REGULATION OF THE ACID-SENSING ION CHANNEL 1 BY PROTEIN KINASE C AND MATRIPTASE

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Acid-sensing ion channel 1 (ASIC1) is a member of the Epithelial Na⁺ channel/Degenerin (ENaC/Deg) family of ion channels. Like the other members of this family, it is sensitive to the diuretic amiloride. It was previously shown that both normal astrocytes and glioma cells express ASIC1 and some ENaC subunits. However, only high-grade glioma cells exhibit an inward Na⁺ current that is sensitive to amiloride and to psalmotoxin 1 (PcTX-1), a specific blocker of ASIC1. Amiloride, PcTX-1, and the knockdown of ASIC1 reduce glioma cell migration. The exact composition of the amiloride-sensitive channel and the molecular mechanisms that regulate its constitutive-expression in glioma cells are not known. In patch clamp experiments, inclusion of PKC in the patch pipette inhibited the inward Na⁺ current of a cultured glioma cell. This suggests that PKC may phosphorylate a component of the glioma channel, like ASIC1. It has been shown that serine proteases can regulate ENaC/Deg subunits. Matriptase, a trypsin-like type II transmembrane serine protease is overexpressed in many cancers. We have found that matriptase expression is higher in glioma cells than in normal astrocytes. Matriptase activates the ENaC channel, and ASIC1 can also be modulated by proteases. Therefore, in two separate studies I tested the following hypotheses about the regulation of ASIC1: a) ASIC1 is inhibited by PKC, and b) matriptase can regulate ASIC1 function through proteolytic cleavage. I found that PKC activation or inhibition both inhibit ASIC1 function, and that two consensus PKC phosphorylation sites (S40 and S499) on
ASIC1 are critical sites mediating the modulation of ASIC1 by PKC. In addition, matriptase can cleave ASIC1 protein in three sites in the extracellular loop, R145, K185, and K384, inhibiting its function. The effect of matriptase on ASIC1 is specific, because matriptase does not affect the function of or cleave ASIC2. The modulation of ASIC1 by PKC and by matriptase could lead to a deeper understanding of PKC regulation and/or matriptase cleavage of this channel in glioma cells.
DEDICATION

I dedicate this dissertation to my husband David, my parents, Liljana and Kristaq, and my sister, Bona.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Acid-Sensing Ion Channel 1 (ASIC1)</td>
<td>1</td>
</tr>
<tr>
<td>ASIC1 Structure and Function</td>
<td>4</td>
</tr>
<tr>
<td>Regulation of ASIC1 by Modulators</td>
<td>17</td>
</tr>
<tr>
<td>Cellular Localization of ASIC1</td>
<td>32</td>
</tr>
<tr>
<td>Physiological and Pathophysiological Functions of ASIC1</td>
<td>35</td>
</tr>
<tr>
<td>Protein Kinase C</td>
<td>38</td>
</tr>
<tr>
<td>Matriptase</td>
<td>43</td>
</tr>
<tr>
<td>Amiloride-Sensitive Channel in Glioblastoma Multiforme</td>
<td>49</td>
</tr>
<tr>
<td>Syntaxin 1A</td>
<td>50</td>
</tr>
<tr>
<td>PKC</td>
<td>51</td>
</tr>
<tr>
<td>Composition of the Amiloride-Sensitive Channel</td>
<td>52</td>
</tr>
<tr>
<td>Purpose</td>
<td>53</td>
</tr>
<tr>
<td>TWO PKC CONSENSUS SITES ON HUMAN ACID-SENSING ION CHANNEL 1b (hASIC1b)</td>
<td>54</td>
</tr>
<tr>
<td>DIFFERENTIALLY REGULATE ITS FUNCTION</td>
<td></td>
</tr>
<tr>
<td>PROTEOLYTIC CLEAVAGE OF THE HUMAN ACID-SENSING ION CHANNEL 1</td>
<td>105</td>
</tr>
<tr>
<td>BY THE SERINE PROTEASE MATRIPTASE</td>
<td></td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>156</td>
</tr>
<tr>
<td>LIST OF REFERENCES</td>
<td>170</td>
</tr>
</tbody>
</table>
APPENDICES

A  IACUC APPROVAL....................................................................................181
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TWO PKC CONSENSUS SITES ON HUMAN ACID-SENSING ION CHANNEL 1b (hASIC1b) DIFFERENTIALLY REGULATE ITS FUNCTION</td>
<td></td>
</tr>
<tr>
<td>1 pH Values for Half-Maximal Activation and Hill Numbers for Wild Type hASIC1b and hASIC1b Phosphorylation Mutants</td>
<td>103</td>
</tr>
<tr>
<td>2 Properties of the pH4.0-Induced Currents of hASIC1b Before and After PMA</td>
<td>104</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figures</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1  Phylogenetic Tree of the Epithelial Sodium Channel/Degenerin (ENaC/Deg) Subunits</td>
<td>3</td>
</tr>
<tr>
<td>2  Characteristics of the ASIC1 Current</td>
<td>6</td>
</tr>
<tr>
<td>3  Subunit Architecture of ASIC1</td>
<td>8</td>
</tr>
<tr>
<td>4  Modulators of ASIC1 Function</td>
<td>32</td>
</tr>
<tr>
<td>5  Domain Structure of PKC Family Members</td>
<td>42</td>
</tr>
<tr>
<td>6  Domain Structures of Matriptase and HAI-1</td>
<td>44</td>
</tr>
</tbody>
</table>

TWO PKC CONSENSUS SITES ON HUMAN ACID-SENSING ION CHANNEL 1b (hASIC1b) DIFFERENTIALLY REGULATE ITS FUNCTION

<table>
<thead>
<tr>
<th>Figures</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1  Alignment of Acid Sensing Ion Channel 1 (ASIC1) Isoforms from Different Species</td>
<td>89</td>
</tr>
<tr>
<td>2  Mutations in PKC Consensus Phosphorylation Sites on hASIC1b Affect its Function in <em>Xenopus</em> Oocytes</td>
<td>91</td>
</tr>
<tr>
<td>3  hASIC1b Expression in <em>Xenopus</em> Oocytes</td>
<td>93</td>
</tr>
<tr>
<td>4  The PKC Activators Phorbol Myristate Acetate (PMA) and Phorbol Dibutyrate (PdBu) Reduce the Acid-Activated Current of WT hASIC1b and S499A hASIC1b, but Not That of S40A hASIC1b</td>
<td>94</td>
</tr>
<tr>
<td>5  PMA Inhibits the Peak Acid-Induced Current of hASIC1b in Transfected CHO-K1 Cells</td>
<td>96</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>6  The PKC Inhibitor Chelerythrine Abolishes the Effect of PMA on WT and S499A hASIC1b</td>
<td>97</td>
</tr>
<tr>
<td>7  Effect of PKC Inhibitors on the Acid-Activated Currents of WT, S40A, S499A, or S40A/S499A hASIC1b</td>
<td>99</td>
</tr>
<tr>
<td>8  PMA and Chelerythrine Have No Effect on the Total Expression or Surface Expression of hASIC1b</td>
<td>101</td>
</tr>
<tr>
<td>PROTEOLYTIC CLEAVAGE OF THE HUMAN ACID-SENSING ION CHANNEL 1</td>
<td></td>
</tr>
<tr>
<td>1  RT-PCR and Western Blot Detection of Matriptase in Glioma Cell Lines and Fresh GBM Tissues</td>
<td>139</td>
</tr>
<tr>
<td>2  Matriptase Decreases the Peak Acid-Activated Current of ASIC1, But Not That of ASIC2 in <em>Xenopus</em> Oocytes</td>
<td>140</td>
</tr>
<tr>
<td>3  Matriptase Does Not Change the pH Activation Curve or Inhibition by PcTX-1 of ASIC1</td>
<td>143</td>
</tr>
<tr>
<td>4  Detection of Matriptase-Specific Cleavage Fragments of GFP-Tagged ASIC1 But Not of Tagged ASIC2 in <em>Xenopus</em> Oocytes</td>
<td>145</td>
</tr>
<tr>
<td>5  Detection of Cleavage Fragments of HA-Tagged ASIC1 But Not of HA-Tagged ASIC2 in <em>Xenopus</em> Oocytes</td>
<td>148</td>
</tr>
<tr>
<td>6  Matriptase Cleaves ASIC1-GFP and GFP-ASIC1, But Not ASIC2-GFP in Transfected CHO Cells</td>
<td>150</td>
</tr>
<tr>
<td>7  Identification and Confirmation of the Matriptase Sites on ASIC1</td>
<td>152</td>
</tr>
</tbody>
</table>
INTRODUCTION

ACID-SENSING ION CHANNEL 1 (ASIC1)

In 1980, a H⁺-activated current was discovered in neurons from rat spinal and trigeminal nerve ganglia (1). The current appeared when the extracellular pH decreased to 6.9 or lower, and was due to an increased permeability of the membrane to Na⁺ and K⁺. This current depolarized the neuronal membrane, was sensitive to amiloride, and its amplitude saturated at pH ~5.4 (1). Seventeen years later a H⁺-gated channel was cloned from rat brain and named Acid-Sensing Ion Channel (ASIC)(2). ASIC was a 526 amino acid protein that formed a simple ligand-gated channel with a current similar to the one described in the rat sensory neurons: it activated by rapid extracellular acidification, and it was permeable to Na⁺ > Ca²⁺ > K⁺ and sensitive to amiloride (2).

Since the cloning of the first H⁺-gated channel, known as ASIC1, additional ASIC proteins (ASIC2, ASIC3, and ASIC4) have been identified in mammals (2,3), amphibians (4), fish (4-6), birds (5), and nematodes (7), and studied by expression in heterologous systems. ASIC2 was first cloned from human and rat brain by two different groups, which had given it two different names, MDEG(8) (for mammalian degenerin) and BNC1(3) (for brain Na⁺ channel 1). This ion channel is activated by the same mutations (G430 to bulky amino acids) that cause a gain of function in the C.Elegans nematode degenerins, resulting in neurodegeneration (8). It was later shown that MDEG is also a H⁺-gated channel. MDEG requires more acidic pH values for activation (9), and the gain of function mutations shift the pH dependence of activation to a much higher pH (from
pH$_{50}$ of 4.1 to 6.9) (10). ASIC3 was first named DRASIC (dorsal root ASIC), for the
dorsal root ganglia neurons from which it was cloned (11) and displays a biphasic H$^+$-gated current with a sustained component, similar to what had been observed earlier in some of the sensory neurons (12); finally, ASIC4 was cloned from rat neurons and named SPASIC (spinal cord ASIC), but it does not form a functional H$^+$-gated channel (13).

Sequence homology and amiloride sensitivity place ASICs in the Epithelial Na$^+$ Channel/Degenerin (ENaC/Deg) family. All members of the ENaC/Deg family share subunit organization and Na$^+$ permeability (14). The phylogenetic tree of the ENaC/Deg subunits shows the position of ASICs relative to some of the most relevant members of this family: the ENaCs expressed mainly in vertebrates, the mechanosensitive MEC and DEG channels expressed in nematodes, the pickpocket (PPK) and ripped pocket (RPK) proteins expressed in Drosophila, and the peptide-gated FaNaCs (FMRFamide-gated Na$^+$ channels) expressed in mollusks (Figure 1)(14). The ENaC/Deg subunits are involved in many diverse functions such as sensing proton gradients (ASICs), regulation of Na$^+$ homeostasis (ENaCs), mechanosensation (DEGs, PPKs), salt-taste perception (RPKs), and sensing peptide neurotransmitters (FaNaCs)(14).
Figure 1. Phylogenetic tree of the Epithelial Sodium Channel/Degenerin (ENaC/Deg) subunits. Sequence alignments and phylogenetic tree were done using ClustalW. This is a phylogram, showing common ancestry. The branch lengths are proportional to the amount of inferred evolutionary change (15).
ASIC1 Structure and Function

Characteristic of the ASIC1 Current

The characteristics of the ASIC1 current have been studied extensively by expression of ASIC1 in heterologous expression systems such as oocytes of the South African clawed frog *Xenopus laevis*. This is the model system that I have used for my graduate studies. First, the mRNA for ASIC1 is injected in *Xenopus* oocytes, which translate it into a functional ASIC1 protein. Then, as early as one day after RNA injection, ASIC1 currents due to the channels at the oocyte plasma membrane can be measured by two-electrode voltage clamp (16). *Xenopus* oocytes are a good expression system for studying heterologous membrane proteins, because they do not express many endogenous membrane proteins that could interfere with the protein of interest, and they are easily obtained and handled (16,17). Although oocytes do have a few endogenous ion channels and transporters, the heterologously expressed ion channels can be distinguished from the endogenous ones because most of the time the endogenous currents are small compared to the currents of the expressed channels. In addition, the expressed channels often display unique pharmacological and biophysical characteristics (16).

To measure ion channel currents with two-electrode voltage-clamp, the following two glass microelectrodes impale the oocyte: the voltage electrode, which monitors the oocyte membrane potential, and the current electrode, which injects current needed to hold the membrane voltage clamped at a certain value (16). A commonly used holding potential is -60mV (17). In this configuration, whole-cell currents from the ion channel of interest can be measured and studied. Because ASIC1 is a H⁺-gated ion channel, it is
activated by changing the pH of the extracellular solution from a normal pH value (pH 7.4) to an acidic pH (pH 4.0 for maximal activation) (Figure 2A).

In a normal saline extracellular solution, where Na\(^+\) is the major cation, ASIC1 channels elicit a fast-activating inward current that quickly inactivates, despite the continuous presence of low extracellular pH. Figure 2B shows an example of this. Non-injected oocytes do not exhibit an endogenous H\(^+\)-activated current that could interfere with the expressed ASIC protein (not shown). The extracellular pH to which the channels are exposed before the switch to an acidic pH is called the *conditioning pH*, while the acidic pH that activates the channels is called the *activation pH*. The portion of the current from the baseline to the peak is the *activation* of the channel. Channel activation is pH-dependent, with larger peak currents obtained at lower activation pHs. After the *peak amplitude* has been reached, the current quickly returns to baseline. During this phase the channels are *inactivating* or *desensitizing*, two terms used interchangeably. The rate of desensitization of ASIC1 (and of ASIC2, but not of ASIC3) is pH-dependent, increasing at higher proton concentrations (18). ASIC currents are characterized by the peak amplitude and the time course/kinetics of activation and inactivation. ASIC1 activates and inactivates with \(\tau_{\text{act}} = 5.8–13.7\) ms, and \(\tau_{\text{inact}} = 1.2–4\) s at pH 6 for rASIC1a, and \(\tau_{\text{act}} = 9.9\) ms and \(\tau_{\text{inact}} = 0.9–1.7\) s at pH 6 for rASIC1b. The pH of half-maximal activation (pH\(_{50}\)) is 6.2-6.8 for rASIC1a and 5.1-6.2 for rASIC1b (19-21).

Inactivated ASIC1 channels cannot be activated again; they must be exposed to pH7.4 for at least 10s, during which they undergo a conformational change from the desensitized state to a closed state (18). Only from this closed state, can they activate in
the presence of $H^+$. The duration of the exposure of desensitized channels to pH7.4 to allow complete recovery of the $H^+$-induced current is the \textit{time course of recovery from inactivation} (18).

Another characteristic of the ASIC1 currents is a run-down, a decreased response to successive applications of low extracellular pH (Figure 2C) called tachyphylaxis. It is a unique feature of homomeric ASIC1a channels. ASIC2 and ASIC3 do not exhibit this phenomenon (22). Increasing the time interval between successive low pH stimulations or increasing the conditioning pH to 8.0 does not affect it; however, tachyphylaxis is pH dependent. The more acidic the stimulation pH, the faster the decrease in ASIC1 currents (22). This is not due to endocytosis of the channels, a reduction in single channel conductance or channel open time. Tachyphylaxis is due to a reduced number of open channels, caused by a prolonged inactive state, which is promoted by $H^+$ permeating during the open state (22).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure2.png}
\caption{Characteristics of the ASIC1 current A) Two-electrode voltage clamp configuration, showing the oocyte impaled by the voltage electrode (V) and the current electrode (I), and the pH7.4 and pH4.0 solutions. B) Example of acid-induced whole-cell current from an oocyte expressing ASIC1, showing conditioning pH (1), activation pH (2), channel activation (3), peak amplitude (4), and channel inactivation/desensitization (5). C) Successive applications of pH (with pH7.4 in between) cause rundown of ASIC1 current.}
\end{figure}
Steady-state inactivation refers to ASIC1 inactivation that occurs at conditioning pH values that are not sufficiently acidic to activate the channels. At lower conditioning pHs, a smaller fraction of ASIC1 can be activated, resulting in a reduced peak current. Steady-state inactivation may occur rapidly; gradual changes to acidic pH may therefore fail to activate ASIC1 channels. Therefore, the change from conditioning pH to activation pH must be achieved within a few milliseconds.

Architecture of ASIC1 Subunits

The topology of each ASIC subunit is similar to the other ENaC/Deg members, and it consists of short intracellular N- and C-termini, two transmembrane domains, and a large extracellular loop(14). In mice, rats, and humans, ASIC1 subunits exist as two splice variants, ASIC1a and ASIC1b (23). The human ASIC1a and ASIC1b differ in the region prior to the second transmembrane domain. Human ASIC1a contains a 46-amino acid (aa) insertion, resulting in a larger protein of 574 aa (24). The rat and mouse ASIC1b differ in only two amino acids and both contain a unique insertion in the N-terminus compared to the respective ASIC1a isoforms (23). Therefore, the rat and mouse ASIC1b isoforms encode larger proteins (559 aa) than the ASIC1a isoforms (526 aa). There is potential for confusion due to the names of the isoforms in various species. hASIC1b is most similar to the rat and mouse ASIC1a, which have been used for most of the studies on ASIC1, while the hASIC1a is unlike any of the other human or rodent ASIC1 isoforms.

The crystal structure of chicken ASIC1 at 1.9 Å was published in 2007, confirming the predicted topology of an ENaC/Deg subunit, and showing that three
ASIC1 subunits form the ASIC1 channel (25); however, removal of the 25 N-terminal and 64 C-terminal residues was required for crystallization, and the truncated channel did not express acid-activated currents. In addition, the channel was crystallized at low pH, suggesting that the crystal structure most likely represents the desensitized ASIC1 (25).

The crystal structure showed that each ASIC1 subunit resembles an upright forearm, wrist, and clenched hand (25) (Figure 3). The transmembrane domains constitute the forearm, and are composed of two $\alpha$-helices, the ends of which connect to long $\beta$-strands in the large extracellular domain, forming the wrist. The wrist joins to a seven-strand $\beta$-sheet called the palm domain. The palm connects to the thumb domain, which is itself composed of two $\alpha$-helices. Two short $\alpha$-helices and some non-$\alpha$, non-$\beta$ structures are located above the palm domain, and form the knuckle and finger domains, respectively. All of these domains (thumb, finger, knuckle, and palm) surround a small five-strand $\beta$-ball (25).

**Figure 3. Subunit architecture of ASIC1** (25). Each subunit resembles an upright forearm, wrist and clenched hand and it consists of two transmembrane domain (TM1, TM2), short intracellular N- and C- termini, and a large extracellular loop.
**pH Sensing, Gating, and Ion Permeation Pathway**

The thumb, β-ball, and finger of one subunit and residues from the palm of an adjacent subunit form a highly negatively charged cavity about 45Å from the transmembrane domains. This acidic pocket contains the ASIC1 pH sensor. Several aspartate (Asp) and glutamate (Glu) residues located in this pocket form carboxyl-carboxylate interactions and are conserved among ASICs but not among non-H⁺-gated members of the ENaC/Deg family, such as the ENaCs (25). The crystal structure suggested that the pH sensor of ASIC1 is distributed over many residues in different subunits, and that four carboxyl-carboxylate pairs in the acidic pocket (Asp 238-Asp 350, Glu 239-Asp 346, Glu220-Asp408, and Glu 80-Glu 417 in chicken ASIC1) are crucial for H⁺ sensing (25). However, it does not appear that any individual carboxyl-carboxylate pair in the acidic pocket is crucial for proton sensing (26). Mutation of some of the carboxyl-carboxylate pair residues decreases the affinity of ASIC1 for protons, but it does not abolish proton sensing (25,26). In addition, two regions in the extracellular loop (amino acids 87-197 and 323-431) localizing to the thumb, β-ball, palm and knuckle domains have been defined as sufficient for proton sensing (27). It may be that substitutions of more than one carboxyl-carboxylate pair would render ASICs insensitive.

Apart from the four carboxyl-carboxylate pairs identified from the crystal structure, other residues participate in pH sensing. Fifteen Asp and Glu residues in the extracellular domain are involved in the pH-dependent gating of hASIC1b. The calculated pKa values of all the Asp and Glu residues in the extracellular domain were used to estimate which residues would be protonated during channel activation and inactivation. The residues that either sense pH, or influence pH sensing, have been
confirmed by mutagenesis and functional analysis. In agreement with the crystal
structure information, they are localized to the thumb, β-ball, and palm domains (27,28). The interactions between the thumb and the β-ball are likely to be critical for activation, while the palm domain might play an important role for ASIC1 inactivation (28). The pH sensor of ASIC1 is therefore not just a cluster of residues that can get protonated, but is distributed over many residues in different subunits.

Several other residues that do not participate in the acidic pocket also affect the proton affinity of ASIC1. Glu63 in the TM1, His72 and His73 in the post-TM1 wrist, and Asp78 in the palm domain affect pH sensitivity of rat ASIC1a and are conserved among ASICs (26). The wrist region post-TM1 may be important for pH sensing (4). The post-TM1 region is different in ASICs of different species, and differences in this region account for differences in the ASICs’ pH sensitivities. For example, frog ASIC1 has a higher affinity for protons than rat or human ASIC1a, which is reflected in a pH$_{50}$ of activation of 7.0 compared to 6.2-6.3 for the rat and human ASIC1a (4). The negatively charged amino acids, which have been proposed to form the pH sensor in the extracellular domain of ASIC1 are conserved between frog and human or rat ASIC1. However, a short linker region between TM1 and the wrist domain contains the important residue L85, which is responsible for the decreased proton affinity of rat and human ASIC1 (4). The post-TM1 region also confers proton-sensitivity to lamprey ASIC1. Lamprey ASIC1 does not respond to protons, but mutation of two residues in the post-TM1 region to the equivalent residues in rat ASIC1a (Q77L, and T85L) converts lamprey ASIC1 into a proton-activated channel with a pH$_{50}$ of 7.2 (29).
The gate of the ASIC1 channel is likely located in the N-terminus. The wrist β-strands connecting the extracellular domain to the transmembrane domains are important for conformational changes associated with channel gating (25). The residues located in the transition between TM1 and the extracellular domain (Leu 71-Pro 73), and TM2 and the extracellular domain (Lys 423-Glu 426) are likely to mediate gating conformational changes (25).

