channels failed the statistical test for being modified by MTSES, whereas R80C channels are modified at both pH 6 and pH 8 (*indicates $p<0.001$ versus WT cells).


A
D78C
R80C
pH 6.0
pH 6.0
pH 8.0
5 min.
MTSES
500 ms
B
pH 6.0
C
τ (ms)
300
200
100
0
NA
6.0
8.0
*
*

significant (Fig. 25C). I conclude that, unlike residue 79, residue 80 is accessible to chemical reagents in both the available and desensitized states. Perhaps residue 78 fails to react because it is inaccessible to chemical reagents in both states; alternatively, MTS reagents could react but fail to change desensitization rate.

Results Summary

In this chapter, I found that a residue extracellular to TM1 undergoes a conformational change during desensitization. This residue, E79, became inaccessible to chemical modification in the desensitized state. Adjacent residues, D78 and R80, showed different patterns of accessibility: D78 was unreactive in either state, and R80 reacted in both available and desensitized states. Additionally, mutations of E79 caused shifts in steady state desensitization. The E79A mutants desensitized at 10 fold lower proton concentration. This shift was also accompanied by a change in the Hill slope of the steady state desensitization curve. All of this data argues that E79 is a critical residue for controlling desensitization in ASICs.

Chapter 6

Discussion

Chapter 6

Activity of acid-sensing ion channels under conditions that accompany stroke (Chapter 3)

My data shows that many ionic changes during ischemia will enhance the activation of ASICs. This activation could then lead to cell death. Changes in pH provide the stimulus to open ASICs during ischemia. Decreased calcium, increased membrane potential, and increased zinc can all act to alter the level of activation. Decreased extracellular calcium will increase the amount of activation by shifting the shape and location of the activation curve. Increases in membrane potential will have an effect on the voltage-dependent Mg^{++} block in these channels. Zinc released from synaptic vesicles will have enhancing effects on ASIC1a/2a channels and an inhibitory effect on homomeric ASIC1a. This study shows that there are many other relevant changes other than pH that will act to enhance ASICs under ischemic conditions.

ASICs are related to ion channels found in *C. elegans* called degenerins that cause cell death when hyperactive. This is one factor leading to the hypothesis that ASICs play a role in neuronal death during ischemia. Degenerin channels can cause cell death when a mutation causes permanent activation of the channel. This activation kills the mechanosensory neurons that express these mutant channels, supporting the idea that excessive activation of ASICs could cause cell death.

The decrease in extracellular calcium during ischemia can be striking, dropping more than ten-fold to 0.1 mM (Siemkowicz and Hansen 1981). Previous work has shown that protons and calcium compete to gate ASICs (Immke and McCleskey 2003), so it is no surprise that I observed enhancement of ASIC activation by decreasing calcium. Decreased extracellular calcium had two effects on activation, changing both shape and

pH range of activation. Both of these effects made ASICs more sensitive to protons. Lower calcium concentrations made the activation curve considerably steeper over the foot of the curve. This steeper section overlies the physiological pH range of ischemia, suggesting external calcium concentrations will play a crucial role in the channels sensitivity to pH changes during ischemia.

Although shifting of activation by calcium has previously been observed in ASICs, this is the first time that calcium has been shown to change the shape of the activation curve. Previous studies on the effect of calcium on ASIC1a have not seen this behavior (Babini, Paukert et al. 2002). These differences may arise from techniques used for solution exchange, and in the cell systems used. Previous studies were performed in oocytes, which are ill suited to study some properties of this channel, especially activation. ASIC1a desensitizes very quickly at the peak of its activation curve (<100 ms), and also undergoes substantial rundown at these pHs. The relatively slow solution exchange in oocytes exacerbates these two issues when measuring activation curves. Since desensitization is occurring faster than solution exchange, many channels will be desensitized before others can be activated. This will lead to artificially low peak amplitudes. To measure my activation curves, the cells were lifted off of the bottom of the dish, which greatly speeds solution exchange. Furthermore, for pH <5.5, currents were always normalized to a pH 5.7 stimulus done 20 sec prior, which eliminated most of the effects of run down.

Decreased calcium not only affects the range of activation of ASICs, but also affects the conductance of ASICs (Immke and McCleskey 2003; Paukert, Babini et al. 2004). When calcium is lowered, the apparent single channel conductance of the

channels increases. The proposed mechanism for this effect is an open channel block by calcium. A higher conductance would contribute to the enhancement of ASIC current by low extracellular calcium. This may underlie some of the enhancement I see, although the shifting and steepening of the activation curve likely causes the majority.

The voltage-dependent block of ASICs by Mg^{++} is qualitatively similar to that seen in NMDA receptors. The affinity for Mg^{++} would be reduced under the depolarized state that occurs during ischemia, but it is unclear what the physiological relevance this site would have, given its relatively low affinity for Mg^{++} (12 mM at 0 mV), and its relatively weak voltage dependence ($\delta = 0.23$). Comparatively NMDA receptors have δ values near 0.8, and K(0) near 4 mM (Wollmuth, Kuner et al. 1998). This higher δ value means that it will be a better coincidence detector since there is a stronger effect of voltage on the binding site. Nonetheless, this binding site in ASICs may play an important role in ischemic cell death, since increased extracellular Mg^{++} has been shown to be neuroprotective in some ischemic models (Zhu, Meloni et al. 2004). Inhibition of ASICs could be one mechanism behind its neuroprotective effects.

The effects I observed of zinc on ASIC1a and ASIC1a/2a heteromers are consistent with previous studies (Baron, Schaefer et al. 2001; Chu, Wemmie et al. 2004). Neither of these previous studies looked at the effects of zinc under different calcium concentrations. Since calcium levels decrease during ischemia, it is important to look at the effect of zinc under these conditions. Zinc had identical actions under these lower calcium concentrations. Studies have suggested that synaptically released zinc may play a role in ischemic cell death from seizures and stroke (Frederickson, Hernandez et al. 1989; Weiss, Sensi et al. 2000; Lee, Zipfel et al. 2002). ASIC1a/2a channels would be an

apparent target for this zinc. On the other hand, zinc would inhibit ASIC1a channels, which are thought to be more important for ischemia induced neuronal death.

ASIC2a is upregulated in neurons that survive an ischemic episode (Johnson, Jin et al. 2001). This has led to speculation that this upregulation of heteromeric channels is a neuroprotective mechanism. Indeed, heteromeric ASIC1a/2a, and ASIC2a homomers are less sensitive to pH and are much less calcium permeable (Bassilana, Champigny et al. 1997).

A recent study demonstrated the important role of ASICs in ischemic cell death (Xiong, Zhu et al. 2004). It showed significantly less damage caused by stroke when ASIC1a was either blocked or knocked out in mice. They used both amiloride and the ASIC1a antagonist PcTx1 to inhibit channels. Both antagonists were effective in reducing cellular death caused by the infarction. The fact the PcTx1 was a potent neuroprotectant led them to conclude that homomeric ASIC1a, and not ASIC1a/2a, is the channel that is responsible for ischemic cell death.

This is in contrast to my results suggesting that ASIC1a/2a may be more active during ischemia. I showed that ASIC1a/2a will pass sustained currents, whereas ASIC1a desensitizes fully. In addition, if significant zinc is released then ASIC1a/2a activity will be enhanced whereas ASIC1a will be depressed. Both of these results suggest that ASIC1a/2a could be more active than ASIC1a. One possibility for this discrepancy is that both channels contribute to ischemic cell death, and by blocking ASIC1a they eliminated a large component of the excitotoxicity. It is also likely that other factors may act to enhance ASIC1a sustained currents in vivo. Chemicals like arachidonic acid and RFamide peptides have indeed been shown to do this (Allen and Attwell 2002). ASIC1a

is more sensitive to pH than ASIC1a/2a so it will likely play a larger role in excitotoxicity if it can remain active.

This work shows that there are at least three separate divalent binding sites in ASIC1a that have different affinities for different divalent ions. Calcium has effects on the channel that are independent of magnesium and zinc. This calcium binding site is involved in activation of the channel, and is likely formed in part by the proton binding site for activation. Calcium's effects could be caused by multiple sites. A calcium binding site has been located and characterized in ASIC1a, and is formed by two acidic residues just outside of the putative pore region (Paukert, Babini et al. 2004). This site seems to be responsible for the observed open channel block by calcium. This site does not remove calcium's effects on gating the channel however, suggesting there are at least two calcium binding sites. Although this site cannot explain all of the effects I saw with calcium on activation, it will definitely contribute to the increase in current observed when extracellular calcium drops. It is hard to explain the change in shape of the activation curve based on this one site, which further suggests that there may be at least two calcium binding sites.

The second site identified is the magnesium binding site which is partially inside the electric field of the channel's pore. There are a couple of characteristics that this site has that suggest it is a separate site. The fact that calcium block has no voltage dependence suggests that calcium does not bind with significant affinity to this site. Since this site is within the pore, calcium may be able to better permeate at this site. One reason for this might be the lower hydration energy of calcium versus magnesium (Hille 2001). The high energy required for magnesium to dehydrate makes it difficult for it to

permeate pores where its hydrated size is too large. Calcium may simply permeate, whereas magnesium gets stuck. It is probable that Mg^{++} is binding just outside the outer vestibule. This would be the last point before total dehydration. Calcium would likely pause briefly there, whereas Mg^{++} would bind more strongly.

At very hyperpolarized potentials (<-140 mV) there is a relaxation of the negative slope in the I-V curve. This suggests that Mg^{++} is permeating at these potentials. The energy provided by the extreme driving force may be great enough that the Mg^{++} can dehydrate and pass through.