The large extracellular domain of ASIC1 contains several cysteine residues that are conserved in ASICs, ENaCs, DEGs, and FaNaC channels. In the ASIC1 crystal structure, these residues form seven disulfide bonds, five of which are located in the thumb, and the rest in the palm and β-ball. The disulfide bonds provide rigidity to the ASIC structure (25). The disulfide bridges of the thumb domain are particularly likely to facilitate transduction of conformational changes in the extracellular loop to the transmembrane domains (25).

It is hypothesized that upon proton binding to a proton-binding site in the extracellular domain, the thumb undergoes a conformational change that gets transmitted to the pore through non-covalent interactions of residues at the base of the thumb (25). Aromatic residues at the base of the thumb (Trp 287) and in the post-TM1 region (Tyr 71) appear to be essential for proton activation of ASIC; mutation of these residues shifts the activation pH to more acidic values (30). It has been proposed that these two residues interact during gating, possibly allowing functional coupling between the extracellular domain and the pore of the channel. Although it seems that the gate of the channel is in the N-terminus of ASIC1, it has been shown that the pre-TM2 region of mASIC1α also changes its conformation following activation by extracellular acidification (31).
One proposed mechanism for ASIC1 gating is the release of a blocking Ca\textsuperscript{2+} ion from the channel outer pore. The hypothesis is that protons and Ca\textsuperscript{2+} compete for binding to this site (32). Substitution of conserved residues Glu425 and Asp432 at the beginning of the second transmembrane domain in the outer channel pore may remove the Ca\textsuperscript{2+} block of the channel, but it does not constitutively open it or eliminate the H\textsuperscript{+} gating (33). While the above site at the outer entrance of the ion pore mediates the block by Ca\textsuperscript{2+}, complete removal of Ca\textsuperscript{2+} from the extracellular solution can open the Ca\textsuperscript{2+}-site mutants, suggesting the presence of a second Ca\textsuperscript{2+}-binding site in the extracellular loop of ASIC1 that stabilizes the closed conformation of the channels. Removing Ca\textsuperscript{2+} from both of these sites is required for channel opening (33). The Ca\textsuperscript{2+} dependence of ASIC1a is mediated by amino acids 197-323 (27).

Two cation-binding sites have been identified in the low-pH crystal structure of a functional chicken ASIC1. This channel is almost identical to the structure of the non-functional ASIC1, with the exception that in this structure, there are no contacts between the transmembrane domains of neighboring proteins (34). Two Cs\textsuperscript{+}-binding sites have been identified, one localizes to the finger domain in a solvent-filled crevice, and the other to the region beneath the thumb domain (34). The two Cs\textsuperscript{+} ions are coordinated by the carbonyl oxygens of main chain Asp and Glu residues (34), which may form or be located near the proposed Ca\textsuperscript{2+}-binding sites.

No continuous pore is visible in the crystal structure of the desensitized chicken ASIC1 protein; however, there are V-shaped fenestrations at the wrist, close to the extracellular side of the transmembrane domains. These fenestrations may provide a way for ions to get through (25,34). The ion-selective pore of the channel is likely located
between the transmembrane domains, which are hour-glass shaped, and contain aliphatic hydrophobic amino acids on the lipid exposed side, and an interior negative electrostatic potential defined primarily by residues from TM2 (25). The crystal structure of the functional cASIC1a clearly shows that the TM1 helix makes contacts with the lipid bilayer, while TM2 lines the ion channel pore (34). The structure supports other studies that suggest that the pore of the channel is lined by the region pre-TM1, TM2, and a few amino acids in TM1 (19,35-38).

ASICs desensitize upon prolonged exposure to acid, and the desensitization rate of ASIC1 increases when the stimulation pH is more acidic (18). The desensitized channel is bound to protons, but the gate of the channel is closed. The desensitization gate in the ASIC1 crystal structure is a physical block of the transmembrane pore by conserved residue Gly436 on the cytoplasmic side and the carbonyl groups of Asp433 on the extracellular side (34). In particular, the region connecting TM1 to β1 of the palm domain, specifically Cys70, changes its conformation during desensitization (31). Three additional residues in the palm domain (Ser83, Gln84, and Leu85) are responsible for differences in the desensitization of rat ASIC1 and fish ASIC1 (39).

A chloride binding site has been identified in the extracellular loop of ASIC1. The residues coordinating this ion (Lys211, Arg309, and Glu313) are located in the two α-helices that define the thumb domain, and are conserved throughout all ASICs (25). It has been shown that chloride does not affect the pH sensitivity of activation or Na⁺ selectivity of ASIC1, and mutation of the amino acids involved in Cl⁻ binding in the chicken ASIC1a crystal structure does not affect the pH activation dose response (40). Instead, the chloride ion modulates the kinetics of desensitization and tachyphylaxis of
ASIC1a in a concentration-dependent manner. Mutation of each of the amino acids forming the Cl⁻-binding site increases the rate of desensitization and slows the rate of tachyphylaxis (40).

**Subunit-subunit Interactions**

The crystal structures of both the non-functional and functional chicken ASIC1 at low pH show that an ASIC1 channel is a trimer with subunit-subunit interactions localized to three regions (25,34). Atomic Force Microscopy (AFM) imaging reveals that the neutral-pH form of the full-length human ASIC1a is a trimer (41). In the crystal structures, the first region of subunit-subunit interactions is the wrist junction right after TM1 of one subunit with the wrist junction near TM2 of an adjacent subunit. The second region involves interactions between the palm domain of one subunit and the thumb of an adjacent subunit. The third area of interactions is between the knuckle of one subunit and the finger domain of a neighboring subunit (25). There are interactions between the TM1 and TM2 of the same subunit, but also between the TM domains of different subunits within the trimer (25,34).

**Heteromeric ASICs**

ASIC1 subunits can combine with other ASIC or ENaC subunits to form heteromeric channels, which can be identified because of their distinct biophysical characteristics. ASIC1-containing heteromers may display unique proton sensitivities, desensitization kinetics, steady-state desensitization, or ionic permeabilities. For example, ASIC1a/ASIC1b heteromers desensitize more slowly than either homomeric
channel. Although ASIC2 is less sensitive to protons than ASIC1 (ASIC2 pH\textsubscript{50} of activation is 4.5 compared to 6.2 for ASIC1), ASIC1/ASIC2 heteromers show proton sensitivities similar to ASIC1 (18). Unlike the individual homomers, which display pH-dependent desensitization kinetics, the desensitization of ASIC1/ASIC2 is pH-independent, and slower than that of ASIC1 but faster than that of ASIC2 (18).

ASIC1/ASIC3 heteromeric channels display some characteristics of ASIC3 homomers, namely, pH-independent desensitization and high proton sensitivity (18). However, the steady-state desensitization of ASIC1/3 heteromers is shifted to lower pH values and the desensitization rate is faster (18,42). Finally, unlike ASIC1 homomers, ASIC1-containing heteromeric channels (ASIC1/3 and ASIC1/2) do not display tachyphylaxis (22).

ASIC1 heteromers display unique modulation by peptides, cations and proteases. For example, ASIC1/ASIC2 and ASIC1/ASIC3 are not inhibited by the homomeric ASIC1-specific blocker psalmotoxin (PcTX-1) peptide (43). Moreover, ASIC1 homomers and heteromeric ASIC1-containing channels are inhibited by Zn\textsuperscript{2+} (44), but ASIC2 and ASIC3 are not. In fact ASIC2 is activated by high [Zn\textsuperscript{2+}] (45). Heteromeric ASIC1\textsubscript{a} /ASIC2\textsubscript{a} channels are sensitive to trypsin, chymotrypsin, and proteinase K, while heteromeric ASIC1\textsubscript{a}/ASIC3 channels are not sensitive to trypsin, but they are sensitive to chymotrypsin and proteinase K (46).

Although it is clear that combinations of two different ASIC subunits result in functional heteromeric channels, there is some debate about whether the combination of three different subunits (e.g., ASIC1+ASIC2+ASIC3) can form functional channels. Although Benson \textit{et al.} have shown that expression of ASIC1\textsubscript{a}+2\textsubscript{a}+3 is the most likely
combination present in dorsal root ganglion (DRG) neurons (20), expression of three subunits in Chinese Hamster Ovary (CHO) cells generates a mixed population of heteromeric channels probably each composed of two, instead of three, different subunits (18). All ASIC subunits seem to be able to form heteromers as detected by analysis of the closeness of their physical interaction (47).

Heteromeric ASIC channels exist in many different types of cells throughout the nervous system. Different types of acid-evoked currents due to functionally active heteromultimers of ASICs have been recorded from sensory neurons, which express ASIC1, ASIC2, and ASIC3 (20,48). In the brain, heteromeric ASIC1/ASIC2 channels have been identified in some medium spiny neurons of the mouse striatum (49). In cultured rat hippocampal neurons, ASIC currents are generated by a mixture of homomeric ASIC1a channels and heteromeric ASIC1a+2a channels (50), because half of the peak ASIC current is inhibited by PcTX-1, which is specific for homomeric rat ASIC1a channels, while the remaining PcTX-1-resistant ASIC current is increased by 300 μM Zn^{2+} (50), which is a co-activator of ASIC2a-containing channels (45). In these cells, ASIC1a establishes the current amplitude, while ASIC2a controls desensitization, recovery from desensitization, pH sensitivity, and response to modulatory agents (51).

Some data suggest combinations of ASIC1 and other ENaC/Deg subunits such as α, β, γ or δ ENaC form heteromers when expressed in heterologous expression systems. ASIC1 physically interacts with each of the ENaC subunits, and the ASIC1/ENaC channels display some unique characteristics such as decreased I_{K_{a}}/I_{Na^{+}} permeability of the acid-induced current (in the case of ASIC1+ α, β, or δ ENaC), and increased sensitivity of the current to PcTX-1-containing venom (for ASIC1+ α, β, γ or δ ENaC)
Endogenous amiloride-sensitive currents mediated by an ASIC and ENaC heteromeric channel have been recorded in glioma cells (53-56). These currents, similar to ENaC, are constitutively active, but unlike ASIC, are not activated by acid (unpublished observations). Furthermore, the amiloride-sensitive current in glioma is blocked by PcTX-1 (55), which has no effect on the prototypical αβγENaC channels (43), and unlike either ASIC or ENaC, this channel has higher permeability to K⁺ than Na⁺ (55). All of these findings suggest the presence of an endogenous heteromeric ASIC/ENaC channel with unique properties. The exact subunit composition and stoichiometry of endogenous heteromeric acid-sensitive or ASIC-containing channels in neurons or glioma have yet to be determined.

Regulation of ASIC1 by Modulators

Although protons are the only known activator of ASICs, ASIC channel properties such as peak current amplitude, sensitivity to extracellular pH, and activation/desensitization kinetics, can be modulated by several factors.

Cations

Zn²⁺, an endogenous trace element released during neuronal activity (57,58) modulates ASIC currents from homomeric ASIC1a channels or heteromeric ASIC1a-containing channels (44). Nanomolar concentrations of Zn²⁺ dose-dependently inhibit ASIC currents in cultured mouse cortical neurons, as well as acid-induced membrane depolarization and increase in intracellular Ca²⁺. Zn²⁺ does not inhibit ASIC currents in neurons from ASIC1a knockout mice. Zn²⁺ inhibition of ASIC1a requires Lys133 in the
extracellular loop (44). At high micromolar concentrations, Zn$$^{2+}$$ increases the ASIC2a and ASIC1a/ASIC2a current amplitude (45) and slows the inactivation of ASIC-like currents in hippocampal neurons (59).

$$\text{Ca}^{2+}$$ affects ASIC currents if it is present in the low-pH solution used for channel activation. Pretreatment with $$\text{Ca}^{2+}$$ in the conditioning pH followed by the continuous presence of extracellular $$\text{Ca}^{2+}$$ in the activation pH (60) and $$\text{Ca}^{2+}$$ co-application with acidic solution without pretreatment reduce ASIC1 currents (33); the absence of $$\text{Ca}^{2+}$$ from the acidic solution enhances ASIC1 currents (32,61). $$\text{Ca}^{2+}$$ shifts the steady-state inactivation of ASIC1 to more acidic values (62).

$$\text{Cu}^{2+}$$ is another endogenous modulator of ASIC channels in the central nervous system (CNS). $$\text{Cu}^{2+}$$ dose-dependently inhibits the amplitude of ASIC currents in cultured hippocampal and cortical neurons, and slows the desensitization (63). $$\text{Pb}^{2+}$$, a toxic metal ion, inhibits ASIC currents, decreases the acid-mediated increase in intracellular $$\text{Ca}^{2+}$$, and decreases the acid-induced membrane depolarization in CNS neurons (64). $$\text{Pb}^{2+}$$ also inhibits ASIC currents in DRG neurons (65). Extracellular $$\text{Ni}^{2+}$$ (1mM) is another inhibitor of homomeric ASIC1a and heteromeric ASIC1a/ASIC2a channels in transfected CHO cells, and of ASIC currents in hippocampal CA1 neurons. $$\text{Ni}^{2+}$$ exerts fast, reversible effects on the pH sensitivity of the channel, but does not impact activation or desensitization kinetics (66).

**Redox Reagents**

ASIC currents are susceptible to regulation by redox reagents. The reducing agent dithiothreitol (DTT) reversibly potentiates proton-activated currents, while the oxidizing
reagent 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) inhibits ASIC currents in rat sensory ganglia and hippocampal neurons (67). The pH50 of ASIC activation does not appear to be affected by DTT (67). However, DTT has been shown to shift the pH50 of activation of ASIC currents in mouse cortical neurons to a more alkaline pH (68). Redox reagents specifically modulate the ASIC1a subunit. Oxidizing or reducing agents have no effect on the acid-activated current in neurons from ASIC1 knock-out mice, and they only affect the current mediated by homomeric ASIC1a, but not homomeric ASIC1b, ASIC2a, or ASIC3 in transfected CHO cells (68). Two residues in the extracellular domain of ASIC1a mediate the effect of redox reagents. Cys61 is important for modulating ASIC1 by oxidizing reagents, while Lys133 is critical for the effect of reducing reagents (68). DTT and glutathione increased H⁺-gated currents from CHO cells expressing human ASIC1a (hASIC1a) and from mouse hippocampal neurons (69). Similar to its effect on ASIC currents in mouse cortical neurons, DTT shifts the pH-dose response of hASIC1a toward a more neutral pH (from pH50 of 6.54 to 6.69). DTT slows channel desensitization as well (69).

Nitric oxide release is increased with inflammation; this is due to higher inducible nitric oxide synthase (iNOS) activity, resulting in increased NO synthesis (70). NO has a direct effect on ASICs, probably by oxidizing one or more of the cysteine residues in the extracellular loop. The oxidizing agents NO and H₂O₂ potentiate acid-induced currents in rat DRG sensory neurons. NO increases the amplitude of rat ASIC1a expressed in CHO cells, and it affects channel kinetics, decreasing both activation and inactivation time constants (70).
Peptides

**RF-amide-related peptides.** Arginine(R)-phenylalanine(F)-amide-related peptides such as FMRF-amide (Phe-Met-Arg-Phe-NH$_2$) are important neuropeptides in insects, nematodes, mollusks, and annelida, where they affect several significant physiological parameters such as feeding behavior, gut motility, reproduction, blood pressure, and heart rate (71). In mammals, the expression of RF-amide-related peptides in the spinal cord is increased in the setting of chronic inflammation (72). The mollusk amiloride-sensitive channel FaNaC is an FMRF-amide gated channel (73).

RF-amide related peptides cannot activate ASICs; however, they can increase the amplitude and slow the rate of desensitization of ASIC currents in sensory neurons and of ASIC1 and ASIC3 in heterologous expression systems, but with lower potency for ASIC1 than ASIC3 (73). The mammalian RF-related peptides RFRP-1 and RFRP-2, increase the amplitude and slow the desensitization of H$^+$-gated currents in DRG neurons (74). Exogenous and endogenous RFRPs decrease the pH sensitivity of the steady-state desensitization of ASIC1a and enhance mouse ASIC1a activity during conditions that normally would induce steady-state desensitization (75). FMRF-amide slows the desensitization of rat ASIC1/ASIC3 heteromers in *Xenopus* oocytes - an effect that is increased at low extracellular Ca$^{2+}$ concentrations (42).

The amide group of the peptide is critical for the effect on ASIC currents. In DRG neurons this is a direct effect on the channel and not dependent on G proteins (76). RFRPs exert their actions on ASICs when they are used at µM concentrations (77). The effect of FMRF-amides on ASICs is strongly pH-dependent with the half-maximal effect at pH 5.6 to 6.0, and it requires addition of the peptide when the channel is closed (at pH
When applied at the low pH, these peptides do not have an effect (77). The pH-dependence of the RF-peptide’s effect depends on the Arg residue of the peptide (76).

**Dynorphins.** Dynorphin opioid peptides are basic neuropeptides that are very abundant in the CNS. Both dynorphin A and Big dynorphin enhance ASIC1 activity by preventing steady-state desensitization of ASIC1a at pH 7.0 in a dose-dependent manner (78). Unlike the RFRPs, dynorphins have an effect on the ASIC1a steady-state desensitization at nanomolar concentrations. Dynorphins have similar effects on acid-activated currents in cortical neurons (78). The dynorphin effect is specific for homomeric ASIC1 channels. Dynorphins show very little effect on heteromeric ASIC1/ASIC2 or ASIC1/ASIC3 channels (78).

**Psalmotoxin-1(PcTX-1).** PcTX-1 was isolated from the venom of the South American tarantula *Psalmopoeus cambridgei* (43) shortly after the cloning of the first acid-activated channel, ASIC1 (2). PcTX-1 is a 40-amino acid peptide with six cysteines linked by three disulfide bridges. Synthetic PcTX-1 peptide specifically and reversibly inhibits rat ASIC1a with an IC$_{50}$ of 0.9 nM and fails to inhibit rat ASIC1b, 2a and 3 subunits. It also does not inhibit αβγENaC or voltage-gated K$^+$ channels (43). H$^+$-gated currents in rat cerebellar granule cells and in a subpopulation of DRG neurons are inhibited by PcTX-1 as well (43). PcTX-1 inhibits rat ASIC1a by increasing its apparent affinity for H$^+$. This causes steady-state desensitization to occur at resting pH values of 7.4 (79). In the presence of PcTX-1, the pH$_{50}$ of steady-state desensitization of ASIC1a shifts from pH 7.19 to pH 7.46, rendering almost all of the available channels unavailable.
for activation. *P. cambridgei* venom does not inhibit ASIC1a currents when it is applied at a conditioning pH of 7.9 and the shift in the steady-state desensitization pH is smaller at higher extracellular Ca\(^{2+}\) concentrations, because Ca\(^{2+}\) and PcTX-1 compete for binding to the channel (79). Because PcTX-1 increases the H\(^+\) affinity, it activates ASIC1 currents under certain conditions, such as activation from an alkaline conditioning pH of 8.0 or when applied with a low pH solution in the absence of Ca\(^{2+}\) (79). PcTX-1 also promotes opening of rat ASIC1b channels, because it binds to the open state of the ASIC1b channels (80). The time constant for inhibition of ASIC1a currents by PcTX-1 is 52 s, while complete recovery from PcTX-1 inhibition is achieved 350 s after PcTX-1 washout, suggesting a slow unbinding of the toxin from the channel (79). Maximal inhibition by PcTX-1 of rat ASIC1a currents is reached after a 3 min application of 1nM PcTX-1 either before or between the pH drops (81).

PcTX-1 does not compete with other inhibitors (e.g., amiloride, flurbiprofen, ibuprofen, or diclofenac) or potentiators (FMRF-amide) of ASIC1 suggesting that it binds ASIC1a at a unique site. Several different domains in the extracellular loop of ASIC1 are important for PcTX-1 binding and its ability to affect ASIC1 gating (81). Since the elucidation of the crystal structure of chicken ASIC1, homology modeling of the human ASIC1b and molecular dynamics simulations have shown that the binding site of PcTX-1 on the ASIC1 trimer is formed by residues in two different subunits (82).

**Non-steroidal anti-inflammatory Drugs (NSAIDS)**

NSAIDS are common anti-analgesic, anti-inflammatory, and anti-pyretic drugs that inhibit prostaglandin synthesis from arachidonic acid through inhibition of
cyclooxygenases (COXs) (83). NSAIDS abolish the increase in mRNA levels of ASIC subunits in sensory neurons that is caused by inflammation (84). The NSAIDs ibuprofen and flurbiprofen can also directly inhibit ASIC1a-containing channels at high concentrations (350 uM) (84). This dose is in the same range as the concentration required for the analgesic effect of these drugs. The inhibition is fast and reversible and independent of the action on COXs (84). Diclofenac and ibuprofen decrease the amplitude of peak H⁺-induced currents in hippocampal neurons and ASICs expressed in CHO cells and slow the rates of desensitization. Diflunisal is able to inhibit ASICs only when applied simultaneously with the acidic solution. It shifts the steady-state inactivation curve to a more alkaline pH and slows the recovery from desensitization (85).

**Inflammatory Mediators**

Arachidonic acid (AA) is a membrane phospholipid metabolite produced from breakdown of phospholipids by phospholipases. Increased AA production occurs in situations associated with an increase in intracellular [Ca²⁺], such as that occurring with brain ischemia. AA enhances ASIC currents in rat cerebellar Purkinje neurons, without affecting the pH dependence of activation. The mechanism of action of AA on ASIC is not known, but AA might induce a membrane stretch that activates ASICs, since the effect of AA is mimicked by cell swelling in a hypotonic solution (86). However, based on studies of its effect on H⁺-gated currents in sensory neurons from rat DRG and in ASIC1a and ASIC3 transfected cells, membrane stretch caused by cell swelling does not potentiate ASIC currents. Arachidonic acid potentiates ASIC currents even in cell-free
membrane patches and in the presence of inhibitors of AA metabolism, suggesting a direct effect on the channel (87).

Lactate has a similar effect to arachidonic acid on ASIC currents in cerebellar Purkinje neurons (86). Lactate concentrations increase in the extracellular space following ischemia due to the anaerobic metabolism of ischemic cells (88). Lactate also potentiates ASIC1a currents in transfected cells, probably because of chelation of divalent cations, since increasing the concentrations of Ca\(^{2+}\) and Mg\(^{2+}\) abolishes the potentiating effect of lactate (89).

**Intracellular pH**

While a decrease in extracellular pH activates ASICs, changes in intracellular proton concentrations can modulate many aspects of ASIC currents: peak amplitude, pH sensitivity, inactivation, and recovery from desensitization (61). The intracellular alkalyzing agent quinine, which increases the intracellular pH by ~0.5 units, increases the peak amplitude and slows the desensitization of ASIC currents in cultured mouse cortical neurons (61). Intracellular acidification by ~0.5 units either by addition and withdrawal of NH\(_4\)Cl or perfusion of propionate has the opposite effect to alkalization: it decreases the peak amplitude of ASIC currents (61). Intracellular alkalization increases the affinity of ASICs to H\(^+\), because if shifts the activation pH curve to more alkaline pHs. It also shifts the steady-state inactivation curve to more acidic pHs and increases the recovery rate from desensitization (61). Intracellular alkalizing agents potentiate the ASIC-mediated intracellular Ca\(^{2+}\) increase, and the ASIC-induced membrane depolarization of mouse cortical neurons (61).
**Other ASIC1 Modulators**

ASICs are molecular targets of aminoglycosides. The aminoglycosides streptomycin and neomycin reduce the amplitude and slow the desensitization rate of ASIC1 in rat DRG neurons. This effect is concentration-dependent and reversible (90). Micromolar concentrations of diarylamidine anti-protozoals inhibit ASIC currents in hippocampal neurons and accelerate current desensitization. Diarylamidines do not block the other ENaC/Deg family members, ENaCs in oocytes. Diarylamidines block currents of heterologously expressed ASICs in CHO cells with decreasing affinity for ASIC1b, ASIC3, ASIC2a and ASIC1a (91). Puerarin, the main isoflavone in the root of a wild leguminous creeper, decreases the amplitude of ASIC currents in hippocampal neurons and accelerates desensitization rate. It also decreases the amplitude of ASIC1 in CHO cells (92). Finally, increasing temperature has no effect on the peak current amplitude, but it increases the rate of desensitization of ASIC1-like currents in DRG neurons (93).

**Interacting Proteins**

Interacting proteins control the surface expression, the subcellular distribution and/or the function of their target proteins (94). The following proteins interact with ASIC1: PICK-1, annexin-II light chain p11, stomatin, alpha-actinin, and AKAP-150:

The protein interacting with C kinase, PICK-1, interacts with the C-termini of ASIC1 and ASIC2 but not ASIC3 via its PDZ domain (95). PICK-1 and ASIC1 are both expressed in DRG neurons (96). PICK-1 may link ASIC channels to the cytoskeleton, and it may influence the synaptic localization of ASIC channels in hippocampal neurons (94). In fact, ASIC1 and PICK-1 co-localize at synapses in transfected mouse...
hippocampal neurons (95). When PICK-1 and ASIC2 are co-expressed in mammalian cells, the distribution of both proteins changes to a more reticular pattern compared to the diffuse distribution, when each protein is expressed alone (95).

Annexin II light chain p11, a member of the small phospholipid and Ca\(^{2+}\)-binding proteins, binds specifically to the N-terminus of rat ASIC1a, but not to ASIC2 or ASIC3 (97). p11 and ASIC1 interact in a yeast-two hybrid assay, and co-immunoprecipitate from rat DRG neurons. Co-expression of p11 and ASIC1a in CHO cells increases the expression of ASIC1a at the membrane, which is also reflected in increased current amplitude. p11 has no effect on the activation or desensitization of ASIC1a (97).

Stomatin is an integral membrane protein in lipid/protein-rich microdomains and the mammalian homolog of MEC-2. MEC-2 in *C.Elegans* interacts with Deg/ENaC subunits to form a mechanosensory complex (98). Stomatin can interact with ASIC1; however, the functional consequences of this interaction have not been reported. Stomatin interacts with ASIC2 and ASIC3 and modulates their gating; it decreases the current amplitude of ASIC3 without affecting its cell-surface levels, and it increases the desensitization rate of ASIC2 (99). Stomatin inhibits and accelerates the desensitization of ASIC1a+3 currents. In addition, deletion of the SLP3 (stomatin-like protein 3, a stomatin analog) gene in mice changes the physiological activity of ASIC channels in cultured DRG neurons (100).

Alpha-actinin -1 and -4 are actin-binding proteins that cluster membrane proteins and signaling molecules into macromolecular complexes. They also link membrane proteins to the actin cytoskeleton. Alpha actinins -1 and -4 associate with mouse ASIC1a in brain and in cultured cells. The C-termini of ASIC1a and ASIC1b contain a seven
amino acid stretch that is critical for this interaction (101). Alpha actinin-4 does not affect cell surface expression of ASIC1a, but it decreases current density, increases pH sensitivity, and accelerates the recovery from desensitization. In rat hippocampal neurons, ASIC current density (most likely from ASIC1a, ASIC1b, and ASIC2a subunits) is increased, and recovery from desensitization is decreased upon RNAi to alpha-actinin, while the time constant of desensitization is not affected (101).

A kinase anchoring protein 150 (AKAP-150) is an interacting protein that binds to the regulatory subunit of protein kinase A (PKA), and has a role in keeping PKA in an active state. Calcineurin is a protein phosphatase that associates with AKAP and PKA. Both the N- and C-termini of ASIC2a can bind AKAP-150 and calcineurin. The C-terminus of ASIC1a shows better binding than the N-terminus to both AKAP-150 and calcineurin in in vitro protein-protein binding assays. In transfected cortical neurons, the anchoring protein interacts with the C-termini of ASIC2a and ASIC1a and mediates a PKA-dependent phosphorylation of the ASIC channels. Moreover, calcineurin-dependent dephosphorylation inhibits ASIC activity, because inhibition of calcineurin potentiates ASIC1 and ASIC2 currents in transfected CHO cells and ASIC-like currents in cultured mouse cortical neurons.

**Protein Kinases**

Phosphorylation by protein kinases may be another mechanism to modulate ASIC channel properties or cellular localization. Protein kinase C phosphorylates the human ASIC1b subunit in an in vitro phosphorylation assay (102). PKC holoenzyme or a combination of PKCβI and PKCβII inhibit human ASIC1b in planar lipid bilayers. The
PKC effect is most likely a direct effect on the channel. PKC has no effect on channel conductance, but it decreases channel open probability (102). PKC has an opposite effect on ASIC2. It can phosphorylate ASIC2 at T39 in the intracellular N-terminus, increasing ASIC2 current density, without affecting the unitary channel conductance (103). This effect is potentiated by the interaction of the ASIC2 C-terminus with PICK-1(103).

ASIC1 is a specific substrate for PKA phosphorylation. In an in vitro phosphorylation assay, PKA phosphorylates ASIC1 at S479 in the intracellular C-terminus, but it does not phosphorylate ASIC2 or ASIC3. PKA also phosphorylates ASIC1 in heterologous cells and endogenous ASIC1 in brain slices. Phosphorylation of ASIC1 at S479 by PKA reduces PICK-1 binding to ASIC1 and decreases clustering of ASIC1 in hippocampal neurons (104).

Transient global ischemia increases Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CAMKII)-dependent serine phosphorylation of ASIC1 in rat hippocampal neurons, but it has no effect on ASIC2 (105). CAMKII interaction with ASIC1 increases after global ischemia, while no interaction of CAMKII with ASIC2 is observed. N-methyl-D-aspartate receptor (NMDAR) activation and subsequent increase in intracellular Ca\(^{2+}\) are required for the increased phosphorylation of ASIC1 following an ischemic insult. NMDAR activation enhances ASIC currents in hippocampal CA1 neurons and ASIC1 currents in human embryonic kidney 293 (HEK293) cells (105). Ischemia also enhances ASIC currents in hippocampal neurons, an effect that is prevented by CAMKII or NMDAR inhibitors and by mutation of two CAMKII sites on ASIC1, Ser478 and Ser479 (105).
A unique splice-variant of serum/glucocorticoid regulated kinase 1 (SGK1), SGK1.1, which is specifically expressed in the brain, has a functional effect on rat ASIC1a in *Xenopus* oocytes. Unlike the more commonly distributed SGK, which increases ENaC activity, SGK1.1 does not affect ENaC currents. The cellular localization of SGK1.1 and the kinase activity are important for the effect on ASIC1a. SGK1.1 decreases ASIC current in oocytes or in neuronal cell lines that endogenously express ASIC1. ASIC1 is not a substrate for phosphorylation by SGK1.1, and SGK1.1 does not change ASIC1 activation and desensitization kinetics. Instead it decreases the peak amplitude of ASIC1 currents by decreasing ASIC1 surface expression (106).

**Proteases**

Although there is no evidence of protease modulation of ASIC currents *in vivo*, serine proteases can specifically modulate ASIC1 or ASIC1-containing heteromeric ASIC channels in heterologous expression systems (46). The serine protease trypsin inhibits whole-cell human ASIC1b currents in CHO cells in a time and dose-dependent manner (46). When trypsin (200 μg/ml) is added to the conditioning pH solution and incubated for 5 min, it almost completely abolishes ASIC1 currents (46), and cleaves the ASIC1 protein at R145 in the N-terminal part of the extracellular loop (46,107). This effect requires the proteolytic action of trypsin, and it is not reversible. The effect of trypsin on human ASIC1b is specific, because trypsin does not decrease currents from rat ASIC1b, human ASIC2a and rat ASIC3 (46). Despite the lack of effect of trypsin on the amplitude of rat ASIC1b channels, the pH dependence of steady-state inactivation of rat ASIC1b is shifted to a more alkaline pH after trypsin exposure (46); however, trypsin
does not cleave rat ASIC1b (107). Chymotrypsin and proteinase K also affect the human ASIC1b current, and they have no effect on the current of ASIC2a or ASIC3. Similar to trypsin, chymotrypsin and proteinase K affect the steady-state inactivation of rat ASIC1b (46). The effect of proteases on ASICs seems to be a unique effect of serine proteases, because papain, a cysteine protease, and different types of collagenases do not affect ASIC1 function (46).

In addition to decreasing the peak current amplitude, trypsin cleavage changes several properties of the human ASIC1b current. The ASIC1-specific blocker PcTX-1 fails to inhibit the current of human ASIC1b channels exposed to trypsin (46). Trypsin exposure also shifts the pH activation curve and the pH dependence of steady-state inactivation of human ASIC1b to a more acidic pH (46,107). Low concentrations of trypsin decrease the Ca$^{2+}$ permeability of native ASIC1a channels in HEK293 cells but do not change the open probability of Na$^+$ inward currents (108). There may be some expression system differences in the effect of trypsin on ASIC1 or differences between the human ASIC1b and mouse ASIC1a because treatment with 200µg/ml trypsin decreases the peak pH6.0 current of mouse ASIC1a in oocytes by only 20%, but it shifts the activation pH to more acidic values as was shown in experiments with the hASIC1b in CHO cells (107). When oocytes expressing mouse ASIC1a are incubated in pH6.8 in the presence of trypsin, there is very little cleavage product apparent in the plasma membrane fraction, suggesting that trypsin cannot cleave ASIC1 in its desensitized/inactive conformation (107).

Trypsin does not change the desensitization/inactivation kinetics of human ASIC1b, but it decreases the time of recovery from desensitization from about 10 s for
untreated ASIC1 channels, to 2-3 s for the cleaved channels, suggesting that protease exposure increases the number of ASIC1 channels available for activation at a lower pH and renders human ASIC1b better able to respond to quick changes in pH (46). In agreement with the effects of trypsin on heterologously expressed ASIC1, the pH dependence of activation of ASIC currents from cultured hippocampal neurons shifts to a more acidic pH upon exposure of the cells to trypsin. Trypsin-exposed neurons exhibit a larger acid-induced depolarization than untreated neurons when the extracellular pH is decreased from a basal pH of 7.0 (46).
Figure 4. Modulators of ASIC1 function. Relative positions of the different extracellular domains and the transmembrane domains of ASIC1 are shown. The information was obtained from Jasti et al (25). Schematic shows most of the known modulators of ASIC1 function (cations, peptides, other inhibitors, proteases, interacting proteins, and kinases). The arrows pointing directly at the structure show the position of the residues important for the modulator’s action. The sites of kinase phosphorylation are indicated by x. Red- inhibitory effect on ASIC1; green, stimulatory effect on ASIC1.

Cellular Localization of ASIC1

ASIC1 is widely expressed in neurons of the central and peripheral nervous systems. It is the most abundantly expressed ASIC channel in the CNS (109). In mouse embryos, ASIC1 is already expressed in the CNS at embryonic day 12, and its levels do not change into adulthood (110). In the mouse brain ASIC1 expression is detected in the anterior and posterior cingulate cortex, sensory and motor cortex, and in subcortical structures (striatum), while the highest ASIC1 expression is detected in the hippocampus,
especially in the hilus (polymorphic layer) of the dentate gyrus, where inhibitory and
excitatory interneurons, mossy fibers, and CA3 dendrites are located (111). Rat ASIC1a
has been detected in fast-spiking basket cells of the dentate gyrus, a soma-targeting
interneuron, and in oriens lacunosum-moleculare (O-LM) cells in the CA1 region, a
dendrite-targeting interneuron (112).

Strong ASIC1 expression is detected in mouse nucleus accumbens, olfactory bulb,
amygdala, habenula, basolateral amygdaloid nuclei, and granule and Purkinje cells in
cerebellum (111)(2). Although from these studies it seems that ASIC1 expression is
highest in certain brain regions, in one study of ASIC1 localization in the rat CNS,
ASIC1 is detected in all areas of CNS examined: frontal cortex, posterior cortex,
cerebellum, striatum, thalamus, midbrain, entorhinal cortex, pons, medulla, spinal cord,
hippocampus, and cortex (110). Both isoforms of ASIC1 have been detected in the
following regions of the human brain: amygdala, hippocampus, caudate nucleus, corpus
callosum, substantia nigra, thalamus, and subthalamic nucleus (24).

In the spinal cord, mouse ASIC1a is broadly expressed in all the laminae of the
adult spinal cord and contributes to all the ASIC currents recorded from cultured spinal
cord neurons (113). ASIC1a is expressed in dorsal horn neurons of the rat spinal cord,
where homomeric ASIC1a and heteromeric ASIC1a/ASIC2a account for the majority of
the proton-induced currents and mediate acidification-induced action potentials (114).

Within neurons, ASIC1 expression is higher in cell bodies and dendrites than in
axons. Indeed, stronger ASIC1 staining has been detected in gray matter and weaker
staining in white matter. In the cerebellum, there is staining in Purkinje cell dendrites but
not in axons (111). Furthermore, in mouse brain sections, ASIC1 transcripts are detected
mostly in areas with neuronal cell bodies, with strongest expression in the Purkinje and
granule cell layers of the cerebellum, the dentate gyrus, regions CA1-CA4 of the
hippocampus, and the olfactory bulb (24). Perhaps due to the use of different techniques
or antibodies, ASIC1 expression has been detected in axons and dendrites of most
pyramidal cells in the cortex, in all regions in hippocampus, throughout the Purkinje cells
in cerebellar cortex, and in cultured mouse cortical neurons and rat hippocampal neurons
(110).

In the periphery, ASIC1 is expressed in a subset of small and large diameter
sensory neurons from the rat DRG (21)(115). ASIC1 is expressed in DRG neurons that
innervate the skin and colon (21), in cutaneus Pacinian corpuscles (116), in colonic
neurons within thoracolumbar DRG (117), in vagal and spinal sensory ganglia
(118)(119), in lumbar DRG (120), in rat bronchopulmonary C-fiber sensory neurons
(121), and in carotid body glomus cells (122). In the rat retina, ASIC1 has been detected
in the outer and inner nuclear layers, in some ganglion/and/or displaced amacrine cells,
and inner segments of cone photoreceptors (123).

ASIC1 has been detected in non-neuronal cells within the brain such as normal
human astrocytes and glioma cells (54,56,124), rat astrocytes (125), cultured rat
oligodendrocyte lineage cells (OLC), and white matter oligodendroglia (126). Outside
the nervous system, ASIC1 is expressed in bladder epithelium (127), bladder smooth
muscle (127), human intestine (115), and cultured vascular smooth muscle cells (128).
ASIC1 is the most abundant ASIC in chondrocytes, but it is also expressed in osteoblasts
and osteoclasts (129).
Physiological and Pathophysiological Functions of ASIC1

As proton sensors, ASICs function in conditions that are associated with a decrease in extracellular pH. High extracellular proton concentrations occur in various physiological and pathophysiological states. These include synaptic transmission, inflammation, ischemia, infection, and malignant tumors (94). In inflammation, extracellular pH values can decrease to 5.4. In ischemic heart or muscle, the extracellular pH can decrease to pH 5.7. Tumor cells exhibit a lower extracellular pH (6.8) than normal cells, and in bone cancer, DRG neurons that innervate the region where the tumor is present show increased expression of ASIC1a and ASIC1b (94).

In normal neural tissue ASIC1 is important for synaptic function, as ASIC1 is mainly localized in brain structures with high synaptic density. ASIC1a is associated with postsynaptic structures of dendritic spines (colocalizes with the synapse marker post-synaptic density protein 95, PSD-95) at glutamatergic synapses (94). The absence of ASIC1 disturbs long-term potentiation in hippocampus, and homozygous ASIC1 knockout mice exhibit obvious behavioral defects and decreased excitatory post-synaptic potentials. Therefore, ASIC1 is implicated in synaptic plasticity, learning, and memory (130). However, very little ASIC1 is located at synapses as demonstrated by the low colocalization of ASIC1 with PSD-95 (110). Moreover, protons released during synaptic activity do not activate postsynaptic ASIC1 currents in excitatory synapses of cultured hippocampal neurons, and activation of presynaptic ASIC currents does not alter synaptic transmission (110).
Extracellular acidosis activates ASIC1 channels, depolarizing the neuronal membrane and exciting the neurons on which they are expressed. Homomeric ASIC1 can be activated by extracellular proton concentrations in the physiologic pH range (62). Because reduction in pH and local acidosis in inflamed tissues contribute to pain, a role for ASICs has been proposed in nociception (10,131). Elevated hydrogen ion concentrations can excite nociceptors in the skin in an ex vivo rat skin nerve preparation and in other in vitro preparations such as the cornea, gastrointestinal tract and intracranial meninges (94). Moreover, drugs that block ASICs can partially relieve acid-induced pain in humans (132,133).

In rats, CFA (Complete Freund Adjuvant)-induced inflammation of the hind paw dramatically increases ASIC1a/b, ASIC2b and ASIC3 mRNA levels in the ipsilateral DRG neurons, and this induction is blocked by anti-inflammatory drugs (94). CNS ASICs contribute to analgesia as well; the expressions of ASIC1a and ASIC2a increase on the ipsilateral side of the dorsal horn following CFA-induced inflammation (94). ASIC1a-like channels are the predominant acid-sensitive channels in the dorsal horn, and knock-down of ASIC1a, or pharmacological blockade with PcTX-1 or amiloride reduce the pain behavior (thermal and mechanical modalities) in the CFA-induced inflammation and formalin models (134). To determine the role of ASICs in inflamed urinary bladder, the expressions of several ASIC isoforms were examined in the urinary bladder after cyclophosphamide (CYP)-induced cystitis; the expression of ASIC1a mRNA is not altered, but the expressions of ASIC2 and 3 are increased (135).

A role for ASIC1a in neuronal damage during brain ischemia and stroke has been demonstrated as well, and ASIC1a antagonists have been proposed as neuroprotective
agents (94). During ischemia, extracellular pH decreases to 7.0-6.43 (130). The decrease in extracellular pH to 6.5-6.0 under normoxic conditions induces neuronal death in cell cultures and hippocampal slices, cultured brain cortical neurons, and also cultured granular neurons (CGNs) of rat cerebellum (130). In hippocampal and cortex neurons, extracellular acidosis causes an increase in intracellular [Ca\(^{2+}\)] through activation of ASIC1a (136). This Ca\(^{2+}\) increase, which does not depend on glutamate receptors or voltage-dependent Ca\(^{2+}\) channels, destroys neurons. This neuronal cell death can be prevented by ASIC1a blockers or lowering of the extracellular calcium concentration, and does not occur in cultured cortical neurons from the brain of ASIC1a knockout mice (136). Knockout of the ASIC1a gene or intravenous injection of ASIC1a blockers protects animal brain from focal ischemia (136), even when blockers are introduced during the post-ischemic period (137).

In a mouse model of multiple sclerosis (MS) (experimental autoimmune encephalomyelitis- EAE), ASIC1a-null mice have been found to have markedly reduced axonal degeneration and behavioral deficits following an inflammatory lesion compared to wild-type mice (138). Acidosis in a range consistent with the activation of ASIC1a can be measured in the spinal cord of EAE animals. ASIC1a inhibitors may function as a treatment for the axonal degeneration associated with MS (138).

In *C. Elegans*, a model has been proposed in which a mechanosensory complex composed of members of the ENaC/Deg family is linked to both the cytoskeleton and extracellular matrix (94). There is evidence supporting a role for ASICs in mechanosensation. ASIC1 is required for normal visceral mechanosensory function of vagal neurons innervating the stomach, esophagus, and DRG neurons innervating the
It is also required for normal gastric emptying (118). ASIC1 is not required for normal cutaneous mechanosensory function, because loss of ASIC1 does not affect the function of cutaneous mechanoreceptors (118). The presence of ASIC1α appears to provide an inhibitory contribution to the mechanosensory ion channel complex, since disruption of ASIC1α increases the mechanical sensitivity of splanchnic colonic afferents and vagal gastro-oesophageal afferents, and doubles the gastric emptying time (119).

**PROTEIN KINASE C**

Protein kinases are enzymes that regulate the function of other proteins by attaching phosphate groups to Ser, Thr, or Tyr residues, an event known as phosphorylation. The Protein Kinase C (PKC) family is a branch of AGC kinases, which includes Akt/PKB, PKC-related protein kinase (PKN), SGK, cGMP-dependent protein kinase (PKG), and PKA (139). The mammalian PKC family members all evolved from PKC1 in *Saccharomyces Cerivisiae* (140).

PKC was identified in 1977 in bovine cerebellum as a kinase that phosphorylates histone and protamine (141), and it was first thought to be a single protein; today the PKC family comprises ten isoforms with different structures, cofactor requirements, functions, tissue expression and cellular compartmentalization (140). The ten members are divided into three classes, differing in domain compositions and cofactor requirements: the conventional (c) PKC isoforms (α, βI, βII, and γ), that require Ca\(^{2+}\) and diacylglycerol (DAG) for activation; novel (n) PKC isoforms (δ, ε, η, and θ) that require DAG; and atypical (a) PKC isoforms (ζ and ι (mouse homologue is known as λ)) that require neither Ca\(^{2+}\) nor DAG (142). All the isoforms share the same architecture of a C-
terminal serine/threonine protein kinase domain (consisting of motifs required for ATP/substrate-binding and catalysis) linked through a flexible variable region to an N-terminal regulatory domain (143). The regulatory domain autoinhibits the enzyme in the absence of the appropriate second messengers, it targets the enzyme to specific cellular locations, and it mediates protein-protein interactions (144).

The regulatory domain is composed of an inhibitory region (pseudosubstrate site), a C1 domain (one or two copies named C1A and C1B depending on the isozyme), and a C2 or PB1 domain. The C1 domain binds DAG and is also the binding site for the potent tumor-promoting phorbol esters, the functional analogues of DAG. Phorbol esters compete with DAG to bind the C1 domain. The C1 domain also binds to the anionic phospholipid phosphatidylserine (PS) (144). The C2 domain binds anionic lipids as well, but in a Ca$^{2+}$-dependent manner. The C2 domain of conventional PKCs has some specificity for binding PS (144). The mature PKCs reside in the cytosol. Ca$^{2+}$-binding to the C2 domain pre-targets PKC to the plasma membrane where it binds anionic phospholipids. The C2 domain’s high specificity and preference for binding phosphatidylinositol-4,5-bisphosphate (PIP2) also selectively targets the conventional PKCs to the plasma membrane (143). Then the C1 domain binds its ligand DAG, an interaction that is enhanced by PS binding. PS disrupts electrostatic interactions between C1A and C2 domains, freeing the C1A domain, which goes through the lipid bilayer and binds DAG (143).