Lastly, there is the zinc binding site on ASIC1a, and a separate zinc binding site on ASIC2a. Both of these sites have been identified on the respective subunits. The enhancing site on ASIC2a is formed by a pair of histidines, one of which is absent in ASIC1a leading to the subunit specificity of this effect (Baron, Waldmann et al. 2002). The blocking site on ASIC1a has also been identified, and is formed by a lysine in the extracellular domain (Chu, Wemmie et al. 2004). This site is specific to ASIC1a.

In addition to the ionic conditions studied here, other molecules may contribute to ASIC activation during ischemia. Lactate, a product of ischemic tissue, is a known potentiator of ASICs and also enhances acid-evoked currents in cerebellar purkinje neurons (Immke and McCleskey 2001; Allen and Attwell 2002). Besides lactate, other molecules and peptides likely released during ischemia have been shown to enhance ASICs including arachidonic acid, neuropeptide FF (NPFF), and other RF-amide related peptides (RFRPs) (Askwith, Cheng et al. 2000; Allen and Attwell 2002; Ostrovskaya, Moroz et al. 2004).

RFamide peptides include FMRFamide which gates the ASIC relative FaNaCh. Although RFamides do not directly gate ASICs, they can increase peak currents and decrease rates of desensitization (Lingueglia, Deval et al. 2006). There are many RFamide peptides in vertebrates, although their distribution tends to be more peripheral and spinal for NPFF (Panula, Kalso et al. 1999). Some of the RFRPs, do have significant expression in the brain (Yano, Iijima et al. 2003).

The physiological functions of ASICs in central neurons are unclear. The best evidence for the role of ASICs in the CNS comes from studies of ASIC1 knockout mice. The knockouts show mild deficits in LTP, learning, and memory (Wemmie, Chen et al. 2002; Wemmie, Askwith et al. 2003). This suggests that ASICs may play some sort of synaptic role in the brain. Although the physiological role of ASICs in the brain is not well defined, there is mounting evidence that these channels play a pathological role.

Xiong et al. showed that ASICs are a legitimate target for neuroprotection from stroke. Here I show ischemic conditions in addition to pH that alter ASIC activation during such conditions. Although the pH is obviously important for activation of ASICs, calcium concentrations may be equally important. By dropping the calcium concentrations, currents were increased by up to four fold (Fig. 8A). This drop in calcium would have a drastic effect on ASIC activation during stroke or seizure. Depolarization of neurons would further increase ASIC activation by reducing the voltage-dependent magnesium block of ASICs. As I have shown though, magnesium will not compensate for the decreased levels of calcium (Fig. 7B). Lastly, zinc will enhance heteromeric ASIC1a/2a channels under the ionic condition present in ischemia.

All of these ionic changes are what likely cause activation of ASICs during ischemia, which can then lead to cell death.

A region affecting desensitization in ASICs contributes to differences in kinetics between ASIC1a and ASIC3 (Chapter 4)

Since desensitization is a common characteristic of all functional ASICs, it is likely that the proton binding sites and machinery are conserved. Two putative proton binding sites I identified in ASIC3, D78 and E79, are conserved in almost all ASICs that generate a proton sensitive current. Neutralization of either of these residues in ASIC3 led to channels that desensitized much more rapidly than wild-type. Alanine mutants of these residues in ASIC1a had similar desensitization phenotypes to those seen in ASIC3. This argues that these two acidic residues may be an important part of the desensitization machinery for functional ASICs.

D78 and E79 are present in virtually every ASIC that generates a proton-gated current. The single exception is an ASIC from zebrafish (zASIC4.1) (Paukert, Sidi et al. 2004) which has an asparagine in place of the aspartate; zASIC4.1 desensitizes very quickly, similar to D78N mutant reported here (Table I). Conversely, ASICs that do not generate proton gated currents (fASIC1.2, fASIC2, zASIC4.2, rASIC2b, and m/r/hASIC4s) have deviations in this sequence. Although D78 and E79 are very highly conserved in ASICs, they are absent in most other DEG/ENaC family members, suggesting that they are important for the acid sensitivity of ASICs.

Consistent with this, D78A and E79A mutants of ASIC1a had similar phenotypes as the ASIC3 mutants. Both of these mutants desensitized faster than wild-type. Of

these two mutants, E79A had a much larger effect on the kinetics. The E79A mutants in ASIC1a desensitized almost 90% faster than wild-type. The consistency of these mutants provides evidence supporting the idea of a conserved function of these residues in this family.

One interpretation of the data in chapter 4 is that titration of one, or both of these acidic residues—D78 and E79—promotes desensitization. If the negative charge(s) prevent channels from entering the desensitized state, then protonation of these residues would ease the transition. Evidence for this comes from a number of sources: 1) Neutralization of either D78 or E79 speeds desensitization (Fig. 14). 2) Addition of a permanent charge on E79 greatly slows desensitization (Fig. 15). 3) Removal of the charge on E79 reduces the Hill slope for steady state desensitization, arguing that it may form a proton binding site (Fig. 20). None of these arguments is conclusive however, and I can only speculate at this point that this might be the case.