Novel PKC isozymes are not sensitive to Ca$^{2+}$, because they contain a variant of the C2 domain that lacks the Ca$^{2+}$-binding residues. Because novel PKCs are regulated only by DAG, they can directly respond to DAG increases, unlike the conventional
PKCs, which depend on membrane recruitment by the Ca$^{2+}$-activated C2 domain to sense DAG (140). Novel PKCs also have a two-fold higher affinity for DAG than do the conventional PKCs. The high affinity is due to a single residue in the C1b domain, a Trp in the nPKCs instead of a Tyr in the cPKCs (143).

The atypical PKCs contain a variant of the C1 domain that does not bind to DAG or phorbol esters. Lacking a C2 domain, atypical PKCs are not sensitive to Ca$^{2+}$. They contain an N-terminal PB1 domain and a C-terminal post-synaptic density-95/discs large/zonula occludens 1 (PDZ)-binding domain that may influence protein-protein interactions (140). The C-terminal tail of all PKCs is conserved (although only PKC α, ζ and ι contain the PDZ domain) and functions as a phosphorylation-dependent docking site for other regulatory molecules (140,145). All isozymes also contain a pseudosubstrate sequence that occupies the substrate-binding cavity, keeping PKC in an inactive conformation. For conventional and novel PKCs, Ca$^{2+}$-and/or DAG-mediated membrane binding provides the energy required to expel the inhibitory peptide from the active site, allowing PKC to phosphorylate downstream substrates (145).

The kinase domain is functional when it is phosphorylated on two (atypical PKCs) or three (conventional and novel PKCs) conserved sites. Phosphorylations of Ser/Thr or Tyr residues in different PKC isoforms can control activity, cellular localization and therefore function (142), and they are essential for the stability and catalytic activity of PKC. The chaperone HSP90 and the co-chaperone Cdc37 bind to a conserved stretch of residues (PXXP) in the kinase domain of conventional and novel PKCs. HSP90 binding is required for the phosphorylation of PKC. Phosphorylation is an important event because PKC that is not phosphorylated gets ubiquitinated and degraded.
The kinase domain contains three conserved constitutive phosphorylation sites: the activation loop site, and two C-terminal sites in the turn motif and the hydrophobic motif. The atypical PKCs contain the phospho-mimetic Glu residue in the hydrophobic motif instead of the phosphorylation-modified residue (140).

The first event in the maturation of all PKC isozymes is phosphoinositide-dependent kinase 1 (PDK-1) phosphorylation of the activation loop site. This occurs when PKC is in an open conformation (quickly after its synthesis), with the pseudosubstrate unbound from the substrate-binding site and the activation loop exposed (143). The two C-terminal tail sites are then phosphorylated, after which the activation loop may be dephosphorylated, without consequences for the enzyme. Phosphorylation in the turn motif stabilizes the active conformation of the kinase and depends on the mammalian target of rapamycin (mTOR) complex 2 (mTORC2); however, it is not clear whether this is a direct or indirect effect (140). The kinase responsible for phosphorylating the hydrophobic motif site in vivo is not known, but this site can be phosphorylated by several kinases in vitro, including PKC and mTOR. HSP90 binding to the PXXP motif of the conventional and novel PKCs facilitates phosphorylation of the hydrophobic motif (140).

A variety of anchor/scaffolding proteins regulate PKC. Scaffolding proteins target PKC to distinct subcellular locations. Indeed, PKC signaling has been detected in the plasma membrane, cytosol, Golgi apparatus, nucleus, and mitochondria (144). Interactions of PKC with other proteins are complex, because they often depend on the conformation of PKC, which may be unphosphorylated, phosphorylated but inactive, or phosphorylated and active. Receptors for activated C-kinase (RACKs) bind to the C2
domain of PKC and regulate its activity and cellular localization. The C1A domain of PKC\(\beta\)II can interact with pericentrin, a scaffolding protein that localizes PKC\(\beta\)II to the centrosome. This interaction is crucial for cell division. The C1A domain of PKC\(\beta\)II also binds to RING finger protein that interacts with C-kinase (RINCK). This interaction results in the ubiquitination and degradation of PKC. The interaction of the C-terminal tail of PKC\(\alpha\) with PICK has been implicated in learning and memory processes.(144)

**Figure 5. Domain structure of PKC family members (140).** Schematic shows the pseudosubstrate, C1 domain, C2 domain, kinase domain, and carboxyl-terminal tail (CT). The three phosphorylation sites in the kinase domain and CT are indicated by short lines. The effects of Ca\(^{2+}\), diacylglycerol (DG), and PIP\(_2\) depending on the subfamily (conventional, novel, or atypical) are shown as +, stimulatory or -, no effect.

After PKC is activated, it can stay activated a long time (hours to days) when the activating stimulus is removed (140). PKC signaling termination is not as well understood as its activation, but several mechanisms are thought to be involved in the reverse translocation of PKC from membranes. This process depends on the C1 and C2 domains and the catalytic activity of PKC (140). Sustained activation can also downregulate PKC, because in the active conformation PKC is more susceptible to dephosphorylation; dephosphorylated PKC is unstable and gets degraded. Therefore,
prolonged treatment with phorbol esters can cause PKC degradation and is used as a means to deplete the cell of the conventional and novel PKCs (140).

PKC isozymes are expressed in a variety of tissues and cells, where they are involved in many cellular processes and have diverse functions. The highest levels of expression and catalytic activity of PKC are found in the brain, and PKC is involved in learning and memory phenomena (144). PKC γ expression is characteristic of normal CNS tissue, while PKCs α and δ are more abundant in non-neuronal tissues (146). PKC signaling has been implicated in diabetes, cardiovascular disease, pulmonary disorders, central nervous system dysfunctions (such as neurodegeneration), and cancer (144). PKC isoforms play important roles in cell proliferation, differentiation, survival, cell-cell contacts, cell polarity, cell cycle control, and cell migration (147),148).

MATRIPASE

Matriptase (MT-SP1, TADG3, epithin, ST14, and, channel activating protease 3 (CAP3)) was first described in 1993 as a gelatinolytic activity in cultured human breast cancer cells, and was isolated in a complex with its cognate inhibitor, the hepatocyte growth factor activator inhibitor-1 (HAI-1) from human milk (148). Matriptase was cloned from human prostate cancer cells, ovarian cancer cells, and breast cancer cells by three different groups (148). The mouse ortholog of matriptase, epithin, was cloned from thymic cells (148).

Matriptase is an 855 amino acid type II transmembrane protease that belongs to the S1 clan of trypsin-like serine proteases. It contains an extracellular C-terminal catalytic domain and an intracellular N-terminus (149). The N-terminus is composed of a
transmembrane domain and an intracellular 54 aa stretch, most likely involved in protein-protein interactions. Extracellular to the transmembrane domain, there are several different domains: an SEA domain (sea urchin sperm protein, enteropeptidase, and agrin), two CUB domains (C1r/s, Uegf, and Bone morphogenic protein-1), and four LDLRA domains (low density lipoprotein receptor class A domains). Matriptase contains four potential N-linked glycosylation sites (148). The C-terminal end of matriptase contains a serine protease catalytic domain (aa 614-855), which contains the conserved catalytic triad His/Asp/Ser (H656, D711, S805) essential for proteolytic activity. N-linked glycosylations of the first CUB domain and the serine protease domain are required for matriptase activation (149).

![Figure 6. Domain structures of matriptase and HAI-1 (149).](image)

Figure 6. Domain structures of matriptase and HAI-1 (149). The glycosylation sites and the catalytic triad amino acids are indicated with arrowheads.

Like other serine proteases, matriptase is synthesized as an inactive, single-chain zymogen with a mass of 95kDa due to glycosylation. However, this size of matriptase is
never detected in cells because matriptase is cleaved by an unknown protease in the secretory pathway at Gly149 within the SEA domain, resulting in two pieces (aa 1-149 and aa 150-855), which remain associated by non-covalent interactions (150). Mutations of Gly149 result in a matriptase that can be detected at 95 kDa. This first N-terminal processing of matriptase is its maturation step (150). Activation of this single-chain zymogen molecule to the proteolytically active disulfide-linked two-chain matriptase occurs by cleavage at R164 in the serine protease domain, after it reaches the cell surface. This activation site cleavage requires the proteolytic activity of matriptase (150).

Matriptase can be shed from the cell surface by proteolytic cleavage at Lys189 and Lys 204. Mutations in any of the catalytic triad amino acids render matriptase inactive, by making it unable to undergo activation site cleavage at R614 (150).

It is thought that matriptase maturation, activation, and shedding are all influenced by interactions of matriptase with HAI-1, its cognate inhibitor. HAI-1 is a Kunitz-type serine protease inhibitor of 513 amino acids. HAI-1 contains a C-terminal transmembrane domain and a 41 amino acid cytoplasmic tail. External to the membrane, it contains two Kunitz type serine protease inhibitor domains, which are separated by one LDLRA domain (150). In contrast to serpin-type inhibitors that form covalent, irreversible interactions with proteases, Kunitz domains competitively inhibit target proteases. HAI-1 also undergoes an N-terminal cleavage, which removes its signal peptide, and further cleavages (one near the transmembrane domain and the other in the Kunitz domain I) (150). These cleavages result in the shedding of HAI-1 from the plasma membrane. In fact, only matriptase in a complex with HAI-1 has been detected in breast
milk or conditioned media from cell culture systems. HAI-1-free matriptase has not been detected in conditioned media (149).

Although the presence of HAI-1 seems required for the activation and shedding of endogenous matriptase, this does not seem to be the case for matriptase transfected cells. In MDCK cells stably transfected with matriptase, almost all the matriptase is found in the conditioned medium of the cell and very little is present at the cell surface. N-terminal processing at G149 is required for the shedding of matriptase from the cell surface (151). In the same transfection system, matriptase does not require HAI-1 for activation (152).

Matriptase is widely expressed in most epithelial tissues. Matriptase expression has been detected in epidermis, cornea, salivary gland, oral and nasal cavities, thyroid, thymus, esophagus, trachea, bronchioles, alveoli, stomach, pancreas, gallbladder, duodenum, small intestine, colon, rectum, kidney, adrenals, urinary bladder, ureter, seminal vesicles, epididymis, prostate, ovaries, uterus, and vagina (149). It is also expressed in immune cells such as mast cells, monocytes and macrophages (149). This protease is involved in the development of the epidermis, hair follicles and cellular immune system. It is crucial for epidermal barrier formation through proteolytical processing of profilaggrin polyprotein into filaggrin monomers and an S-100 N-terminal protein, which promotes epidermal differentiation (149). Mice lacking matriptase develop to term, but die of fatal dehydration 1-2 days after birth, due to compromised skin and oral epidermal barrier function, which leads to rapid and fatal dehydration (149). The matriptase missense mutation, G827R, which results in an inactive protease, has been identified in humans (153). Patients with this disease suffer from autosomal recessive
ichthyosis with hypotrichosis syndrome, a skin and hair disorder characterized by scaly, itchy skin, sparse hair, and defective desquamation (153). In patients with a homozygous frameshift mutation in the ST14 gene, it has been demonstrated that profilaggrin processing in keratinocytes is impaired, resulting in the autosomal recessive syndrome of congenital ichthyosis, follicular atrophoderma, and hypotrichosis. These patients suffer from scaly skin, enlarged depressions of the pilosebaceous orifices, sparse scalp hair with bald patches and subnormal sweating (148). Matriptase is involved in hair follicle growth and thymocyte development. Loss of matriptase impairs the growth of hair follicles and prevents vibrissae eruption, due to failure to form vibrissal hair canals (149). In the thymus, matriptase increases apoptosis of immature thymocytes, leading to thymocyte depletion (149).

Despite its functions in normal physiological processes, matriptase has been implicated in many cancers. It is consistently expressed in human epithelial tumors of the head, neck, mesothelium, breast, ovary, cervix, prostate, lung, and gastrointestinal tract. Its overexpression in keratinocytes results in spontaneous squamous cell carcinomas (154). In most tumors, matriptase RNA and protein are upregulated, and there is a positive correlation between matriptase expression and tumor grade. The normal breast and prostate epithelia express matriptase. However, in breast and prostate carcinomas, increased matriptase expression correlates with tumor grade and stage. In breast cancers, high matriptase expression is a predictor of poor survival (154). The normal ovarian or cervical epithelia do not express matriptase, but high matriptase expression is detected in stage I ovarian cancer and there is increased matriptase expression with higher cervical cancer grades. In studies that measure matriptase activity
(instead of simply protein or RNA levels) it is reported that a larger proportion of matriptase is free from the inhibitor HAI-1 in cancer cells compared with nontumor cells (148).

In addition to profilaggrin, matriptase has many physiological substrates that get activated upon cleavage. Some of them, such as PAR-2 (protease activated receptor-2), proHGF/SF (pro hepatocyte growth factor/scatter factor), and pro-uPA (pro urokinase plasminogen activator) are implicated in malignant progression. Matriptase is also an activator of the pro-form of the GPI-anchored serine protease prostasin (PRSS8, channel activating protease-1) in vitro, and the two proteases colocalize in the epidermis and in different epithelial cells (148). Matriptase is most likely located upstream of prostasin in vivo, because pro-prostasin cannot autoactivate. Supporting this hypothesis, matriptase-deficient mice lack active prostasin in the epidermis (148).

Matriptase also has other substrates involved in cell migration. Matriptase can cleave fibronectin and laminin, two extracellular matrix components that mediate cell adhesion and migration. Matriptase can also degrade gelatin and collagen type IV (154). Matriptase inhibition in vitro or in xenografted tumors with siRNAs or antisense oligodeoxyribonucleotides most often does not affect the proliferation of cancer cells, but it decreases invasion. Matriptase anti-sense decreases the invasiveness of ovarian cancer cells, while matriptase overexpression in gastric cancer cells enhances lymph node metastases in nude mice. Overexpression of the serine protease inhibitor HAI-1 suppresses the in vitro invasion of glioblastoma cells (155). There could be a role for matriptase in regulating cell proliferation because a matriptase-specific inhibitory drug suppresses the growth of human prostate cancer cells in a xenograft (156).
ENaC/Deg subunits may be either directly or indirectly modulated by matriptase. Matriptase co-localizes with and activates prostasin, a glycosylphosphatidylinositol (GPI)-linked epithelial serine protease that activates ENaC currents in epithelial cells or ENaC expressed in heterologous systems (157). It has been shown that matriptase activates the amiloride-sensitive Na\(^+\) current of \(\alpha\beta\gamma\)ENaC channels expressed in oocytes (158). Although it is not known if ENaC is a direct substrate for matriptase, these data suggest the possibility of other ENaC/Deg members’ modulation by matriptase.

AMILORIDE-SENSITIVE CHANNEL IN GLIOBLASTOMA MULTIFORME (GBM)

GBM are central nervous system (CNS) tumors that arise from glial cells or their progenitors (159). They are the most aggressive form of glioma and account for 65% of all primary malignant brain tumors in adults. They are highly invasive, highly proliferative, and highly vascularized tumors with diffuse margins, which make their complete surgical resection impossible (159). Patients with this devastating disease have a mean survival of three months without surgical resection, or one year after surgical resection (159).

Two years after the cloning of ASIC1, the expression of a basal-activated Na\(^+\) conductance that was completely inhibited by 100 \(\mu\)M amiloride was identified in glioma cells. Amiloride inhibited these currents with a \(K_i\) of \(~30\ \mu\)M (53). ASIC1 mRNA was detected in freshly-resected and low passage non-neoplastic astrocytes (received from patients undergoing surgery for intractable epilepsy), low-grade glioma cells, freshly resected GBM cells, and GBM cell lines (53). However, this amiloride-sensitive
conductance was detected only in the high-grade glioma cells and was absent from the normal astrocytes and low-grade glioma cells, despite the presence of ASIC1 mRNA (53).

In order to identify molecular mechanisms that control the expression of the amiloride-sensitive conductance in glioma cells, the Benos lab has investigated several candidate molecules:

Syntaxin 1A

Syntaxin 1A is an integral membrane protein with one transmembrane domain, a short extracellular C-terminus and intracellular N-terminus. It is a t-SNARE involved in vesicle fusion to membranes (160). Syntaxin 1A is expressed both in glioma cells and in normal astrocytes (54). According to experiments with planar lipid bilayers and expression in *Xenopus* oocytes, syntaxin 1A can inhibit ASIC1, but only when the ASIC2 and γENaC subunits are present (54). This inhibition occurs most likely through a direct interaction of syntaxin with the channel complex. Syntaxin 1A can precipitate *in vitro* translated γENaC (54). ASIC1, γENaC, and ASIC2 are all expressed in normal astrocytes (54,56,124). In glioma cells, although ASIC1 (124) and γENaC (56) are present at the plasma membrane, ASIC2 is not (124). The mechanisms leading to the absence of ASIC2 from the plasma membrane of glioma cells have been studied. ASIC2 protein is not expressed at all in ~60% of glioma cells examined (54,161). This appears to be due to the methylation of the ASIC2 promoter because treatment of cells with a methylation inhibitor restores ASIC2 expression in these cells (161).

Further analysis of the cellular localization of ASIC2 in the glioma cells that do express it has shown that ASIC2 is trapped intracellularly (the endoplasmic reticulum or
cis Golgi) due to interactions with the chaperone Hsc70, and is not present at the plasma membrane (124). Hsc70 is a chaperone that binds to misfolded proteins, and its expression is higher in glioma cells compared to normal astrocytes (124). The molecular chaperone glycerol, the transcriptional activator Na⁺-4-phenylbuturate (NaPB), and Hsc70 siRNA all induce the expression of ASIC2 at the plasma membrane of D54-MG cells and inhibit the amiloride-sensitive current. The whole-cell conductances of U87-MG cells, which do not express ASIC2, and of normal astrocytes, in which ASIC2 traffics to the plasma membrane, are not affected by glycerol or NaPB (124). It has not been shown whether syntaxin 1A is required for the effects of glycerol, NaPB or Hsc70. However, while ASIC2 transfection in U87-MG inhibits the amiloride-sensitive current of glioma, the current is restored by the presence of the syntaxin-binding protein Munc-18 in the cytosol (54). This suggests that the binding of Munc-18 to syntaxin 1A removes the syntaxin block of the channel. Therefore, the hypothesis for the role of syntaxin 1A in the glioma conductance is that in the absence of plasma membrane ASIC2, syntaxin 1A is not able to inhibit the channel complex.

PKC

Human glioma cells show a differential expression of PKC isoforms compared to normal astrocytes. For example, PKCα activity (146) and PKCε and PKCζ expression (162) are increased in malignant glioma, while PKCβ is absent (102,162). In addition, PKC holoenzyme and a combination of PKCβI and βII inhibit the open probability of ASIC1 in planar lipid bilayers. PKCβI and βII, when included in the patch pipette, also inhibit the basal conductance of U87-MG glioma cells in patch clamp experiments (102).
Addition of PKCζ or PKCβII alone has no effect on this conductance (102). These results suggest that PKC may phosphorylate ASIC1. This could contribute to the presence of the constitutive amiloride-sensitive conductance of glioma cells. An amiloride-sensitive whole-cell current can be induced in normal astrocytes pretreated with a specific PKCβ inhibitor, when Munc-18 is inside the cell, in agreement with the hypothesis that both PKC and syntaxin 1A inhibit this channel in normal astrocytes (102).

Composition of the Amiloride-Sensitive Channel

The exact composition of the amiloride-sensitive channel in glioblastoma cells is not known, but the presence of several ENaC/Deg subunits and the characteristics of the glioma current suggest that the glioma channel complex is a heteromeric channel composed of both ENaC and ASIC subunits (54-56). The roles of ASIC1, αENaC, and γENaC have been specifically studied, although other subunits such as ASIC3, ASIC4, or βENaC, are expressed in glioma cells (54) and they may form or modulate the channel complex. However, their specific role in the glioma conductance has not been studied. In addition to the above data with syntaxin 1A, the inhibition of the glioma conductance by the ASIC1-specific inhibitor PcTX-1 (55) strongly suggests that ASIC1 is a component of the glioma channel complex. In agreement with this hypothesis, dominant negative knockdown of ASIC1 inhibits the amiloride-sensitive current in D54-MG cells (56). Knockdown of αENaC or γENaC subunits has the same effect, suggesting that these subunits are also components of the glioma channel complex (56). In addition, an overexpressed GFP-tagged ASIC1 co-immunoprecipitates with αENaC and γENaC in
D54-MG cells, while a GFP-tagged CIC channel does not, suggesting that the interaction of ASIC1-GFP with α and γENaC is specific (56).

**PURPOSE**

The overall purpose of this project is to better understand the regulation of the constitutively active current expressed in glioma cells, and why this current is absent in normal astrocytes. ASIC1 is a central component of the amiloride-sensitive channel in glioma. PKC has been implicated in glioma and as a possible regulator of this channel complex. PKC can phosphorylate ASIC1 in vitro and inhibit its open probability in planar lipid bilayers. Therefore, my immediate goal was to test the hypothesis that PKC can regulate ASIC1 function using Xenopus oocytes as a model system, and to identify the PKC phosphorylation sites on ASIC1. The serine protease matriptase has been implicated in many malignant cancers. Matriptase can activate ENaC channels. Moreover, serine proteases can modulate the function of ASIC1. Therefore, my second immediate goal was to determine the expression of matriptase in glioma, and to test the hypothesis that matriptase can modulate ASIC1 function.
TWO PKC CONSENSUS SITES ON HUMAN ACID-SENSING ION CHANNEL 1b (hASIC1b) DIFFERENTIALLY REGULATE ITS FUNCTION

by

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ABSTRACT

Human acid–sensing ion channel 1b (hASIC1b) is a $\text{H}^+$-gated amiloride-sensitive cation channel. We have previously shown that glioma cells exhibit an amiloride-sensitive cation conductance. Amiloride and the ASIC1 blocker, psalmotoxin-1, decrease the migration and proliferation of glioma cells. Protein kinase C (PKC) also abolishes the amiloride-sensitive conductance of glioma cells and inhibits hASIC1b open probability in planar lipid bilayers. In addition, hASIC1b’s C-terminus has been shown to interact with protein interacting with C kinase 1 (PICK1), which targets PKC to the plasma membrane. Therefore, we tested the hypothesis that PKC regulation of hASIC1b at specific PKC consensus sites inhibits hASIC1b function. We mutated three consensus PKC phosphorylation sites (T26, S40, and S499) in hASIC1b to alanine (A) to prevent phosphorylation, and to glutamic acid (E) or aspartic acid (D), to mimic phosphorylation. Our data suggest that S40 and S499 are critical sites mediating modulation of hASIC1b by PKC. We expressed mutant hASIC1b constructs in *Xenopus* oocytes and measured acid-activated currents by two–electrode voltage clamp. T26A and T26E did not exhibit acid-activated currents. S40A was indistinguishable from wild-type (WT), while S40E, S499A, and S499D currents were decreased. The PKC activators PMA and PdBu inhibited WT hASIC1b and S499A, and PMA had no effect on S40A or on WT hASIC1b in oocytes pretreated with the PKC inhibitor chelerythrine. Chelerythrine inhibited WT hASIC1b and S40A, but had no effect on S499A or on S40A/S499A. The PKC activators or inhibitor did not affect the surface expression of WT hASIC1b. These data show that the two PKC consensus sites S40 and S499 differentially regulate hASIC1b and mediate the effects of PKC activation or PKC inhibition on hASIC1b. This will result in a deeper
understanding of PKC regulation of this channel in glioma cells, information which may help in designing potentially beneficial therapies in their treatment.

Keywords: two-electrode voltage clamp, *Xenopus laevis* oocytes, PMA, chelerythrine, mutagenesis
INTRODUCTION

Acid-sensing ion channels (ASICs) are activated by extracellular protons and belong to the epithelial sodium channel/degenerin (ENaC/Deg) family of ion channels. Four ASIC genes (ASIC1-4) have been cloned, and ASICs 1-3 have splice variants (19, 28, 29, 31, 42). ASICs are permeable to Na⁺ but also to other mono- and divalent cations like Ca²⁺ (in the case of ASIC1), Li⁺, K⁺, and H⁺ (41, 42). ASIC subunits are expressed in peripheral nervous system neurons, where they have been implicated in nociception during tissue acidosis and in mechanosensation (42). In the central nervous system (CNS), ASIC1 has been mostly studied in mouse and rat brains where it is abundantly expressed, and ASIC1a, ASIC1b, and ASIC2 have been found to be expressed in human brain (17). Mouse ASIC1a modulates synaptic plasticity, contributing to learning, memory and fear conditioning (42). ASICs are also expressed in nonneuronal tissue and cells such as the retina, osteoblasts, ear, taste buds, lung, testis, astrocytes and intestine, where they may sense extracellular acid changes (38, 42). The recent crystallization of chicken ASIC1 at low pH has confirmed the protein structure and has also revealed that ASIC1 forms a homotrimer (26). The protein consists of short intracellular N- and C-termini, two transmembrane-spanning α-helices, and a large extracellular loop (28, 29, 40, 42). Different ASIC subunits are capable of forming heteromultimers with other ASIC subunits and in some cases with other ENaC/DEG subunits (2, 4, 5, 15, 32). The extracellular domain senses protons and interacts with modulators, including proteases, Zn²⁺, Ca²⁺, and redox reagents (42). The cytoplasmic N- and C-termini contain phosphorylation sites and interact with other proteins such as protein interacting with C kinase 1 (PICK1) (14, 25, 42) postsynaptic density protein 95 (PSD-95), abnormal cell
lineage 7b (Lin-7b) (24), and annexin (13), resulting in changes in the current density or cellular localization of ASIC. For example, PICK1 increases the ASIC2a current amplitude by potentiating phosphorylation of ASIC2a at T39 (equivalent to site S40 on hASIC1b) (3). Also, PKC phosphorylation of the C-terminus of ASIC3 (at S523) increases the binding of ASIC3 to Na⁺/H⁺ exchanger regulatory factor 1 (NHERF-1), which in turn increases the current of ASIC3 expressed in Xenopus oocytes (12). Furthermore, PKA phosphorylates hASIC1b at S479, and this phosphorylation interferes with PICK1 binding (30). Berdiev et al. (6) showed that PKC holoenzyme phosphorylates hASIC1b in vitro, and addition of PKC to the intracellular side of hASIC1b in lipid bilayers decreases its open probability. Generally, the direction of the effect of PKC on ion channels is cell-type specific, and channel-subtype specific, with PKC activation resulting either in an increase or in a decrease of the current (11). For example, activation of PKC increased transepithelial Na⁺ transport measured as amiloride-sensitive short-circuit current (Isc) across the skins of two different species of frogs, and inhibited Isc across the bladders of the same animals (10).

We have focused on characterizing the consensus PKC phosphorylation sites of hASIC1b and on describing the effect of PKC activation or inhibition on hASIC1b function because of evidence suggesting this ion channel and PKC are involved in the regulation of an amiloride-sensitive cation conductance in high-grade glioma cells. We have previously shown that this conductance is expressed only in high-grade glioma, and it is absent in normal astrocytes and in low-grade glioma (6-9, 38). These cells express only the hASIC1b splice variant (38), and previous evidence suggests that hASIC1b is a component of the amiloride-sensitive channel complex (7, 38, 39). The physiological
importance for glioma cells to express this amiloride-sensitive conductance becomes clear because inhibition of this conductance affects glioma cell migration and proliferation. Amiloride inhibits glioma cell migration and proliferation (23, 34, 38), while psalmotoxin1 (PcTX-1), an ASIC1 blocker, inhibits the glioma conductance (8) and the regulatory volume increase of glioma cells following a hyperosmotic challenge (33).

Berdiev et al. (6) also showed that addition of PKC abolished the inward current of a glioma cell, and that PKC inhibition is one of the necessary steps required to observe a similar amiloride-sensitive current in normal astrocytes. Therefore, we hypothesize that differential regulation of hASIC1b by PKC may turn this conductance on in glioma cells and off in normal astrocytes. Additionally, the hASIC1b splice variant is the most similar to the rat and mouse ASIC1a (Fig.1), both of which are expressed and play a role in the CNS. Thus, it is important to determine specifically if PKC can regulate hASIC1b function. The presence of a PDZ binding domain in the C-terminus of hASIC1b and the fact that hASIC1b C-terminus interacts with PICK1 (25) also suggest that phosphorylation by PKC might regulate hASIC1b. Moreover, queries with databases that search for motifs in the amino acid sequence of a protein have shown that there are three consensus PKC phosphorylation sites in the cytoplasmic tails of hASIC1b. The aim of this study is to determine if these sites are important for the effect of PKC on hASIC1b currents. In order to study the effect that specific phosphorylation sites have on hASIC1b function, we have heterologously expressed hASIC1b and several phosphorylation null or mimic mutants of hASIC1b in *Xenopus* oocytes and measured acid-activated currents by two-electrode voltage clamp. *Xenopus* oocytes provide a good model system for studying
regulation of proteins by protein kinase C, because they do not exhibit endogenous acid-activated currents (37) and express most of the PKC isoforms (PKC α, βI, βII, γ, δ, ζ and ε) (27, 44). We have found that mutations in the hASIC1b consensus PKC phosphorylation sites decrease hASIC1b functional expression in *Xenopus* oocytes, and that these sites determine if PKC activation or inhibition has an effect on hASIC1b.
MATERIALS AND METHODS

Materials

The PKC activator phorbol-12-myristate-13-acetate (PMA) (Calbiochem, San Diego, CA) was stored as a 1 mM stock in dimethyl sulfoxide (DMSO) (Sigma, Saint Louis, MO) at -80°C. Phorbol-12,13-dibutyrate (PdBu) (Calbiochem) was stored as a 10 mM stock in DMSO. The inactive phorbol ester 4-α-PMA (Promega, Madison, WI) was kept as a 5 mM stock in DMSO at -20°C. Chelerythrine, HCl (Calbiochem or Sigma) was stored as a 2 mM stock in DMSO at -20°C. The PKC inhibitory peptide 19-31 was dissolved in 5% acetic acid at 1 mg/ml and stored at -20°C.

Generation of hASIC1b phosphorylation mutants

The full length sequence of human ASIC1b (GenBankTM accession number NM001095) was entered into the Genetics Computer Group sequence analysis suite (GCG) at UAB and Scansite software (http://scansite.mit.edu) to determine the PKC consensus phosphorylation sites. There are three PKC consensus phosphorylation sites located in the intracellular aspect of hASIC1b: T26, S40, and S499. These sites were mutated to alanine (A), to prevent their phosphorylation, or to glutamic acid (G), or aspartic acid (D), to mimic phosphorylation. Site-directed mutagenesis was performed using the Excite kit for the T26A, S40A, and S499A mutants, or the Quickchange II XL kit (both kits from Stratagene, La Jolla, CA) for the T26E, S40E, S499D, S40A/S499A, S40E/S499A, and S40E/S499D mutants. Each human ASIC1b construct was subjected to PCR with sense and antisense primers with the necessary base changes to result in the desired amino acid mutations. The PCR product was digested overnight at 37°C with Dpn
I to remove non-mutated DNA, and it was transformed into XL-10 Gold *E.coli* following the manufacturer’s instructions. Transformed *E.coli* were plated on Luria Bertani (LB) plates with 50 ug/ml ampicillin and grown for 16h at 37°C. DNA was isolated from 5 ml LB + amp cultures of each colony using a miniprep kit (Qiagen, Valencia, CA) and the presence of the mutations was confirmed by sequencing (Heflin Genetics Center, UAB). The colonies were then grown into 250 ml LB + amp cultures, and the DNA was isolated with a maxiprep kit (Qiagen). The full length hASIC1b was sequenced again after this step.

*Generation of HA-tagged hASIC1b*

To insert the haemagglutinin (HA) tag (YPYDVPDYA) of the influenza virus between the F147 and K148 residues of the extracellular loop of hASIC1b, we used a similar method to Geiser et al. (18). The oligonucleotide primers were obtained PAGE-purified from Integrated DNA Technologies, Inc (Coralville, IA). The forward primer was 63 base pairs long with the 5’ end formed by 36 nucleotides corresponding to the hASIC1b DNA sequence upstream of the insertion of the HA tag followed by the 27 nucleotides encoding the HA tag. The reverse primer was 131 base pairs long with the 5’ end consisting of 104 bases identical to the hASIC1b DNA sequence downstream of the HA tag position. The PCR fragments were obtained by PCR reaction of 3 μg of each of the forward and reverse primers with Taq Polymerase. The primers were denatured at 94°C for 10 min, annealed by decreasing the temperature from 94°C to 60°C for 34 min at a rate of 1°C per min, and extended at 68°C for 10 min. The PCR product was gel-purified with a Qiaquick Gel Extraction Kit (Qiagen). 300 ng of this gel purified PCR
fragment and 100 ng of recipient plasmid DNA (hASIC1b) were used in a mutagenesis reaction using the Quickchange II XL Site-Directed Mutagenesis kit (Stratagene) as described in the kit’s instructions. After transformation of XL-10 Gold *E. Coli*, and isolation of plasmid DNA by miniprep, the presence of the HA tag in the correct position on hASIC1b was confirmed by DNA sequencing.

**cRNA preparation**

The WT hASIC1b and the phosphorylation mutants, or the HA-tagged hASIC1b were linearized with XbaI. After proteinase K digestion, Na-acetate precipitation, and phenol/chloroform followed by chloroform extraction, the purified DNA in water was used for cRNA transcription using the MegaScript High Yield In Vitro Transcription kit (T7) and Cap Analog or ARCA (Ambion, Austin, TX). The quality and size of the synthesized cRNA were checked by denaturing agarose-formaldehyde gel electrophoresis. The cRNA concentration was determined spectroscopically by measuring optical density at 260 nm.

**Isolation of Xenopus laevis oocytes**

Oocytes were isolated surgically from anesthetized female *Xenopus laevis* frogs (Xenopus I, Inc., Dexter, MI) and placed in Ca\(^{2+}\)-free ND96 buffer (96 mM NaCl, 1mM MgCl\(_2\), 2 mM CaCl\(_2\), 2 mM KCl, 5 mM HEPES, pH 7.4). The oocytes were first dissociated manually with forceps into small groups. They were then digested with 2 mg/ml collagenase A (Roche Applied Science, Indianapolis, IN) in Ca\(^{2+}\)-free ND96 pH 7.4 buffer for 75-100 min, after which they were washed with Ca\(^{2+}\)-free ND96 followed
by three washes in ND96 with 2mM CaCl₂. Stage V-VI oocytes were incubated (before and after injection) at 18°C in ND96 buffer with 10 mM sodium pyruvate (Sigma) and 10 mg/ml gentamycin (Lonza, Basel, Switzerland). These experimental procedures are in accordance with and were approved by the Institutional Animal Care and Use Committee (IACUC) rules at University of Alabama at Birmingham.

*Expression of hASIC1b and phosphorylation mutants in Xenopus oocytes*

Oocytes were injected 18-24 hours post collagenase digestion with 11.5 ng of a 0.5 µg/µl cRNA dilution. We used a nanoliter injector (World Precision Instruments, Inc., Sarasota, FL) and 10 µL microdispensers (Drummond Scientific, Broomall, PA) pulled with a vertical Kopf Model 700D micropipette puller (David Kopf Instruments, Tujunga, CA). The cRNAs used to express each WT or phosphorylation mutant construct in oocytes were *in vitro* transcribed 3-4 different times, and were not all from one batch of cRNA, with the exception of the S40A/S499E and S40E/S499D cRNAs. Also, acid-activated currents were measured in oocytes injected with cRNAs for the same construct, *in vitro* transcribed in different days, to ensure that the results are not affected by cRNAs of different batches. Experiments were performed at room temperature 1-4 days post-injection.

*Two-electrode voltage clamp experiments*

Two-electrode voltage clamp experiments were performed using a Digidata 1200 Series interface and a Dagan TEV 200 or Geneclamp 500B amplifier with a steady-state restore switch modification (Axon Instruments, Sunnyvale, CA). The oocyte was placed
in a recording chamber with bath volume of 500 µl and impaled with two electrodes. The micropipettes were made from VWR calibrated (100 µl) borosilicate glass capillaries (World Precision Instruments, Inc.) pulled with a Kopf Model 700D vertical pipette puller (Kopf Instruments) and had resistances of 0.5-2.0 MΩ when filled with 3M KCl and inserted into the bath solution. A SF-77B Perfusion Fast-Step (Warner Instruments, LLC, Hamden, CT) set at 3 steps per position was used for rapid exchange of solution bathing the oocyte from ND96 pH7.4 to ND96 pH4.0 (with 5 mM MES substituted for 5 mM HEPES), to activate the heterologously expressed hASIC1b channel. The protocol used exposed the oocyte to pH 7.4 solution for 13 s, to pH 4.0 for 13 s, and again to pH 7.4 for 13 s to allow for complete recovery of the acid-activated channel. Before recording acid-activated currents, the clamp gain and stability were set for each oocyte using capacitive transients of a step protocol from -40 mV to -50 mV. The oocytes were clamped at -60 mV for all the experiments. The flow of solutions out of the perfusion fast-step was controlled with a VC-6 Six Valve Controller (Warner Instruments) and the solution flow rate was 1-2 ml/min.

For pH activation curves the solution flowing out of one barrel of the perfusion system was ND96 pH 7.4, while the solution flowing out of the second barrel was changed to ND96 pH 7.0, 6.5, 6.0, 5.5, 5.0, and 4.0 sequentially. These solutions were buffered with 5 mM HEPES (for pH 7.4 – pH 6.0) or 5 mM MES (for pH 5.5 – pH 4.0). Acid-activated currents at each pH were normalized to the pH 4.0 peak current of the oocyte. The pooled normalized values were fitted to the sigmoidal dose-response (variable slope) equation in GraphPad Prism 3 to obtain the pH50’s and Hill coefficients.
For the PKC activator experiments, five sweeps of acid-activated currents (activated with ND96 pH 4.0) were recorded, the flow of solutions was stopped, and the oocyte was unclamped before and during addition of PMA or PdBu to the bath solution to a final 1 μM concentration for 5 min (21). After the PMA incubation period, the oocyte was clamped at -60mV and another five acid-activated peaks were recorded. As controls we used 5 min of no treatment, DMSO (1:1000, the equivalent of DMSO in the PMA experiment) or 4-α-PMA, an inactive PMA analog, at 1μM final concentration. The peak acid-induced current recorded after addition of a treatment (no treatment, DMSO, 4-α-PMA, PMA, or PdBu) was normalized to the current from the last sweep before treatment. In some experiments, oocytes were pre-incubated in ND96 pH 7.4 with 1μM chelerythrine for 1 h before recording. The PKC inhibitory peptide 19-31 was injected in the oocytes 1h before recording at a final concentration of 6 μM.

Data were acquired with pClamp 8.0 software at 200 Hz and analyzed with Clampfit 9.0. After acquisition, data were filtered with an 8-pole Bessel filter with a 10 Hz -3dB cutoff, and baselines were subtracted.

Cell Culture and Transfection

Chinese Hamster Ovary-K1 (CHO-K1) cells were maintained in continuous culture in 50:50 Dulbecco's modified Eagle's medium/F-12 medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone.) For electrophysiological recordings, cells were split into 6-well tissue culture dishes and transiently transfected using four μg of pBi-eGFP-hASIC1b plasmid DNA and 10 μl of Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocols. After transfection, cells
were replated using PBS/EDTA onto flame sterilized glass coverslips and patched within 24-48h.

*Patch clamp*

Micropipettes were prepared using a Narashigi PP-83 two-stage micropipette puller and were filled with 120 mM KCl, 5 mM NaCl, 10 mM HEPES, 0.4 mM CaCl₂, 2 mM MgCl₂, 1 mM EGTA, 2 mM MgATP, pH 7.4. Pipette electrical resistance was 3–5 MΩ. Outside-out patches were formed by abutting the pipette tip to the cell surface, applying suction to form a GΩ seal, rupturing the membrane with increased suction, and slowly removing the pipette from the cell surface to form an outside out patch. The patch/pipette tip was moved just in front of the barrels of a VC-77MCS perfusion system from Warner Instruments. Signals were recorded on a PC running pClamp 9 using an Axopatch 200B patch clamp amplifier and a DigiData 1320 digitizer. The signal was sampled at 5 kHz and low pass filtered at 5 kHz with the 200B’s 4-pole Bessel filter.

Currents were recorded by holding the membrane voltage at -60 mV and perfusing with Krebs buffer (130 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM D-glucose, and 10 mM HEPES, pH 7.4). Acid-induced currents were evoked by pH 6.0 Krebs buffer, in which 5 mM HEPES was replaced with 5 mM MES and the pH was adjusted with HCl.

For PMA experiments, 100 nM PMA was added to the 7.4 and 6.0 solutions, with an equivalent amount of (1/10000 v/v) DMSO being added to 7.4 and 6.0 control solutions. An approximately 30s protocol was used: 5s pH 7.4, 10s pH 6.0, 15s pH 7.4. Little to no desensitization of the current was observed with this protocol for this
activation pH. Cells were patched on a Nikon TE200 with an epifluorescent attachment, allowing for visualization of GFP in transfected cells. Outside out patches showing less than 100 pA of acid induced current were excluded to allow for an acceptable dynamic range. In these conditions, no acid induced currents were observed in nonfluorescent cells.

**Whole oocyte membrane preparation**

To assess the whole oocyte expression of WT hASIC1b and each phosphorylation mutant, we used a modified procedure of Turk et al. (36). Ten oocytes per group were rinsed in sterile ND96 pH 7.4 and homogenized in lysis buffer (20 µl per oocyte) (100 mM NaCl, 20 mM Tris-HCl, pH 7.6, 1 % Triton X-100, and 1 Complete® protease inhibitor tablet (Roche Applied Science) per 50 ml) in a 1.5 ml tube with an Eppendorf® micropestle. The homogenized samples were centrifuged for 10 min at 12,000 rpm and the middle liquid layer was retained for gel electrophoresis. Protein concentrations were determined with a BCA assay kit (Pierce, Rockford, IL).

**Electrophoresis and immunoblotting**

Equivalent amounts of protein (30 µg) from the oocyte total membrane preps were boiled in 1X Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue) at 95°C for 5 min and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with an 8% separating gel and a 4% stacking gel. Proteins were electrotransferred to methanol-treated Polyvinylidene Difluoride (PVDF) membranes (BioRad, Hercules, CA) for 1.5 h at 100 V at 4°C in a
Tris-glycine buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol). The membranes were blocked in 5% nonfat dry milk in Tris buffered saline with Tween (TBST) buffer (100 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween) for 1 hour at room temperature. The membranes were incubated overnight at 4°C with rabbit polyclonal antibody to ASIC1 (Alomone Labs, Jerusalem, Israel) at 1:200 or with mouse anti-actin (Chemicon, Billerica, MA) at 1:5000 in 2% milk in TBST. Primary antibody incubation was followed by three washes of 15 min each with TBST. A goat anti-rabbit IgG-HRP antibody (Pierce) diluted 1:10,000 or goat anti-mouse IgG-HRP (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:20,000 in 2% milk in TBST was used to detect the primary antibodies for 1 h at room temperature. After three 15 min washes with TBST, the membranes were treated with SuperSignal West Pico Substrate (Pierce) and exposed to Kodak Biomax XAR film (Perkin Elmer, Waltham, MA).

**Single oocyte chemiluminescence**

In order to quantify the expression of hASIC1b at the plasma membrane of oocytes in control or PKC modulated conditions, we used the method of single oocyte chemiluminescence (SOC) as described by Zerangue et al. (45). Oocytes expressing HA-tagged WT hASIC1b were treated with PMA (1 µM, 5 min), PdBu (1 µM, 5 min), chelerythrine (1 µM, 1h) or both chelerythrine (1 µM 1h) and PMA (1 µM, 5 min), as was done for recording the peak pH4.0-activated currents. They were then fixed in 4% paraformaldehyde in ND96 pH7.4 for 15 min at 4°C. All subsequent steps were performed at 4°C. The oocytes were washed 3 times, 5 min each time in ND96 pH7.4, and incubated in blocking solution (1% BSA in ND96 pH7.4) overnight. They were then
incubated in rat anti-HA antibody (Clone 3F10, Roche) at 1 μg/ml for 2h. They were washed twice in blocking solution 30 min each time and incubated in goat anti-rat HRP-conjugated secondary antibody (Pierce) at 1:400 for 1h. The secondary antibody was washed twice 30 min each time with blocking solution and the oocytes were finally washed with ND96 pH7.4 for 10 min. Each oocyte was then placed in a 1.5 ml Eppendorf tube and all the solution was removed. 50 μL of ELISA Supersignal West Femto Chemiluminescence substrate (Pierce) were added and luminescence was measured with a Turner TD20/20 luminometer (Turner BioSystems, Inc., Sunnyvale, CA).

Data analysis and statistics

Data are presented as means and standard deviations (SD). Statistical significance was determined with a two-tailed paired or unpaired Student’s t-test (in Excel) for two-group comparisons, or one-way ANOVA followed by Tukey’s post-hoc test for comparisons of three or more groups (GraphPad Prism 3). A statistically significant difference was accepted if p<0.05. 95% confidence intervals (CI) were also determined for each mean (GraphPad Prism 3).
RESULTS

Mutations in hASIC1b consensus PKC phosphorylation sites and hASIC1b expression

We have analyzed the amino acid sequence of hASIC1b for PKC phosphorylation motifs with the GCG (UAB) and Scansite (MIT) software. This analysis resulted in several consensus PKC phosphorylation sites; however, we decided to concentrate on the three amino acids that are located on the intracellular N and C termini of hASIC1b: T26, S40, and S499 (Fig. 1) because phosphorylation of membrane proteins is an intracellular event (43). We used site-directed mutagenesis to generate phosphorylation mutants at each of these three sites. Mutations to alanine (A) prevent phosphorylation, and mutations to glutamic acid (E) or aspartic acid (D) can often mimic a phosphorylated amino acid.