The mutation of E79 to an aspartate conserves the charge of the amino acid, yet desensitization is very fast. This indicates that the size of this residue is also crucial to its function. Interestingly though, this mutant had almost no shift in its steady state desensitization (Table III). It appears as though the negative charge preserves the pH dependence of desensitization.

The pKa of the side chain carboxylic acids on glutamates and aspartates is around 4 (Voet and Voet 1995). Desensitization occurs between pH 7.4 and 6.8, which is very far from the pKa of the side chains of these amino acids. These pKas are deceptive however, since they are calculated from the free amino acid in solution. In a folded protein, it is much more difficult to say what the pKa of a side chain is since the

surrounding environment can greatly alter the pKa. Nearby negative charges and hydrophobic regions can raise the pKa of acidic residues to stabilize them, so it is not unreasonable to suggest that acidic residues are titrated within this pH range. Both acidic (Chen, Pfuhl et al. 2000) and basic (Schulte and Fakler 2000) amino acids have been shown to have "anomalous" pKa values near neutral in the context of a protein. It is difficult to speculate on the environment surrounding D78 and E79 since we do not know the structure of the region.

It is interesting to note, however, that D78 and E79 are two adjacent acidic residues. Depending on the orientation of the side chains this may cause an increase in their pKa. It is apparent that E79 has a larger role in desensitization when compared with D78. Perhaps D78 acts to modify E79 by shifting its pKa towards more basic values. This would be consistent with the fact that a negative charge at D78 conserved the desensitization phenotype.

ASIC4 has a proline at residue 79 instead of the conserved glutamate (Fig. 11). ASIC4 has never been shown to be acid-gated, and the proline at this site may be one reason why. It is possible that this subunit is acid-gated, and its desensitization properties make it difficult to detect. The E79P mutant of ASIC3 had greatly shifted pH dependence of desensitization, with a pH_{50} of 7.8 (Table III); all the channels are desensitized at pH 7.4 in this mutant. Since this is the pH of standard extracellular solution, all ASIC4 channels may be simply desensitized if attempting to record currents under these conditions. This is indeed the case with reports on ASIC4 (Grunder, Geissler et al. 2000). It would be interesting to see if this were the case, and given the right conditions, ASIC4 could yield acid sensitive currents. Of course this begs the question of

what ASIC4 responds to *in vivo*, since it apparently is not responsive to acid under physiological conditions.

The alanine scan of residues surrounding D78 and E79 shows that most of this region can affect desensitization in ASICs (Fig. 13). Since this region plays a role in determining desensitization kinetics, variability in this sequence may underlie desensitization kinetics differences between subunits. The mutation of S82 in ASIC3 suggested that this might be the case. If there is a serine at this position (WT), the channel desensitizes relatively quickly; an alanine at this position yields slowly desensitizing channels. Both ASIC1a and ASIC2a have an alanine at this position, and both desensitize slower than ASIC3. This suggests that this residue might be a key regulator of desensitization kinetics. Indeed, mutation of the alanine in ASIC1a to a serine sped up desensitization. Serine and alanine are very similar in their size, and only differ in the presence of a hydroxyl group. One could speculate that either the hydrophilicity or hydrogen bonding capabilities of the hydroxyl group are important. At this point we do not know what is so special about this hydroxyl group. Interestingly, this residue is conserved as either an alanine, or a serine or threonine in all functional ASICs.

The adjacent residue (E81 in ASIC3) has higher variability among different subunits: it is an alanine in ASIC1a, and a valine in ASIC2a. Mutations of this residue in ASIC3 caused either increases (alanine) or decreases (valine) in the speed of desensitization (Fig. 14). The valine corresponds well with the slow time constant for desensitization of ASIC2a, but the faster desensitization of the alanine mutant does not fit with the slower time course of ASIC1a. Interestingly, this valine mutation in ASIC3 was

not desensitization specific. It affected both activation curves and steady state desensitization curves (Table III), shifting them in opposite direction. The end result of these shifts were channels that generated very large sustained currents since activation was enhanced, and desensitization was inhibited. This residue likely plays an important role in the phenotype of each subunit.

Swapping four residues between ASIC1a and ASIC3 conferred desensitization properties of the opposite channel. The ASIC1a-Quad mutant (ASIC1a with ASIC3 residues 80-83) was most striking. Desensitization kinetics of this mutant looked almost identical to wtASIC3. The opposite mutation in ASIC3 was not as remarkable, but still was profoundly different from wtASIC3. This mutant desensitized much slower than wtASIC3, but did not closely resemble ASIC1a. This indicates that other residues further influence desensitization kinetics in ASICs.