We first assessed the effect of these phosphorylation site mutations on the functional expression of each construct in *Xenopus* oocytes. Oocytes were injected with WT hASIC1b, or each phosphorylation site mutant cRNA. Electrophysiological recordings were obtained 1-4 days post-injection. The oocytes were voltage-clamped at -60 mV, and acid-activated currents were recorded by rapidly switching the solution bathing the oocyte from ND96 pH 7.4 to ND96 pH 4.0. Representative traces of these recordings are shown in Fig. 2A. Uninjected oocytes did not exhibit any acid-activated currents (not shown and also there is no acid-activated current in oocytes injected with two of the hASIC1b mutants, Fig. 2). The peak pH 4.0 current ($I_{pH4.0}$) of each mutant was normalized to the average peak pH 4.0 current of WT hASIC1b constructs recorded from the same batch of oocytes on the same day post-injection (1.01 (SD 0.39); n = 85; 95% CI [0.925, 1.10])(Fig. 2B). Mutation of S40 to A had no effect on the functional expression of hASIC1b, as S40A exhibited average peak pH4.0-activated currents that
are not statistically different than WT hASIC1b (0.88 (SD 0.63); n = 24; p = ns, one-way ANOVA; 95% CI [0.609,1.15]; p<0.05 versus S499D). Mutation of S40 to E, a phosphorylation mimic, resulted in a decrease in $I_{pH4.0}$ of hASIC1b (0.39 (SD 0.42); n = 28; p <0.001; 95% CI [0.231, 0.552]). At site S499, mutation to either A or D also resulted in a decrease in $I_{pH4.0}$ (0.43 (SD 0.31); n = 38; p < 0.001; 95% CI [0.327, 0.532] for S499A and 0.58 (SD 0.15); n = 14; p < 0.01; 95% CI [0.498, 0.670] for S499D). The double mutant construct S40A/S499A exhibited a reduced functional expression as well (0.39 (SD 0.46); n = 19; p< 0.001; 95% CI [0.170, 0.161]). The S40E/S499A mutation had an even greater effect (0.0877 (SD 0.137); n = 17; p<0.001; 95% CI [0.0172, 0.158]; p<0.05 versus S499D). The S40E/S499D mutation also expressed a reduced $I_{pH4.0}$ compared to WT (0.330 (SD 0.271); n = 17; p<0.001; 95% CI [0.191, 0.470]). We were not able to measure any acid-activated currents in oocytes expressing the T26A (n = 8) or the T26E (n =11) hASIC1b constructs. The translation of these constructs in oocytes was confirmed by immunoblotting of total membranes of uninjected oocytes or oocytes injected with each of the ASIC1b constructs with an ASIC1 antibody (Fig.3).

Because ASIC1 is a ligand-gated channel, activated by H+, we determined if the mutations in the consensus PKC phosphorylation sites have any effect on the affinity of the channel for protons. The oocytes were injected with cRNA for each hASIC1b mutant and acid-activated currents were recorded at different activation pHs ranging from pH 7.0 to pH 4.0 (Fig.2C). The oocyte was sequentially exposed to solutions of higher then to lower pH. To determine if hASIC1b current exhibited rundown, i.e., a diminution of the peak current with repeated acid challenges, two consecutive activation curves were taken and no significant rundown of the current was observed (not shown). The peak acid-
activated currents at each pH are normalized to peak $I_{pH4.0}$ (Fig. 2C). The $pH_{50}$ values of activation and the Hill coefficients were determined for each individual oocyte by fitting the normalized $I/I_{pH4.0}$ at each test pH to the sigmoidal dose-response (variable rate) equation in Prism 3. The pH activation curve shown is the average of all the oocytes. The means and standard deviations of the $pH_{50}$'s and Hill coefficients are indicated in Table 1. The $pH_{50}$ values of the hASIC1b phosphorylation mutants were not significantly different than the $pH_{50}$ of WT hASIC1b. However, there was a statistically significant difference in the Hill coefficients (Table 1).

**Effect of the PKC activator PMA on the acid-activated currents of hASIC1b is mediated by site S40.**

We examined the effect of PKC activation with the phorbol esters PMA or PdBu on hASIC1b currents. PMA and PdBu are cell-permeable diacylglycerol (DAG) analogs that activate classical and novel PKC isoforms. Oocytes were injected with WT hASIC1b, S40A hASIC1b, or S499A hASIC1b to examine the importance of the amino acids that are consensus PKC phosphorylation sites. Acid-activated currents were recorded in the same oocyte before and after 1 µM PMA or 1 µM PdBu addition to the bath solution for 5 min. Incubation of the oocyte with PMA decreased the peak pH4.0-activated current of WT hASIC1b (0.70 (SD 0.20); n = 29; p = 0.0008; two-tailed paired t-test of peak pH 4.0 currents before and after treatment). PdBu also inhibited the WT hASIC1b current (0.67 (SD 0.12); n = 6; p = 0.010; two-tailed paired t-test). Incubation of WT hASIC1b-injected oocytes in the bath solution (ND96 pH7.4) for 5 min without a treatment (n = 27), with DMSO (1:1000) (n = 10), or with the inactive PMA analog 4-α-
PMA (1 μM) (n = 12) had no effect on the acid-activated currents (Fig. 4A). The 95% CIs for the means of the ratio of peak pH4.0 current after treatment to peak pH4.0 current before treatment for the WT hASIC1b construct are [0.940, 1.09] for 5 min of no treatment, [0.852, 1.05] for DMSO, [0.856, 1.19] for 4-α-PMA, and [0.626, 0.783] for PMA, and [0.544, 0.792] for PdBu. A statistically significant effect of PMA or PdBu on hASIC1b currents was not observed in oocytes expressing S40A hASIC1b (0.87 (SD 0.13); n = 8; p = 0.0607, two-tailed paired t-test for the PMA treatment; 0.80 (SD 0.059); n = 4; p = 0.10, for the PdBu treatment) (Fig. 4B). The 95% CIs for the ratios of peak pH 4.0 current after treatment to the peak pH 4.0 current before treatment for the S40A construct are [0.799, 1.05] for 5 min of no treatment, [0.768, 0.984], for DMSO, [0.922, 1.00] for 4-α-PMA, [0.766, 0.978] for PMA, and [0.7, 0.89] for PdBu. The S499A hASIC1b construct responded to activation of endogenous oocyte PKC with PMA or PdBu in a similar manner to WT hASIC1b, decreasing by a similar amount (0.64 (SD 0.24); n = 8; p = 0.0333, two-tailed paired t-test for the PMA treatment, and 0.55 (SD 0.23); n = 3; p = 0.064, two-tailed paired t-test for the PdBu effect) (Fig. 4C). The 95% CIs for the mean ratios of peak pH4.0 current after treatment to peak pH4.0 current before treatment for the WT channel are [0.914, 1.09] for 5 min of no treatment, [0.645, 1.15] for DMSO, [0.844, 1.01] for 4-α-PMA, [0.461, 0.824] for PMA.

Because PKC can have cell-type specific effects (11), we repeated the experiment of activating PKC with PMA in CHO cells transfected with a WT hASIC1b-eGFP bicistronic vector. Acid-activated currents were recorded before and after superfusion of 100 nM PMA in the chamber. To normalize for differences in patch size and current density, the peak acid-activated currents were normalized to the first peak current for the
patch. Three peaks before PMA and three peaks 5-7 minutes after PMA were averaged to assay the effect of PMA on the acid induced currents. Treatment with 100 nM PMA reduced the peak acid-activated current of WT hASIC1b in outside-out patches to 0.605 (SD 0.0697), a decrease which was statistically significant compared to the normalized current values before PMA (Fig. 5). This is consistent with our findings in the TEV system with *Xenopus* oocytes.

We also determined changes in channel-gating kinetics upon PKC activation with PMA of the hASIC1b constructs expressed in *Xenopus* oocytes. We determined the peak half-width (defined as the time (ms) between the two points that are 50% of the peak current amplitude from the baseline), ½ activation time (or rise time (ms), defined as the time from 0% to the 50% of the peak amplitude), and ½ inactivation time (or decay time (ms), defined as the time from 100% to the 50% of the peak amplitude). A summary of these parameters for each construct (WT, S40A, and S499A) is shown in Table 2. The half-width and inactivation time of WT hASIC1b decreased after PMA treatment. PMA had no effect on the half-width, activation time, and inactivation time of the S40A acid-induced currents. The half-width of the S499A current was also decreased upon application of PMA.

We were able to prevent the inhibitory effect of PMA on the acid-induced currents of WT hASIC1b or S499A hASIC1b expressed in oocytes, by pretreating the oocytes in 1µM chelerythrine (Fig. 6). Chelerythrine is a potent and specific PKC antagonist that inhibits the catalytic domain of PKC and does not interfere with DAG, and therefore also with PMA binding. These data suggest that the effect of PMA on WT or S499A hASIC1b is mediated by PKC and is not an artifact of non-specific PMA
effects. This is also supported by the lack of effect of the inactive PMA analog 4-α-PMA (Fig.4A, 4B, 4C). PKC activation has an inhibitory effect on the functional but not total protein expression of hASIC1b, and this effect is mediated by site S40 of hASIC1b.

The PKC inhibitor chelerythrine decreases the acid-activated current of WT hASIC1b and S40A but not of S499A hASIC1b.

Because activation of PKC with PMA decreased the acid-activated current of hASIC1b, and the presence of site S40 was necessary to observe this effect, we inhibited PKC with chelerythrine and measured acid-activated currents in control oocytes, and chelerythrine-treated oocytes, which were injected with WT hASIC1b, S40A hASIC1b, or S499A hASIC1b. Chelerythrine was applied for 1h at 1μM in ND96 pH7.4. Control oocytes were incubated in ND96 pH7.4 with no drugs for 1h. The peak IpH4.0 were normalized to the average peak IpH4.0 of control untreated oocytes expressing the appropriate hASIC1b construct being studied. PKC inhibition with chelerythrine decreased the acid-activated currents of WT hASIC1b (0.54 (SD 0.36); n = 41; p<0.0001; two-tailed unpaired t-test) (Fig. 7A), and of S40A hASIC1b (0.56 (SD 0.38); n = 20; p = 0.00888) (Fig.7B). The 95% CIs for the normalized control WT hASIC1b peak pH 4.0 currents are [0.873, 1.13] for the control, and [0.431, 0.656] for the chelerythrine-treated WT hASIC1b, and [0.726, 1.27] and [0.394, 0.734] for the control and chelerythrine-treated S40A, respectively. Another PKC inhibitor, the PKC inhibitory peptide 19-31 (PKC-IP19-31), when injected in a 50 nl volume for an approximate final 6 μM concentration in the oocyte, also inhibited the acid-activated currents of WT hASIC1b-expressing oocytes (0.68 (SD (0.23); n = 18; p=0.011, two-tailed unpaired t-
test to control; 95% CI of PKC-IP 19-31 [0.561, 0.792]; 95% CI of control [0.786, 1.21]) (Fig. 7A). Control oocytes were injected with 50 nl vehicle (5 % acetic acid) only.

Chelerythrine- treated oocytes expressing S499A hASIC1b exhibited currents that were not different to the control oocytes (0.88 (SD 0.68); n = 17; p = 0.586) (Fig. 7C), with [0.693,1.31] and [0.531, 1.23] 95% CIs for the control S499A and chelerythrine-treated S499A respectively. Chelerythrine did not have a statistically significant effect on the S40A/S499A double mutant (Fig. 7D) (0.86 (SD 0.34); n = 27; p = 0.18, two-tailed t-test versus control; 95% CI of chelerythrine group [0.727, 0.995], 95% CI of control group [0.83, 1.17]). These data suggest that site S499 is mediating the inhibitory effect of PKC inhibition on the functional expression of hASIC1b. Some basal level of PKC activity seems to be necessary for the amplitude of acid-activated currents of WT hASIC1b. This could be due to a basal phosphorylation at site S499, because when this site is mutated to A, there is no inhibition in the amplitude of hASIC1b acid-activated currents, after chelerythrine treatment.

**Activation or inhibition of PKC has no effect on the total protein expression or on the surface expression of WT hASIC1b.**

Because PKC activation or inhibition could have an effect on total expression of hASIC1b or on hASIC1b expression at the plasma membrane, we detected total membrane levels and plasma membrane levels of hASIC1b. The total expression of hASIC1b was assessed by immunoblots with an ASIC1 antibody, to detect whole oocyte WT hASIC1b, S40A, and S499A protein expression levels from membrane preps of control oocytes and in oocytes treated with 1 μM PMA for 5 min as in the TEV
experiments (Fig. 8A). No change in total protein expression of hASIC1b was observed due to PKC activation with PMA. Protein expression analysis with a western blot of whole lysates of oocytes expressing WT hASIC1b, S40A, or S499A, under control conditions or PKC inhibition with chelerythrine, showed that treating oocytes with 1 µM chelerythrine for 1h did not affect the total level of hASIC1b protein expression (Fig. 8B).

Next we assessed the surface expression of WT hASIC1b with single oocyte chemiluminescence in control oocytes or in oocytes treated with 1µM PMA or 1 µM PdBu for 5 min. The luminescence of each oocyte was normalized to the average luminescence of control oocytes injected with WT HA-tagged hASIC1b (1.00 (SD 0.863); n=31; 95%CI [0.683, 1.32]) (Fig. 8C). The WT HA-hASIC1b construct exhibited whole cell peak pH4.0-activated currents that were significantly lower than those obtained in oocytes expressing the untagged WT hASIC1b; however, it responded to PMA treatment in the same way as the untagged WT hASIC1b (not shown). The luminescence from uninjected oocytes or oocytes injected with untagged WT hASIC1b was minimal (Fig.8C). The PKC activators PMA and PdBu did not have a significant effect on the surface expression of WT-HA hASIC1b (normalized luminescence value is 0.863 (SD 0.637); n=27; 95% CI [0.611, 1.12] for PMA, and 0.862 (SD 0.592); n=26; 95% CI [0.623, 1.10] for PdBu). The PKC inhibitor chelerythrine also had no effect on the surface expression of WT HA hASIC1b (1.07 (SD 0.841); n=27; 95% CI [0.738, 1.40]). In some TEV experiments we pretreated oocytes with chelerythrine to inhibit endogenous oocyte PKC and then tested the effect of PMA on I_{pH4.0}. The luminescence experiment shows that there is no effect of chelerythrine and PMA on the surface
expression of WT hASIC1b (0.793 (SD0.561); n=23; 95%CI [0.550, 1.04]) (Fig. 8C). Since there was an effect of these drugs on the WT hASIC1b current, but no effect on its surface expression, we did not test the effect of these treatments on the surface expression of mutant hASIC1b channels. We conclude that the decrease in the peak pH4.0-currents of WT hASIC1b upon activation or inhibition of PKC is not due to a change in the total protein expression or expression of the channel at the plasma membrane.
DISCUSSION

In this study, we test the hypothesis that the PKC consensus phosphorylation sites on human ASIC1b and PKC affect the functional expression of hASIC1b. We use molecular biological and electrophysiological techniques to better understand how hASIC1b is regulated by PKC. There are three intracellular PKC consensus phosphorylation sites on hASIC1b, and using site-directed mutagenesis, they were mutated to A to prevent phosphorylation and to E/D to mimic phosphorylation. The cRNAs encoding each phosphorylation mutant were expressed in *Xenopus* oocytes and acid-activated currents at pH 4.0 were measured by TEV. We have found that mutating site T26 either to A or to E results in non-functional channels, as we are not able to measure any acid-activated currents in oocytes expressing either T26A or T26E. A similar observation has been reported for the similar residue T92 in αENaC (20).

Mutation of T92 to A results in a significant reduction of α-ENaC $I_{Na^+}$ compared to $I_{Na^+}$ of WT α-ENaC expressed in oocytes, without affecting its surface expression (20). Two conserved residues involved in gating of ENaC (GH) are located at positions 94-95 in α-ENaC and 28-29 in hASIC1b (Fig.1), suggesting that non-conservative mutations of T26 might negatively affect the gating of the channel, because of the proximity of this amino acid to the GH region (20). We did not pursue the study of the T26A and T26E mutants further, because they do not express acid-activated currents.

Site S40 seems to mediate the inhibitory effect of PKC on hASIC1b. When S40 is mutated to A, an amino acid with a non-phosphorylatable CH$_3$ side chain, the peak $I_{pH4.0}$ is similar to that of WT; however, the S40E mutant, which mimics phosphorylation because the COO- group of E mimics the negative charge of an added phosphate (PO$_4^{3-}$),
expresses a reduced $I_{\text{pH}4.0}$ compared to WT. This result suggests that phosphorylation at S40 inhibits the functional expression of hASIC1b. In agreement with this idea, PKC activators (PMA or PdBu) inhibit the current of WT and of S499A hASIC1b, but they have no statistically significant effect on S40A hASIC1b, suggesting that the S40 site mediates inhibition of hASIC1b currents upon PKC activation. There is evidence for direct modulation of hASIC1b kinetics as well as for trafficking of related channels in response to PKC. Previous experiments in planar lipid bilayers showed that PKC addition to the intracellular side of hASIC1b decreased its open probability and had no effect on the channel conductance (6). We can also speculate from data on the channel gating kinetics before and after addition of PMA (Table 2) that the observed reduction in the magnitude of whole-cell pH4.0-activated currents could be due to changes in the gating of the channel. PMA does not affect the half width, activation time, or inactivation time of S40A hASIC1b pH4.0-activated current, but it decreases the half-width of WT and S499A, and the inactivation time of WT hASIC1b. Another possibility is that the PKC activators cause membrane retrieval of the channel, therefore decreasing its functional expression. This has been reported for other membrane proteins expressed in oocytes like the $\alpha\beta\gamma$ rENaC (1), type II Na$^+$-Pi cotransporters (16), the glutamate transporter EAAC1 (35), and the anion exchanger SLC26A6 (22). The effects of PMA on membrane retrieval could be specific to the membrane protein expressed in oocytes or general endocytosis of the plasma membrane (1). Because the mutation in the PKC consensus site S40 is able to prevent the inhibitory effect of PMA or PdBu on hASIC1b, this is probably a specific effect on hASIC1b, rather than general membrane endocytosis. Moreover, measurements of the amount of WT hASIC1b channel expressed at the plasma membrane by single
oocyte chemiluminescence show that there is no effect of PMA or PdBu on the surface expression of hASIC1b (Fig. 8C).

While the S40 site seems to mediate the effect of PMA on hASIC1b, the S499 site mediates the effect of the PKC inhibitor chelerythrine on hASIC1b. Pretreatment of oocytes with this PKC inhibitor decreases the peak $I_{pH4.0}$ of WT (Fig. 7), without decreasing its surface expression (Fig. 8C). This suggests that PKC activity could maintain a basal level of phosphorylation of the channel. Chelerythrine has the same effect on S40A hASIC1b, but no effect is observed on the S499A hASIC1b peak $I_{pH4.0}$. If there is basal phosphorylation of the S499 site and if this phosphorylation is necessary for either trafficking of the channel to the plasma membrane or for a certain level of channel conductance, then mutating this site to A would decrease the amplitude of the current observed by TEV. In agreement with this hypothesis, the S499A and S40A/S499A mutants exhibit a reduced peak $I_{pH4.0}$ compared to the WT. One would then expect the phosphorylation mimic S499D to have a peak $I_{pH4.0}$ similar to the WT channel. However, S499D hASIC1b also exhibits a slightly but statistically significant reduced current compared to WT. A discrepancy between phosphorylation of an amino acid and its substitution with a phosphorylation mimic has been reported previously for rat ASIC2a. PKC phosphorylation at site T39 on rat ASIC2a stimulated the peak current, but the phosphorylation mimic mutation T39D did not significantly increase it as expected (3).

If there is basal phosphorylation at S499, then in control untreated oocytes, basal PKC activity would allow for phosphorylation at S499 in the WT and S40A hASIC1b. In chelerythrine-treated oocytes, PKC is inhibited and unable to phosphorylate the S499 site
located on WT or on S40A hASIC1b. The observed decrease in the peak pH4.0 currents of WT and S40A upon treatment with a PKC inhibitor is consistent with this idea. Additionally, PKC inhibition with chelerythrine does not affect the S499A hASIC1b. These data are somewhat at odds with Leonard et al. who found little phosphorylation by PKC in a hASIC1b C-terminus peptide containing aa 459-528 (30), but in this study the focus was phosphorylation by PKA, and there was no positive control showing in vitro phosphorylation by PKC.

Although further experiments determining the phosphorylation status of hASIC1b at each site are needed to support these interpretations, this work shows that the S40 and S499 sites mediate the effect of PKC activation and of PKC inhibition on hASIC1b, and that the effects of chelerythrine and PMA on the hASIC1b current are not due to a decreased surface expression of the channel. This modulation of hASIC1b by PKC may be relevant for physiological and pathophysiological situations involving ASIC1 function.
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REFERENCES


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<td>ELSMVKIPSKASAKYLAKKFNKSEQYIGENILVLDIFFEVLNYETIEQKKAYEIAGLLG............</td>
</tr>
<tr>
<td>hASIC1a</td>
<td>305</td>
<td>ELSMVKIPSKASAKYLAKKFNKSEQYIGENILVLDIFFEVLNYETIEQKKAYEIAGLLG............</td>
</tr>
<tr>
<td>hASIC1b</td>
<td>307</td>
<td>HGHGVAPYHPKAGCSLLSHEGPPPQRPFPKPCCLGDIGGQMGLFIGASILTVLELFDYAYEVIKHRLCRR</td>
</tr>
<tr>
<td>hASIC1a</td>
<td>307</td>
<td>HGHGVAPYHPKAGCSLLSHEGPPPQRPFPKPCCLGDIGGQMGLFIGASILTVLELFDYAYEVIKHRLCRR</td>
</tr>
<tr>
<td>hASIC1b</td>
<td>336</td>
<td>GKCQKEAKRSSADKYVALSISDLQHRNPQCSLQHPAMTAAYLN1PHHPARGTFEDFTC 559</td>
</tr>
<tr>
<td>hASIC1a</td>
<td>336</td>
<td>GKCQKEAKRSSADKYVALSISDLQHRNPQCSLQHPAMTAAYLN1PHHPARGTFEDFTC 559</td>
</tr>
<tr>
<td>hASIC1b</td>
<td>347</td>
<td>GKCQKEAKRSSADKYVALSISDLQHRNPQCSLQHPAMTAAYLN1PHHPARGTFEDFTC 526</td>
</tr>
<tr>
<td>hASIC1a</td>
<td>347</td>
<td>GKCQKEAKRSSADKYVALSISDLQHRNPQCSLQHPAMTAAYLN1PHHPARGTFEDFTC 526</td>
</tr>
</tbody>
</table>

Figure 1. Alignment of Acid Sensing Ion Channel 1 (ASIC1) isoforms from different species. Sequence alignment of ASIC1 isoforms from human (hASIC1a, NM_020039;
hASIC1b, NM_001095), mouse (mASIC1a, NM_009597; mASIC1b, AB208022), and rat (rASIC1a, NM_024154; rASIC1b, AJ309926) was done with Clustal IW, and shading was done with the Boxshade program (http://www.ch.embnet.org/). Identical amino acids are shown as white letters on black background, and conserved amino acids are shown as black letters on gray background. The transmembrane domains’ position was obtained from Jasti et al. (26) and is indicated by a black bar. The amino acid sequence of hASIC1b was analyzed with GCG (UAB) and Scansite (MIT) software for consensus PKC phosphorylation sites. The three consensus PKC phosphorylation sites on hASIC1b are indicated by stars and they are T26, S40 (on the cytoplasmic N-terminus) and S499 (on the cytoplasmic C-terminus).
Figure 2. Mutations in PKC consensus phosphorylation sites on hASIC1b affect its...
function in Xenopus oocytes. 11.5 ng of cRNA for wild type (WT) hASIC1b or hASIC1b PKC phosphorylation mutants was expressed in Xenopus oocytes. Acid-activated currents at pH 4.0 were measured by two-electrode voltage clamp at 1-4 days post-injection as described in MATERIALS AND METHODS. (A) Representative traces of acid-activated currents of wild type hASIC1b and each hASIC1b mutant. (B) The peak pH 4.0 current for each phosphorylation mutant was normalized to the average peak pH 4.0 current recorded on the same day in oocytes of the same batch expressing wild type hASIC1b. This is the summary of 3-6 experiments and values are means + SD. The number of individual oocytes recorded is shown in parentheses on top of each bar. p values were determined with a one-way ANOVA with Tukey’s post-hoc test. *p< 0.01 †p< 0.001 versus WT. NC = no acid-activated current. (C) pH activation curves for WT hASIC1b and the hASIC1b phosphorylation mutants. Peak acid-activated currents were recorded by switching the extracellular solution to pH 7.0, 6.5, 6.0, 5.5, 5, and 4.0 sequentially as described in MATERIALS AND METHODS and were normalized to the peak pH4.0 current. The normalized values were fitted to the Hill equation to obtain the pH 50s and Hill coefficients. (n = 13 oocytes for WT, n = 10 for S40A, n = 11 for S499A, n = 8 for S40A/S499A, n = 8 for S40E, n = 8 for S499D, n = 5 for S40E/S499A, n = 5 for S40E/S499D) The T26A (n = 8) and T26E (n = 8) constructs did not exhibit any acid-activated current at any pH (data not shown).
Figure 3. hASIC1b expression in Xenopus oocytes. Oocytes were injected with 11.5 ng RNA for WT hASIC1b or each hASIC1b mutant, and total membranes were isolated as described in MATERIALS AND METHODS. 30 μg protein were resolved by SDS-PAGE, transferred to a PVDF membrane, and immunoblotted with an antibody to ASIC1 (1:200) or to actin (1:5000) as a loading control. Blot is representative of 2-4 experiments comparing WT hASIC1b expression to that of the hASIC1b phosphorylation mutants.
A  Wild Type

B  S40A

C  S499A

Before PMA  After 5 min PMA

pH 4.0

4 µA  5s

No treatment DMSO 4-α-PMA PMA PdBu

p=0.37 (27)  p=0.35 (10)  p=0.63 (12)  p=0.0008 (29)  p=0.010 (6)

p=0.27 (11)  p=0.14 (8)  p=0.24 (7)  p=0.061 (8)  p=0.10 (4)

p=0.84 (8)  p=0.37 (8)  p=0.058 (6)  p=0.033 (8)  p=0.064 (3)

Before PMA  After 5 min PMA

pH 4.0

0.5 µA  5s
Figure 4. The PKC activators phorbol myristate acetate (PMA) and phorbol dibutyrate (PdBu) reduce the acid-activated current of WT hASIC1b and S499A hASIC1b, but not that of S40A hASIC1b. Oocytes were injected with RNA for WT hASIC1b (A), S40A (B), or S499A (C). Acid-activated currents were measured by two-electrode voltage clamp before and after addition of 1 μM PMA or 1 μM PdBu to the bath for 5 minutes, to activate endogenous oocyte PKC. The negative controls used were either no treatment, vehicle (DMSO 1:1000), or an inactive PMA analog (4-α-PMA, 1 μM) added to the bath for five minutes between current measurements. The bar graph values are fractions of the peak pH4.0 current recorded after the treatment over the peak pH4.0 current recorded before the treatment. Representative acid-activated current traces before (black line) and after (grey line) 1 μM PMA was added to the bath are also shown to the right of each graph. The numbers in parentheses on top of each bar graph indicate the number of oocytes. p values were determined by a two-tailed paired Student’s t-test comparing the sets of peak pH 4.0 values before and after each corresponding treatment. Data are means ± SD.
Figure 5. PMA inhibits the peak acid-induced current of hASIC1b in transfected CHO-K1 cells. (A) In outside out patches of CHO-K1 cells transfected with a bicistronic plasmid encoding WT hASIC1b and eGFP, acid induced currents were observed. Treatment with 100 nM PMA significantly reduced the current. (B) For analysis, the currents were normalized to address expression variability and patch size. Data are shown as the average normalized current for three pulses prior to PMA application and three pulses five minutes post PMA application; n=3 cells and analyzed with a paired Student’s t-test.
Figure 6. The PKC inhibitor chelerythrine abolishes the effect of PMA on WT and S499A hASIC1b. (A) Oocytes expressing WT hASIC1b were pretreated with the PKC inhibitor chelerythrine (1 μM) for 1h, and peak pH4.0 currents were measured before and after 5 min of 1 μM PMA, 1 μM 4-α-PMA, DMSO (1:1000), or no treatment. Representative pH 4.0-activated currents recorded by TEV before (black line) and after addition of 1 μM PMA to the bath for 5 min (grey line), in oocytes expressing WT hASIC1b, pre-treated with 1 μM chelerythrine for 1 h. (B) Oocytes expressing S499A hASIC1b were pretreated with 1 μM chelerythrine overnight. Peak pH 4.0 activated currents were measured before and after addition of 1 μM PMA to the bath. The peak pH 4.0 current recorded after the 5 min of treatment was normalized to the peak.
pH 4.0 current recorded before treatment on the same oocyte. The effect of PMA on control untreated oocytes is also shown. Bar graphs represent mean normalized values + SD. The p values shown on top of each bar were determined with a two-tailed paired Student’s t-test on sets of before and after peak pH 4.0 current values for each treatment. The number of oocytes is shown in parentheses.
Figure 7. Effect of PKC inhibitors on the acid-activated currents of WT, S40A, S499A, or S40A/S499A hASIC1b. Oocytes were injected with 11.5 ng RNA for hASIC1b wt, S40A, S499A, or S40A/S499A. Two to three days post-injection they were pretreated with 1 μM chelerythrine for 1h (A-D), or injected with the PKC inhibitory peptide 19-31 (PKC IP 19-31) for a 6 μM final concentration for 1h (A). pH 4.0-activated hASIC1b currents were recorded by TEV. The bar graph values are average peak pH 4.0 currents from chelerythrine-treated oocytes or oocytes injected with the PKC IP 19-31 normalized to the average peak pH 4.0 currents from untreated oocytes or oocytes injected with vehicle for PKC IP 19-31 (5% acetic acid). Data are summaries of 3-6 experiments and values are means of individual oocytes + SD. The number of
oocytes is shown in parentheses. The p values were determined using a two-tailed unpaired Student’s t-test versus control.
Figure 8. PMA and chelerythrine have no effect on the total expression or surface
expression of hASIC1b. (A) and (B) Immunoblots showing hASIC1b expression with and without PMA (A) or chelerythrine (B) treatment in total membranes prepared as described in MATERIALS AND METHODS 3 days post-injection of oocytes with RNA for wt, S40A, or S499A hASIC1b. U indicates uninjected oocytes. Equivalent amounts of protein (30 μg) were loaded on each lane, and separated by SDS-PAGE. The proteins were transferred to PVDF membranes, which were blotted for hASIC1 (1:200) and actin (1:5000) as a loading control. as shown by the actin immunoblot; these experiments were repeated 2-4 times. (C) Mean normalized luminescence of oocytes expressing WT-HA hASIC1b. There is no statistically significant difference between WT hASIC1b surface expression in control or treated oocytes (p=0.628, one-way ANOVA). This is a summary of 3 experiments. The numbers in parentheses on top of each bar indicate the number of individual oocytes measured.
Table 1  pH values for half-maximal activation and Hill numbers for wild type hASIC1b and hASIC1b phosphorylation mutants.

<table>
<thead>
<tr>
<th></th>
<th>pH&lt;sub&gt;50&lt;/sub&gt; (SD)</th>
<th>Hill Coefficient (SD)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>6.17 (0.186)</td>
<td>1.78 (0.405)</td>
<td>13</td>
</tr>
<tr>
<td>S40A</td>
<td>6.49 (1.17)</td>
<td>1.83 (0.704)</td>
<td>10</td>
</tr>
<tr>
<td>S499A</td>
<td>6.11 (0.141)</td>
<td>2.03 (0.736)</td>
<td>11</td>
</tr>
<tr>
<td>S40A/S499A</td>
<td>6.11 (0.0542)</td>
<td>2.77 (0.595) *</td>
<td>8</td>
</tr>
<tr>
<td>S40E</td>
<td>6.18 (0.0292)</td>
<td>3.21 (0.717) **†</td>
<td>8</td>
</tr>
<tr>
<td>S499D</td>
<td>6.17 (0.0732)</td>
<td>2.39 (0.633)</td>
<td>8</td>
</tr>
<tr>
<td>S40E/S499D</td>
<td>6.26 (0.0247)</td>
<td>4.30 (0.435) **†‡ *†</td>
<td>5</td>
</tr>
<tr>
<td>S40E/S499D</td>
<td>6.24 (0.0917)</td>
<td>3.37 (0.498) **†</td>
<td>5</td>
</tr>
</tbody>
</table>

The data are means and standard deviations of n individual oocytes. The pH<sub>50</sub> values and Hill coefficients were obtained by fitting the normalized pH response values to the Hill equation on GraphPad Prism 3 for each individual oocyte. The pH<sub>50</sub> values are not statistically different (p = 0.71, one-way ANOVA). The Hill coefficients were statistically analyzed with a one-way ANOVA followed by a Tukey’s post-hoc test: *, p < 0.05 versus WT and S40A; **, p < 0.01 versus S499A; †, p < 0.001 versus WT, and S40A; ‡, p < 0.001 versus S499A, and S499D; *†, p< 0.01.
Table 2. Properties of the pH4.0-induced currents of hASIC1b before and after PMA

<table>
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<th>No treatment</th>
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<th>4-α-PMA</th>
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<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Half-width (ms)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>711 (SD 326)</td>
<td>681 (SD 289)</td>
<td>2013 (SD 903)</td>
<td>1793 (SD 970)</td>
</tr>
<tr>
<td>n</td>
<td>22</td>
<td></td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>0.449</td>
<td></td>
<td>0.0879</td>
<td></td>
</tr>
<tr>
<td>S40A</td>
<td>795 (SD 337)</td>
<td>719 (SD 232)</td>
<td>870 (SD 153)</td>
<td>906 (SD 209)</td>
</tr>
<tr>
<td>n</td>
<td>9</td>
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<td>7</td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>0.133</td>
<td></td>
<td>0.632</td>
<td></td>
</tr>
<tr>
<td>S499A</td>
<td>673 (SD 157)</td>
<td>677 (SD 195)</td>
<td>750 (SD 294)</td>
<td>689 (SD 222)</td>
</tr>
<tr>
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<td>7</td>
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<tr>
<td>p</td>
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<td>τ1/2activation (ms)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>185 (SD 87)</td>
<td>188 (SD 66)</td>
<td>163 (SD 46)</td>
<td>155 (SD 40)</td>
</tr>
<tr>
<td>n</td>
<td>21</td>
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<td>8</td>
<td></td>
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<tr>
<td>p</td>
<td>0.435</td>
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<tr>
<td>S40A</td>
<td>149 (SD 101)</td>
<td>163 (SD 66)</td>
<td>146 (SD 43)</td>
<td>155 (SD 40)</td>
</tr>
<tr>
<td>n</td>
<td>9</td>
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<td>5</td>
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<tr>
<td>p</td>
<td>0.76</td>
<td></td>
<td>0.727</td>
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</tr>
<tr>
<td>S499A</td>
<td>108 (SD 45)</td>
<td>128 (SD 67)</td>
<td>185 (SD 108)</td>
<td>153 (SD 78)</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td></td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>0.217</td>
<td></td>
<td>0.55</td>
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</tr>
<tr>
<td>τ1/2inactivation (ms)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>387 (SD 167)</td>
<td>364 (SD 129)</td>
<td>1426 (SD 671)</td>
<td>1264 (SD 636)</td>
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<td>p</td>
<td>0.392</td>
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<td>0.167</td>
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</tr>
<tr>
<td>S40A</td>
<td>500 (SD 200)</td>
<td>474 (SD 160)</td>
<td>625 (SD 112)</td>
<td>668 (SD 196)</td>
</tr>
<tr>
<td>n</td>
<td>8</td>
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<tr>
<td>p</td>
<td>0.242</td>
<td></td>
<td>0.551</td>
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<tr>
<td>S499A</td>
<td>496 (SD 143)</td>
<td>486 (SD 195)</td>
<td>507 (SD 188)</td>
<td>483 (SD 158)</td>
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<tr>
<td>n</td>
<td>8</td>
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<tr>
<td>p</td>
<td>0.839</td>
<td></td>
<td>0.377</td>
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Values are means ± standard deviations; Half width – time between the two points that are 50% of the peak current amplitude from the baseline; τ1/2activation – time from 0% to 50% of the peak amplitude during activation; τ1/2inactivation – time from 100% to 50% of the peak amplitude during inactivation; PMA, phorbol-12-myristate-13-acetate. Bold font numbers emphasize statistically significant p-values obtained with two-tailed paired Student’s t-tests between the before and after pairs for each construct each condition.
PROTEOLYTIC CLEAVAGE OF THE HUMAN ACID-SENSING ION CHANNEL 1
BY THE SERINE PROTEASE MATRIPTASE

by

Edlira B. Clark, Biljana Jovov, Arun K. Rooj, Catherine M. Fuller, Dale J. Benos

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Format adapted for dissertation
ABSTRACT

Acid-Sensing Ion Channel 1 (ASIC1) is a H⁺-gated channel of the amiloride-sensitive Epithelial Na⁺ Channel/ Degenerin (ENaC/Deg) family. ASIC1 is mostly expressed in the central and peripheral nervous system neurons. ENaC and ASIC function is regulated by several serine proteases. The type II transmembrane serine protease matriptase activates the prototypical αβγENaC channel, but we have found that matriptase is expressed in glioma cells, and its expression is higher in glioma compared to normal astrocytes. Therefore, the goal of this study was to test the hypothesis that matriptase regulates ASIC1 function. Matriptase decreased the acid-activated ASIC1 current as measured by two-electrode voltage clamp in Xenopus oocytes, and cleaved ASIC1 expressed in oocytes or CHO K1 cells. Inactive S805A matriptase had no effect on either the current or the cleavage of ASIC1. The effect of matriptase on ASIC1 was specific, because it did not affect the function of ASIC2, and no matriptase-specific ASIC2 fragments were detected in oocytes or in CHO cells. Three matriptase recognition sites were identified in ASIC1 (R145, K185, and K384). Site-directed mutagenesis of these sites prevented matriptase cleavage of ASIC1. Our results show that matriptase is expressed in glioma cells, and that matriptase specifically cleaves ASIC1 in heterologous expression systems.
INTRODUCTION

Acid Sensing Ion Channels (ASICs) are H⁺-gated members of the Epithelial Na⁺ Channel/Degenerin (ENaC/Deg) family of amiloride-sensitive ion channels (1). So far, four ASIC genes have been cloned, ASIC1-ASIC4 (2). Human ASIC1 exists as two isoforms, ASIC1a and ASIC1b, with the hASIC1a containing an extra 43 amino acids in the region just before the start of the second transmembrane domain (3). The recent crystallization of chicken ASIC1 has revealed that ASIC1 is likely to be a trimer. Each ASIC1 subunit consists of short intracellular N- and C- termini, two transmembrane domains, and a large extracellular loop, which in ASICs, contains the pH sensing region (4). ASICs are mainly expressed in the neurons of the central and peripheral nervous systems, where they have been implicated in physiological functions such as learning, memory, and pathologies such as neurodegenerative diseases and ischemia (1). ASIC1 is also expressed in glioblastoma multiforme (GBM) cells, highly invasive and proliferative primary brain tumors, where it plays an important role in cell migration and proliferation (5-8).

Several serine proteases can modulate endogenous ENaC currents and ENaC/Deg subunits in in vitro expression systems (9). The large extracellular loops of ASICs and ENaCs contain many arginines and lysines which form putative sites for cleavage by serine proteases. For example, proteolytic cleavage of αENaC and γENaC subunits by furin convertases occurs in the trans-Golgi apparatus during ENaC maturation. These channels may be clipped further by secreted serine proteases (e.g., trypsin), or membrane-bound proteases (e.g., prostasin). Non-cleaved αβγENaC channels also traffic to the plasma membrane, where they provide a pool of “near-silent” channels, ready to be
cleaved into active ones (9-13). The exact mechanism of how cleavage activates ENaC is incompletely understood, but one hypothesis is that cleavage removes inhibitory peptides from αENaC and γENaC (12). An alternative mechanism of ENaC activation by cleavage has been proposed by Hu et. al, involving loss of the αENaC N-terminus (including the first transmembrane domain) from the channel complex (14). Cleavage by proteases does not change the number of ENaC channels at the surface, but rather increases channel open probability, and is a mechanism for regulating ENaC activity (13). The sites for cleavage of ENaCs by many proteases such as prostasin, furin, and trypsin have been identified and are located in the N terminal part of the extracellular loop (12).

ASIC1 homomers or ASIC1-containing heteromeric ASICs can also be modified by serine proteases such as trypsin, chymotrypsin, and proteinase K. Protease treatment disrupted the \textit{P. cambridgei} venom block of heterologously expressed ASIC1 channels, decreased the peak acid-activated current, and shifted the pH$_{50}$ of activation to a more acidic pH (15,16). Because ASICs inactivate quickly upon exposure to a low pH, this shift could be important in extracellular acidosis, where a sustained decrease in extracellular pH could inactivate ASICs. The only known site of ASIC cleavage by proteases that has been identified is the trypsin cleavage site at R145 (16).

Recently, the serine protease matriptase (also known as channel activating protease 3 or CAP3) has been identified as modulator of ENaC activity in vitro. When matriptase was co-expressed with αβγENaC subunits in \textit{Xenopus laevis} oocytes it caused a 10-fold increase in I$_{Na}$ (17,18). However, this functional effect of matriptase on ENaC was not correlated with the presence of ENaC proteolytic cleavage fragments.
Matriptase is an 80-90 kDa type II transmembrane protease that belongs to the S1 family of trypsin-like serine proteases. It contains an extracellular C-terminal catalytic domain and an intracellular N-terminus. It was first described as a gelatinolytic activity in cultured breast cancer cells and was isolated in a complex with its cognate inhibitor, hepatocyte growth factor activator inhibitor-1 (HAI-1) from human milk (19). Matriptase is synthesized as an inactive, single-chain zymogen. Its activation is complex; it is first cleaved in the secretory pathway by an unknown protease at Gly149, and later at R614 in the serine protease domain, after it reaches the surface. This last cleavage converts matriptase into the active protease. Mutations in any of the catalytic triad (H656, D711, S805) amino acids render matriptase inactive, by making it unable to undergo the activation site cleavage at R614 (20,21).

Matriptase is an epithelial protease and has important physiological functions. It is crucial for epidermal barrier formation and is involved in hair follicle growth and thymocyte development. In addition, matriptase has been implicated in many epithelial cancers. It is consistently expressed in human epithelial tumors of the head, neck, mesothelium, breast, ovary, cervix, prostate, lungs, and gastrointestinal tract, and its overexpression in keratinocytes results in spontaneous squamous cell carcinomas. In most tumors matriptase RNA and protein are upregulated, and there is a positive correlation between matriptase expression and tumor grade (20,22). Interestingly, matriptase inhibition in vitro or in xenografted tumors with siRNAs or antisense oligodeoxyribonucleotides decreased invasion (20,22,23). Additionally, overexpression of the serine protease inhibitor HAI-1 suppressed the in vitro invasion of glioblastoma
cells (24). However, the target of HAI-1 in these assays is not known, because HAI-1 inhibits several other serine proteases in addition to matriptase.

The goal of this study was to test the hypothesis that matriptase can modulate the activity of ASIC1 channels through proteolytic cleavage. Matriptase cleaved ASIC1 when the two were co-expressed in *Xenopus laevis* oocytes or Chinese hamster ovary (CHO) cells. Matriptase decreased ASIC1 function, but had no effect on the function of ASIC2. The effects of matriptase on the function and proteolytic cleavage of ASIC1 could be prevented by mutagenesis of three matriptase recognition sites on the ASIC1 extracellular loop.
EXPERIMENTAL PROCEDURES

RNA extraction and reverse transcription-PCR

Total RNAs were isolated from freshly-excised human tissues (obtained from Birmingham Neurosurgery Brain Tissue Bank under Institutional Review Board approval) using TRIzol (Invitrogen), following the manufacturer’s instructions as described previously (5). Total RNAs from human primary cells or human cell lines were isolated with the RNeasy RNA extraction kit (Qiagen) as specified by the manufacturer. The integrity and quality of the isolated RNAs were checked with denaturing agarose-formaldehyde gel electrophoresis. The RT-PCR reaction was done using a One-Step RT-PCR kit (Qiagen) with 500ng RNA and 0.6 µM of each primer. The matriptase forward and reverse primer sequences were 5’-CACAAGGAGTCGGCTGTGAC-3’ (forward) and 5’-GAGGGTAGGTGCCACACAA-3’ (reverse). Standard RT-PCR conditions were used: 50°C, 30 min × 1 cycle; 95°C, 15 min × 1 cycle; 94°C, 1 min, 54°C, 1 min, 72°C, 1 min × 35 cycles; 72°C, 10 min ×1 cycle. The RT-PCR product was visualized by electrophoresis in a 2% agarose gel. A negative control with no RNA in the RT-PCR reaction was included with each experiment to guard against contamination.

Cell culture and transfections

Primary non-tumor human astrocytes isolated from astrogliosis regions, and primary human GBMs were obtained from the University of Alabama at Birmingham, Neurosurgery Brain Tissue Bank (Institutional Review Board approval X050415007). The cell lines used (U87MG, D54MG, SKMG, and U251 MG, CHO K1) have been
described previously (7,8,25). All cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 (1:1) (Hyclone) with 10% fetal bovine serum (Hyclone), and were maintained in a 95% O₂, 5% CO₂ humidified incubator at 37°C.