The Canessa lab showed that three residues that are adjacent to the area identified in chapter 4 play a role in determining species specific differences between ASIC1 from fish and rats (Coric, Zhang et al. 2003). Three residues differ between ASIC1 in rats and in toadfish (SQL and PLM respectively), which correspond to H83-Q84-L85 in ASIC3. The fish ASIC1 desensitizes much quicker than rat ASIC1. By swapping the sequences between these two species, they were able to create rapidly desensitizing rat ASICs, and slowly desensitizing fish ASICs. My study extends these findings to show that this same region can determine differences between subunits. In addition this region influences more than just rates of desensitization, but also has effects on pH sensitivity.

At this point it is unclear what the role of these four residues is. One possibility is that they simply modulate the properties of the upstream conserved acidic residues.

Another is that they affect the conformational change that happens during desensitization (Chapter 5). Evidence from mutagenesis and the conservation of sequence suggests that residues surrounding D78 and E79 contribute to the desensitization phenotypes of each subunit.

Evidence has shown that the pH dependence of desensitization plays a crucial role in determining whether or not ASICs can pass sustained currents (Yagi, Wenk et al. 2006). The overlap of activation and desensitization allows "non-desensitizing" currents. These currents are created by channels being able to flicker between closed, open and desensitized states. Although these currents tend to be a very small percentage of the total current, they can be substantial provided that there is high enough expression. This is indeed the case with ASIC3 in sensory neurons (Benson, Eckert et al. 1999).

Different ASICs have different abilities to pass sustained currents. This region outside TM1 influences the pH sensitivity of desensitization. Perhaps it is alterations in this sequence that accounts for differences in sustained currents. For instance, ASIC2a/3 heteromers are able to pass larger sustained currents over a broader pH range than ASIC3. ASIC1a/2a channels are able to pass sustained currents, whereas ASIC1a homomers are not. There seems to be some property that ASIC2a has which enables it to pass sustained currents better. One possible reason could be that ASIC2a has a valine at position 81. This mutation in ASIC3 greatly shifted steady state desensitization in such a way that now sustained currents were a very large fraction of the total current. It is possible that this is one reason ASIC2a heteromers create larger sustained currents.

Clearly the extra-TM1 region is important for desensitization. A further screen would likely yield other regions that may interact with this one to affect desensitization.

This region is an important beginning for uncovering the mechanism and molecular details of desensitization.

A conformational change outside the first transmembrane domain that accompanies desensitization of acid-sensing ion channel (ASIC) 3 (Chapter 5)

Chapter 5 extended the previous chapter to show that there is a conformational change within the extra-TM1 region accompanying desensitization. Using substituted cysteine accessibility, I showed evidence that desensitization of ASIC3 involves a conformation change in the extracellular domain. The evidence is that MTSES can react at residue 79 when the channel is closed (pH 8) but not when it is desensitized (pH 7 and below). Thus, residue 79 appears to get buried away from the extracellular space during desensitization. The neighboring residues, 78 and 80, do not exhibit this state dependent change in accessibility.

The idea of a conformational change accompanying desensitization is not new. Askwith et al. showed that there is a very large temperature dependence of desensitization in ASIC3 (Askwith, Benson et al. 2001). This relatively high Q10 (4.4) suggests that there is a reasonable conformational change needed to put the channel into the desensitized state. A recent study by Pfister et al. showed that a pore lining residue on the intracellular end of TM1 appears to move during gating (Pfister, Gautschi et al. 2006). Whether or not it is through activation or desensitization cannot be determined. The accessibility of this residue is affected following desensitization, but it is unclear whether the conformation change was due to activation or desensitization.

A similar argument can be made about this study: that perhaps the conformation change occurred during activation. There is evidence that strongly suggests that the conformational change at E79 occurs during desensitization rather than activation: 1) Modification of E79C by MTS reagents only alters desensitization, without affecting activation. 2) E79 point mutants alter desensitization properties, without affecting activation properties. 3) Modification of E79C is greatly inhibited at pH 7.4 where there is negligible activation of the channel, yet substantial desensitization. All of these data suggest that the conformational change observed at E79 is due to desensitization and not activation. If the conformational change were to occur during activation, then I would expect modification and mutations to affect activation properties.

D78A, E79A and R80A mutants each desensitize rapidly compared to wild-type channels, yet only E79 mutants have significantly altered steady state desensitization. In principle, there is no surprise that rate and steady state occupancy do not predict each other because the two are controlled by different energies. The faster rate indicates a decrease in the transition state energy barrier between the open and desensitized states. The 10-fold lower proton concentrations necessary for steady state desensitization of E79A indicates stabilization of the desensitized state, a decrease in the energy of the desensitized state. The two unique properties of residue 79—only residue 79 changes accessibility during desensitization and only residue 79 mutations stabilize the desensitized state—seem likely to be related.

The steady state desensitization curve is very steep in wild-type channels (Hill slope = 7) and this decreases by a factor of 2 in the E79A mutant. Even such a large decrease in Hill slope in a mutant cannot be interpreted unambiguously because Hill

slope is a function of agonist number, agonist affinity, and the efficiency of gating conformation change (Colquhoun 1998).

Additionally, the Hill slope could be artificially steep in these channels. If activation promotes desensitization, even in a proton independent manner, then the pH dependence of desensitization will be at least partially based on the pH dependence of activation.

With most ligand gated ion channels, activation and desensitization are linked. A ligand binds at one site, which causes both activation and desensitization. Other channels have more complex ligands, requiring more complex ligand binding sites. ASICs on the other hand use protons as ligands which are easily bound with reasonable affinity to many amino acids. The dependence of desensitization on activation is not as easily assumed. Activation in the absence of desensitization has been shown at alkaline pHs when ASICs are activated by removal of extracellular calcium (Immke and McCleskey 2003). In this case, there is no desensitization because of the lack of protons. This shows that desensitization requires protons, unlike activation. Both activation and desensitization are affected by extracellular calcium (Babini, Paukert et al. 2002) and protons but it seems that they are affected differently. This suggests that the proton binding sites for each process are different.

The E79A mutant further showed that activation and desensitization are separable. The pH dependence of desensitization was shifted by almost an entire pH unit, without any effects on activation. In these channels, desensitization is clearly present without activation. This argues that channels do not need to go through the open state to desensitize. One caveat with this argument however, is that if desensitization is a very

forward-biased reaction, then we might still be getting desensitization from open channels. Even with a very low probability of opening the desensitized stated would be favored, and channels would tend to accumulate in this state. The activation may not be measurable, especially using whole cell patch clamp. So we cannot rule out entirely that desensitization can occur without activation.

On the other hand, some mutations did affect both activation and desensitization. L77A mutants and E81V mutants had both activation and steady state desensitization curves shifted. In the case of L77A, both were shifted equally to the right towards lower pH values. With E81V, the curves were shifted in opposite directions, creating more overlap between activation and desensitization. The fact that one mutation can alter both processes does not necessarily mean that they are linked. By disrupting the channel structure, you could be disrupting both processes independently. The residue L77 is very highly conserved across all DEG/ENaC family members as a hydrophobic residue (I, V, L, or M). The extreme conservation within the family suggests that it plays a very important conserved role. As mentioned previously, E81 is not conserved, and may play a role in distinguishing desensitization kinetics between different subunits. This data also implies that this residue may act to alter the pH sensitivity of activation and desensitization of different subunits.

The speculation that E79 is titrated during desensitization is supported by the fact that it gets buried. Perhaps E79 cannot be buried easily when it is charged. Neutralization of the carboxylic acid might allow the conformational change to become more favorable. Addition of permanently charged MTS reagents to this residue blocked the channels from fully desensitizing. Addition of similarly sized molecules with a lesser

charge had a much milder effect. This suggests that charges cannot be buried easily when attached at this position. If the native glutamate were to get protonated, then the transition to the desensitized state would be facilitated.

D78 and E79 are present in virtually every ASIC that generates a proton-gated current. (The one exception is an ASIC from zebrafish (zASIC4.1) (Paukert, Sidi et al. 2004) that has an asparagine in place of the aspartate at 78 and does indeed make acid-gated currents.) ASICs that do not generate proton gated currents (fASIC1.2, fASIC2, zASIC4.2, rASIC2b, and m/r/hASIC4s) all have deviations in the D78-E79 motif. Although D78 and E79 are very highly conserved in ASICs, they are absent in most other DEG/ENaC family members, their acid-insensitive cousins. This pattern suggested that the residues might be important for the acid-induced opening of ASICs, so it came as a surprise to find large effects on desensitization without any on channel opening. This supports the idea that desensitization can occur without channel opening (Korkushco, Krishtal et al. 1983) and also implies strong evolutionary conservation of residues that are devoted to acid-induced desensitization kinetics. This, in turn, implies an essential biological role for ASIC desensitization. A possibility previously raised in the literature is that, under physiological conditions, desensitization tightly limits the effective range of pH sensitivity to the narrow window of overlap between activation and desensitization curves (Yagi, Wenk et al. 2006).

Future Directions

This section attempts to address some of the unanswered questions raised by the data in this thesis. Each aim briefly highlights an unanswered question or unaddressed problem of the experiments performed, and outlines possible experiments.

Aim 1: Investigate activation of ASICs in cultured CNS neurons.

The experiments done on ischemic conditions affecting CNS ASICS were all performed in cultured cell lines transfected with cloned channels. Although this is a great way to study the individual channels, the experimental setup is very refined. Often the channels will behave very differently in a more native environment where there are many more accessory proteins and regulatory elements. In addition, cell death studies could be performed investigating what role pH, calcium, magnesium, and zinc play on neuronal death and whether they act through ASICs.

The main idea behind this aim is to move the experimental system to cultured CNS cells, such as hippocampal neurons. These cells would better represent the cellular environment that ASICs would be in. Activation curves of native acid-sensing channels from cultured hippocampal neurons could be measured as in Chapter 3 using whole cell patch clamp. These curves can be measured in the presence of normal (1.2 mM) or ischemic (0.1 mM) calcium to see how calcium affects the activation of these native channels. In addition to measuring acid-evoked currents, the membrane potential could also be recorded from neurons. This may give a better indication of what effect the pH and calcium conditions would have on the cell.

In addition to looking at transient activation of the native pH-evoked currents, this system would also be useful for looking at sustained activation of ASICs by low pH. Experiments in chapter 3 suggested that ASIC1a may not be able to pass sustained currents. This could be tested in cultured neurons as well. Relative activation of ASIC1a and ASIC1a/2a could be dissected with PcTx1, which is a toxin that selectively inhibits ASIC1a homomers.

If calcium influx through these channels is important for cell death, then it might also be important to run longer experiments on neurons looking at calcium influx. Sustained calcium elevation could easily be measured using calcium imaging on neurons exposed to various pH and calcium conditions.

Primary cell cultures are still far from physiological. The relatively harsh treatment that the neurons endure during the culturing process, plus the use of artificial growth media, will create a system that is still far from physiological. A more physiological way to perform some of these experiments would be in an acute slice preparation. In this prep, many of the synaptic contacts are preserved, and cells are much closer to their native state. One major issue however is that solution exchange becomes more difficult. This kind of preparation could be well suited to study sustained currents created by ASICs, since slow pH changes would be easily accomplished.

Aim 2: Characterize properties of E79 that are important for its role in desensitization.

Chapter 4 showed a region that affected desensitization in ASICs when mutations were introduced. Within this region residue E79 was the most influential residue. There

are some hints as to what it may do, but more work is needed to clarify the role of this residue.

If E79 gets buried, then mutations like E79F, or E79Y could be interesting to look at. The larger size of these two residues would help describe the importance of size at this position. One could hypothesize that desensitization might be slowed by these mutations, since E79 needs to be buried for desensitization to occur. On the other hand though, these two residues lack the negative charge that appears to slow desensitization. It is difficult to predict what effect these two mutants will have, but the outcome may yield important information about the properties of this residue.

The conformational change that E79 undergoes was not extensively characterized. I showed that it moves, but not where or how. This is a difficult question to address without more structural information. As this information becomes available for ASICs then this question becomes more accessible. More data could be collected on MTS accessibility of other residues within this region. If more residues behave like E79, then we may gain some insight into what is going on, but the interpretations are still very limited.

Combining the E79C mutation with other mutants may better address the issue of whether the conformational change occurs during activation or desensitization. Two mutations shifted activation of ASIC3. Combining one of these two mutations with E79C may lead to channels that have greater separation in activation and desensitization. This could help us separate whether activation or desensitization prevents modification of E79C. Alternately, we may get channels that can activate with very little desensitization (E81V). In this case we could apply MTS compounds during the large sustained currents

to test whether E79C is accessible in the activated state as we predict. Of course both of these would be dependent on whether or not the double mutants behave as predicted.

Aim 3: Look at desensitization of ASIC2a.

The experiments in chapter 4 showed that a set of four residues greatly influenced desensitization kinetics between ASIC1a and ASIC3. These four residues are conserved within each subunit. The sequence is ASIC2a is different from both ASIC1a and ASIC3. There are many obvious differences between ASIC2a, ASIC1a and ASIC3. The residue that stands out within this stretch is the homologous residue to E81 in ASIC3, which is a valine in ASIC2a. The E81V mutant in ASIC3 desensitized very slowly, like ASIC2a.

The first step in this aim would be to make mutants of ASIC2a. The point mutants V81E, and A82S would help compare these analogous residues with those mutants made in ASIC3. As in chapter 4 with ASIC1a, the same quadruple mutants could be made with ASIC2a (VVAQ→RESH) and ASIC3 (RESH→VVAQ) to exchange the short sequence. Testing these mutants may further support the idea that these four residues are critical in determining subunit specific desensitization properties.

Aim 4: Identify other residues involved in desensitization.

Previously I mentioned that the extracellular domain of ASICs has 27 conserved acidic residues, of which I only mutated 6. Mutating the rest of these would likely uncover further effects with either activation or desensitization. This very high conservation of negative charge must be serving some functional role. One problem is that many of these charges may be redundant, so phenotypes may not be observed with

point mutants that are part of a binding site. This is especially true considering the steep Hill slopes of activation and desensitization predict that there are likely a large number of binding sites. Making alanine mutants of these residues in ASIC3 would be a good way to uncover further sequences that are involved in desensitization.

Additionally, the residue E63 was never pursued. The E63A mutant desensitized more slowly than WT, and this residue is very highly conserved. The residue itself, along with adjacent residues could be pursued for its role in desensitization. E63 is just outside TM1, and since TM1 is thought to move during desensitization, this location may undergo some movement during desensitization. Making a cysteine mutant of E63 would provide a quick test as to whether or not this residue changes accessibility during desensitization, as E79 appears to do.

Chapter 7

Summary and Conclusions

Chapter 7

Although the physiological role ASICs play is debatable, their steep pH dependence makes them ideal pH sensors. Here I show properties of these channels that affect their ability to sense pH. Their activation properties are greatly influenced by the external ionic environment. I also located a region of the channel that appears to be involved in controlling the rate and sensitivity of desensitization. The interplay of desensitization and activation is crucial to the ability of these channels to act as sensors.

Although it is unknown what these channels are doing in the brain, there is a growing body of evidence suggesting they may be harmful under pathological conditions. The main reason is that ASICs are activated by low pH, which is a product of ischemia. In this work, I showed that other factors present during ischemia can enhance activation of these channels. The most important of these is calcium, which drops dramatically during ischemia. The four fold enhancement I saw of activation under these low calcium concentrations suggests that this could be a huge contributor to the neuronal cell death from ischemia. Increased levels of zinc and depolarizations induced by ischemia would also lead to increased activation of ASICs, either through enhancement of ASIC2 containing channels, or by reducing a voltage dependent block by magnesium. Zinc enhances heteromeric ASIC1a/2a channels, whereas it inhibits ASIC1a homomers. Magnesium has a voltage dependent block of both ASIC1a and ASIC1a/2a channels. This block is weakly voltage dependent ($\delta = 0.23$) and has a relatively low affinity ($K = 4$ mM at -70 mV)

Comparing ASIC1a homomers to ASIC1a/2a heteromers, I found that the heteromers are able to pass sustained currents, whereas homomers desensitize completely

within a few seconds. This suggests that the heteromers might be more detrimental during ischemia if ASIC1a homomers desensitize in vivo during ischemia.

This data further supports the idea that ASICs are a legitimate target for neuroprotection during ischemia. The evidence shows that ASICs should be significantly activated by many changes brought on by ischemia.

ASIC desensitization has also been shown to be important to the physiological function of these channels. I've shown that there are two acidic residues in ASIC3 that are important for desensitization. Mutation of either D78 or E79 to an alanine resulted in rapidly desensitizing channels. E79 also regulates the sensitivity of desensitization to pH, which is very important in determining the channel's ability to generate sustained currents. E79A mutants had greatly shifted steady state desensitization with no effects on activation.

Evidence suggests that titration of these two conserved residues aids desensitization. One interpretation is that protons bind to one or both of these residues, and neutralizes them. This neutralization of charge would ease the transition into the desensitized state. This speculation is based on the fact that neutralizing either of these charges accelerates desensitization. The neutralization of E79 also decreases the Hill slope of steady state desensitization, which could be interpreted as the loss of a proton binding site. Addition of highly charged MTS reagents to E79C mutants greatly reduced the ability of these channels to desensitize. They not only desensitized more slowly when modified, but their desensitization was incomplete.

Mutations of D78 to alternate amino acids showed the importance of charge at this position. Mutations to asparagines resulted in quickly desensitizing channels,

whereas a glutamate at this position yielded normal desensitization. A cysteine mutation of this residue was not modified by MTS reagents in a detectable manner.

These two residues are conserved in virtually every ASIC that generates a proton gated current. Consequently I also mutated them in ASIC1a. These mutants had a similar phenotype to that seen in ASIC3. They desensitized quickly, with E79A having a greater phenotype than D78A in this subunit. This showed that these conserved residues appear to have a conserved function in ASICs.

Residues adjacent to D78 and E79 were also shown to influence both speed and pH sensitivity of desensitization. An alanine scan of the region revealed that mutations in residues T75-S82 all affected the kinetics of desensitization. Many also affected the pH sensitivity of steady state desensitization.

Although there are some very highly conserved residues within this region, there are also some variable residues that I showed act to determine the desensitization kinetics of different subtypes of ASICs. Changing four residues in ASIC1a can make its desensitization look very similar to ASIC3. These residues immediately follow E79, and are likely key modulators of desensitization between different subunits. One key residue within these four is S82 in ASIC3 and A82 in ASIC1a. If there is an alanine at this position, then channels desensitized slowly. A serine yields quickly desensitizing channels. This pattern is consistent among different ASICs.

The modification of E79C mutants with MTS reagents was highly state dependent. Reaction occurred quickly when the channels were available to be opened, but was negligible when channels were desensitized. This showed that there was a conformational change during desensitization that made this residue inaccessible to

modification. Adjacent residues showed different patterns of accessibility, with D78C mutants not reacting to MTS in either state, and R80C mutants reacting equally well whether desensitized or not.

Chapters 4 and 5 not only show that a region outside TM1 is important for desensitization, but they give some mechanistic information about what happens during desensitization. Evidence is strong that E79 needs to be buried in order to allow the channels to pass into the desensitized state.

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