For transfections, Chinese hamster ovary (CHO) K1 cells were split into six-well tissue culture dishes and were transiently transfected with 2 µg of each plasmid DNA and 10 µL Lipofectamine 2000 reagent (Invitrogen) per well following the manufacturer’s protocol. Whole-cell lysates were obtained 48h post-transfection as described below.

Plasmids, tagged constructs, and site-directed mutagenesis

The plasmids used contain the DNA sequence of human ASIC1b (NM_001095), human ASIC2b (NM_001094), and human matriptase (NM_021978). N- or C- terminal fusions of eGFP on hASIC1b and hASIC2b were prepared as described previously (26). The channel sequences were modified with PCR by the addition of 5’ XhoI and 3’ BamHI restriction sites using *Pfu* Polymerase (Stratagene). The constructs were then subcloned into the pEGFP -N1 or pEGFP-C1 vectors (Clontech). The DNA sequences coding for the eGFP fusion channel proteins were subcloned using NheI and AflII for the C-terminal tags and using NheI and BamHI for the N-terminal tag, into the pcDNA 3.1+ vectors (Invitrogen). The ASIC1-HA construct with hemagglutinin (HA) in the extracellular loop between F147 and K148 and the ASIC2-eYFP construct have been described previously (25,26). For the ASIC2-HA construct, a new BstBI site was added in the extracellular loop of ASIC2 with PCR, and an HA tag was inserted into the BsTB1 site at T239.
Site-directed mutagenesis to introduce point mutations in the matriptase sites on ASIC1 was carried out as described previously using the Quickchange II XL Mutagenesis kit (Stratagene), sense and antisense primers, and ASIC1-EGFP-N1 in pcDNA3.1+ as template DNA (25). The primers contained the necessary base changes to mutate the desired arginines (R) or lysines (K) to alanines (A).

The catalytically inactive S805A-matriptase-pcDNA3.1+ was generated using the Quickchange II XL Site-directed mutagenesis kit (Stratagene) and primers containing the appropriate base pairs (18). All the generated mutations were confirmed by DNA sequencing (Heflin Genetics Center, UAB).

Expression in Xenopus oocytes

Oocytes were isolated surgically from anesthetized female *Xenopus laevis* frogs and were collagenase-treated, sorted, and maintained as described previously (25). These procedures are in accordance with and were approved by the Institutional Animal Care and Use Committee of UAB. cRNA preparations and injections into oocytes have also been previously described (25). cRNAs were transcribed using the High Yield In Vitro Transcription kit and CAP analog (Ambion). Each oocyte was injected with 12ng of channel cRNA in 50 nl H2O with matriptase or with S805A-matriptase cRNAs as indicated in the experiments.

Electrophysiological analysis

Two-electrode voltage clamp at a holding potential of -60mV was performed at room temperature 1-4 days post-injection as described previously (25). Oocytes were
placed in a recording chamber (500 µl) and impaled with two glass microelectrodes filled with 3M KCl, with resistances 0.5-2MΩ. Whole-cell currents were recorded with a Geneclamp 500B amplifier with a steady-state restore switch modification (Axon Instruments). A SF-77B Perfusion Faststep (Warner Instruments) was used to rapidly exchange the solution bathing the oocyte from ND96 pH7.4 (96 mM NaCl, 1mM MgCl₂, 2 mM CaCl₂, 2 mM KCl, and 5 mM HEPES) to ND96 pH4.0 (same as ND96 ph7.4, but with 5mM MES instead of the HEPES). The oocyte was exposed to pH7.4 for 13s, pH4.0 for 13s to maximally activate the channels, and again to pH7.4 for 13s to allow for recovery of the acid-activated channels. To obtain the pH activation curves, oocytes were exposed to sequentially lower activation pHs (pH7.0, 6.5, 6.0, 5.5, 5.0 and 4.0). The peak current at each activation pH was normalized to the peak pH4.0 current within each oocyte. The normalized values were fit to the Hill equation, \( I = \frac{I_{max}}{[1+(10^{PH50}/10^{-\text{pH}})^n_H]} \), where \( I_{max} \) is the maximal current (current at pH4.0), \( \text{pH50} \) is the pH at which half the maximal current is obtained, and \( n_H \) is the Hill coefficient. \( \text{pH50} \)'s and Hill coefficients were obtained separately for each oocyte by means of a brute-force algorithm. Predicted values for the normalized currents across the range of experimental pH values were obtained by means of the Hill equation and the sum-squared error between predicted and observed currents for each combination was computed. The Hill coefficient and \( \text{pH50} \) combination that minimized the sum-squared error was then selected.
Reagents

Recombinant human matriptase/ST14 catalytic domain was obtained from R&D Systems, Inc. and contained an N-terminal sequence corresponding to the beginning of the catalytic domain with V615. Purified and folded psalmotoxin (PcTX-1) peptide was obtained from Peptides International in the trifluoroacetate form and with formed disulfide bonds. The cell-permeable proteasome inhibitor MG132 was from Calbiochem.

Whole-cell lysates and immunoblot analysis

Mammalian cell lines growing on tissue-culture dishes were washed twice with ice-cold phosphate-buffered saline (PBS) and incubated in lysis buffer (150 mM NaCl, 5mM EDTA, 50 mM Tris pH7.5, 1% Triton-X-100, Complete® protease inhibitor cocktail (Roche)) at 4°C for at least 30 min. The cells were scraped off the dishes; the lysate was transferred to a microcentrifuge tube, and was passed through a 21 gauge needle 5-7 times. After centrifugation at 12,000 rpm for 20 min at 4°C, the supernatants were saved, and 30-50 µg of lysate were used for Western blot. All experiments were repeated at least three times.

Oocyte whole-cell lysates were obtained by lysing oocytes 2-3 days post-injection in 20 µL of Triton homogenization buffer per oocyte as described previously (25). After protein assay, equal amounts of protein (50 µg) were used for Western blot. All experiments were repeated at least three times.

The lysates were boiled at 95°C for 7 min in 1X Laemmli sample buffer (62.5 mM Tris HCl (pH6.8), 25% glycerol,2% SDS, 10% β-mercaptoethanol, and 0.01% bromophenol blue) and subjected to 8% (for matriptase blots only) or 12% SDS-PAGE
with 4% stacking gels. The separated proteins were transferred to Immobilon-P transfer polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were blocked with 10% nonfat dry milk in Tris buffered saline with Tween (TBS-T) (100 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween-20) for 30 min to 1h at RT or overnight at 4°C and probed with the appropriate antibodies in 3% milk in TBS-T overnight at 4°C or 2h at RT. The following antibodies were used: mouse anti-matriptase/ST14 monoclonal antibody (R&D Systems) at 1:500, mouse anti-GFP monoclonal antibody (Abgent) at 1:2000; and rat anti-HA antibody (Roche) at 1:2000. The blots were washed with TBS-T and incubated in goat anti-mouse horseradish peroxidase (HRP)-conjugated antibody (Jackson Immuno-Research Laboratories, 1:10,000) or in goat anti-rat HRP-conjugated antibody (Thermo Scientific, 1:10,000) as appropriate in 5% milk in TBS-T. The blots were developed in SuperSignal West Pico Substrate (Thermo Scientific) and exposed to X-ray film (Denville).

Data analysis and statistics

Data analysis was done with Clampfit 9 (Molecular Devices) or Excel. Data are presented as mean ± SD or mean ± SE as noted. Statistical significance was set at p<0.05 and statistical tests were done with Excel or GraphPad Prism 5. Two-tailed t-tests (unpaired or paired) or comparisons of multiple groups with post-hoc tests were chosen as appropriate to determine the p values.
RESULTS

Detection of matriptase RNA and protein in glioma cells

Matriptase expression has been reported in numerous epithelial tissues, both normal and cancerous (20). However, in studies where matriptase localization was determined by Northern blot analysis of rodent tissues, matriptase RNA was not detected in the normal brain (17,27). ENaC/Deg subunits are candidate substrates for matriptase, because matriptase increased the amiloride-sensitive current of αβγENaC, when the two were co-expressed in Xenopus oocytes (18). Because we have previously shown that glioma cells express ENaC/Deg subunits, and because matriptase is involved in malignant progression, we looked for matriptase expression in high-grade glioma cells. RT-PCR results showed that matriptase mRNA was expressed in two glioma cell lines (U251, SKMG), in three different freshly-excised GBMs, and two different freshly-resected grade III anaplastic astrocytomas. Matriptase RNA was not detected either in primary human astrocytes (from astrogliosis regions), or in astrogliosis tissue (Fig. 1A). Matriptase protein expression in glioma was confirmed by western blot of glioma cell lines and human primary GBM lysates. Matriptase was detected at approximately 75kDa. Even though we could not detect matriptase mRNA in the primary human normal astrocytes, we could detect matriptase protein there (Fig.1B); therefore, we cannot rule out that very low levels of matriptase mRNA that are undetected with standard RT-PCR, are expressed in normal astrocytes. Western blot data show that matriptase protein expression was lower in the normal astrocytes compared to the primary human GBMs or glioma cell lines (Fig. 1B).
Matriptase inhibits the function of ASIC1 and has no effect on the function of ASIC2 in Xenopus oocytes.

In order to test the hypothesis that matriptase regulates ASIC1, we assessed ASIC1 function by measuring the whole-cell peak pH4.0-activated current of oocytes expressing ASIC1 +/- matriptase. Unlike its effect on αβγENaC, matriptase decreased the peak pH4.0 current of ASIC1. A similar effect of proteases on ASIC1 has been reported for trypsin (15). A catalytically inactive matriptase, with a mutation in one of the catalytic triad amino acids (S805 to A), did not have an effect on the maximum ASIC1 current (Fig.2A).

A dose-response of the effect of matriptase was obtained by measuring ASIC1 whole-cell currents at pH4.0 in oocytes injected with 12ng ASIC1 cRNA and increasing amounts of matriptase cRNA (0-12ng) as indicated in Fig. 2B. The injection of 2ng matriptase with ASIC1 did not have a statistically significant effect on the peak pH4.0 current. However, ASIC1 current decreased significantly when 4ng or higher amounts of matriptase cRNA were used. The inactive S805A matriptase did not have a statistically significant effect on ASIC1, even at the highest concentration used (Fig.2B).

In order to determine whether the effect of matriptase on ASIC1 was specific, we injected oocytes with another human ASIC family member, ASIC2. Matriptase had no effect on the whole-cell peak current of ASIC2, suggesting some specificity of its action on ASIC1 (Fig.2C).

When the purified recombinant matriptase catalytic domain was added to the recording bath (50 ng/ml for 5 min, the same timescale and concentration used in its activity assay by the manufacturer), the current of ASIC1 decreased, while that of ASIC2
did not significantly change (Fig.2D). We used the purified matriptase catalytic domain, because of the shorter time-scale compared to co-injection with ASIC1 followed by a two-day expression. However, it is not possible to compare the amount of matriptase present in the bath and available to cleave the ASIC1 channels to the amount of matriptase protein that the oocyte could express in two days after injection. Addition of recombinant active matriptase to the bath reduced the ASIC1 current by 20%; this decrease was smaller than the one observed when matriptase was co-injected in oocytes with ASIC1, but it was statistically significant. It has been shown previously that 2 µg/ml (about 1 µM) trypsin for 5 min decreased the peak pH4.0 current of ASIC1 by only 10%, while 20µg/ml trypsin decreased it by 30-40% (15). These trypsin concentrations are much higher than the 50ng/ml (about 2nM) used in our experiments for matriptase. Due to the limited amount of purified matriptase catalytic subunit, we were unable to use higher concentrations of matriptase in these experiments. The physical closeness of expressed matriptase versus the added matriptase to the ASIC1 channels, and the fact that matriptase could be more active in its native conformation compared to the purified protein, could also contribute to the result obtained with the catalytically active purified matriptase. Although the purified matriptase decreased ASIC1 peak pH4.0 current, it had no statistically significant effect on the peak current of ASIC2-injected oocytes (Fig.2D).

**Matriptase does not change the pH activation curve or PcTX-1 block of ASIC1**

Matriptase decreased the peak current of ASIC1, and for this decrease to occur, matriptase clearly required a functional catalytic domain (S805A has no effect on
ASIC1), suggesting that matriptase cleaved the ASIC1 channels. Trypsin also cleaves
ASIC1, and it affects the pH activation and channel inhibition by the venom of *P.
cambridgei*. Trypsin shifted the pH activation curve of ASIC1 to a more acidic pH, and
disrupted the venom block of ASIC1 (16). Therefore, we hypothesized that matriptase
would also affect these characteristics of the ASIC1 current. We obtained a pH
activation curve and PcTX-1-sensitivity of ASIC1+matriptase oocytes. For the pH
activation curve, oocytes injected with ASIC1, ASIC1+matriptase, or ASIC1+ matriptase
S805A were subjected to two-electrode voltage clamp and exposed to pH7.4 first,
followed by sequentially lower pHs ranging from pH7.0 to pH4.0, returning to pH7.4
after each activation. The peak current at each activation pH was normalized to the
pH4.0 current for each oocyte. The normalized values were then plotted against the
activation pH to obtain the pH activation curves and were fitted to the Hill equation to
obtain pH50 values and Hill coefficients. As shown in Fig. 3A, there is no difference in
the pH activation curves in ASIC1, ASIC1+matriptase, or ASIC1+matriptase S805A.
The pH50’s and Hill coefficients were also not statistically different (pH50=6.18(SD 0.18),
nH=1.77(SD 0.32), n=10 for ASIC1; pH50=6.20(SD 0.08), nH=1.64(SD 0.41), n=10 for
ASIC1+matriptase; pH50=6.22(SD 0.13), nH=1.71(SD 0.49), n=7 for ASIC1+S805A, p =
ns by one-way ANOVA).

For the PcTX-1 block experiments, PcTX-1 was included in the pH 7.4 solution at
100 nM. PcTX-1 inhibited not only the ASIC1 current, but also the current of
ASIC1+matriptase (Fig. 3B). This is different from trypsin, which removed the venom
block of ASIC1 (15). Because the characteristics of the current (pH activation and
PcTX-1 block) measured in ASIC1+matriptase were not different from those of ASIC1,
but matriptase almost abolished the whole-cell peak current, and its catalytic site was required for this effect, it is likely that the current measured in ASIC1+matriptase oocytes is from a population of noncleaved ASIC1 channels, and that the cleaved channels are non-functional. The other possibility is that the cleaved channels have the same properties as the noncleaved ASIC1. However, the experiments below detecting the presence of ASIC1 cleavage products and determining the location of the possible sites where matriptase cleaves ASIC1 support the hypothesis that the current measured in ASIC1+matriptase is due to a population of noncleaved channels.

*Matriptase-specific fragments for ASIC1 but not ASIC2 are observed in Xenopus oocytes and in transfected CHO K1 cells.*

To determine whether ASIC1 channels are cleaved by matriptase, we obtained whole-cell lysates from oocytes expressing N- and C-terminal eGFP-tagged ASIC1 and matriptase. First, we measured whole-cell peak currents at pH4.0 by two-electrode voltage-clamp of ASIC1-GFP (C-terminal eGFP tag) + matriptase and GFP-ASIC1 (N-terminal eGFP tag) + matriptase, to ensure that the tag did not affect the results. Matriptase decreased the current of ASIC1-GFP and that of GFP-ASIC1 similarly to the non-tagged ASIC1 (Fig. 4A, 4C). Following oocyte lysis and immunoblotting with a GFP antibody, in ASIC1-GFP+matriptase and GFP-ASIC1+matriptase oocytes, we observed several cleavage products, which were not detected in the control lanes (Fig. 4B, 4D). The full length GFP-tagged ASIC1 was detected at approximately 100 kDa, because the GFP tag adds 25 kDa to the 75 kDa ASIC1 protein (see schematics on Fig.4).
For the ASIC1-GFP constructs, five fragments were detected due to matriptase, and they are indicated by arrows in Fig. 4B.

The schematic below Fig.4B shows the approximate positions of the possible cleavage sites that would result in the observed fragments. It also shows the approximate sizes of the fragments. Fragments that are not attached to GFP would not be detectable with the GFP antibody. The two largest fragments at 70-75kDa could result from cleavage in the N-terminal part of the extracellular loop. The two fragments between 37-50 kDa could result from cleavage at a site near the C-terminus of the extracellular loop, and the smallest fragment could be from cleavage at a site very near the second transmembrane domain. Alternatively, the smaller cleavage products could be due to further cleavage of the largest fragments by other endogenous oocyte serine proteases. The blot shown for GFP-ASIC1 shows that there is no full-length GFP-ASIC1 detected in the matriptase lane at this exposure, but some full-length GFP-ASIC1 was detected at longer film exposures (not shown).

A schematic of GFP-ASIC1 is shown below Fig. 4D. It illustrates the same possible cleavage sites as determined from the ASIC1-GFP construct, and the corresponding approximate fragment sizes that would be expected from cleavage at those sites. Two fragments at 35-40 kDa are detected for GFP-ASIC1. Their sizes correspond well to the two N-terminal sites, which would result in the two 70-75 kDa fragments for ASIC1-GFP (Fig. 4B). The schematic shows that larger fragments at 80-90 kDa are also expected for the GFP-ASIC1, but no such fragments are detected in the blot in Fig. 4D. It is possible that the N-terminal 80-90 kDa fragments are cut further into smaller pieces by endogenous serine proteases.
Matriptase had no effect upon the function of ASIC2. A western blot of lysates from oocytes expressing a GFP-tagged or YFP-tagged ASIC2 and matriptase shows that there are no matriptase cleavage products of ASIC2-YFP, and that the full-length ASIC2-YFP protein did not decrease like the full-length ASIC1-GFP or GFP-ASIC1 did (Fig. 4E). These results support the hypothesis that the functional effect of matriptase on the current of ASIC1 is accompanied by a decrease in full-length channels and presence of cleavage products, while the lack of effect on ASIC2 is accompanied by a lack of ASIC2 cleavage products or substantial decrease in the full-length protein.

We also tested the effect of matriptase on an ASIC1 construct with an HA tag in the extracellular loop. The data with the eGFP-tagged ASIC1 and ASIC2 showed that matriptase cleaves ASIC1 but not ASIC2. The ASIC1-HA and ASIC2-HA were used to further confirm these findings. Because the HA tag (at F147 on ASIC1 and T139 on ASIC2) is in a different position than the N or C terminus of the protein, we would detect different ASIC fragments than when using GFP-tagged ASICs. The ASIC1-HA and ASIC2-HA constructs did not differ from the untagged ASIC1 and ASIC2 in the way that they were functionally affected by matriptase. The current of ASIC1-HA + matriptase was decreased compared to control; matriptase had no effect on ASIC2-HA (Fig. 5A, 5C). Western blots with an HA antibody detected full length ASIC1-HA or ASIC2-HA at the expected size of about 75kDa, and only ASIC1-HA was cleaved by matriptase.

A schematic of the ASIC1-HA construct showing the relative position of the HA tag, the positions of the same possible cut sites that were deduced from Fig. 4B, and the approximate sizes of the fragments, is below Fig. 5B. Only the fragments that would contain the HA tag and would thus be detectable by an HA antibody were considered.
The sizes on top of the schematic are for fragments from the N-terminus of ASIC1 to the indicated cleavage site. The size indicated below the schematic is for the fragment from the C-terminus of ASIC1 to the first potential N-terminal cleavage site. This is approximately 50kDa, corresponding well to what is observed in the blot in Fig. 5B. As the schematic shows, there are a few fragments with sizes 45-50 kDa, and we cannot tell the exact cleavage site that results in the 50kDa fragment observed. It is possible that this fragment is from cleavage at the first N-terminal site and represents the C-terminal linked fragment, and the larger N-terminal linked fragments get further cleaved by proteases (especially since the largest N-terminal fragments for GFP-ASIC1 were not seen). The catalytically inactive matriptase S805A did not cleave ASIC1-HA, consistent with its lack of effect on the function of ASIC1. A few non-specific smaller fragments were observed for ASIC1-HA and ASIC2-HA without matriptase (Fig. 5B, 5D). These fragments could be degradation products and were not observed in non-injected oocytes (not shown). In agreement with the ASIC2 functional and western blot data, no ASIC2-HA cleavage products specific to matriptase were detected, further supporting the hypothesis that matriptase does not cleave ASIC2.

In the above experiments ASIC1 and matriptase were heterologously co-expressed in *Xenopus* oocytes, and because there may be differences between heterologous expression systems, we used mammalian CHO K1 cells to test the hypothesis that matriptase cleaves ASIC1 specifically, while not cleaving ASIC2. In these experiments, ASIC1-GFP full-length protein was detected at 100 kDa in transfected CHO cells, and its intensity decreased with increasing amounts of matriptase DNA (Fig. 6A). The relative densitometry values for each band were normalized to the control.
ASIC1-GFP with no matriptase (the first lane on the blot). The GFP antibody detected some non-specific fragments which were present in all the samples transfected with ASIC1-GFP, but were not present in non-transfected CHO cells. There were two faint fragments between 37-50 kDa specific to catalytically active matriptase. These two fragments were present only in the matriptase lanes and not in the control lane or the catalytically inactive matriptase S805A lane.

Because these fragments were difficult to detect, we hypothesized that after cleavage, these fragments were being sent for degradation. We tested this hypothesis by treating the transfected CHO cells with the proteasome inhibitor, MG132 (5 µg/ml for five hours or overnight) 48 hours post-transfection with 2 µg ASIC1-GFP alone, 2 µg ASIC1-GFP + 2 µg matriptase, or 2 µg ASIC1-GFP + 2 µg matriptase S805A. As shown in Fig. 6B, in the samples treated with MG132, the two proteolytic fragments between 37-50 kDa could be clearly detected in the matriptase lane. Fig. 6B shows the normalized densitometry of the full-length bands and the two detected fragments. The non-specific bands present in all the lanes were ignored because they were not specific to matriptase. All the bands were normalized to the signal of the full-length untreated ASIC1-GFP. The graph shows that the intensity of the full-length ASIC1-GFP protein decreases with matriptase, and two fragments labeled as Fragment 1 (F1) and Fragment 2 (F2) appear in the matriptase lane. The intensities of all the bands, including the two fragments, increased when the cells were pretreated with the proteasome inhibitor. The inactive matriptase S805A did not decrease the signal of the full-length ASIC1-GFP.

The same experiment as shown in Fig. 6B was repeated for the N-terminal GFP-tagged ASIC1 (GFP-ASIC1). One fragment was observed for GFP-ASIC1 in the
matriptase lane (Fig. 6C). The non-specific bands that were present in all the lanes for the ASIC1-GFP construct were not detected with the GFP-ASIC1. MG132 pretreatment increased the intensities of all the bands, including the fragment (Fig. 6C densitometry). No fragments were observed either in the control or the matriptase S805A in both the untreated and the MG132-treated samples.

The effect of matriptase on a C-terminal GFP-tagged ASIC2 (ASIC2-GFP) in CHO cells was tested in order to determine if the effect of matriptase on ASIC1 was specific. Surprisingly, a substantial decrease in the full length ASIC2-GFP was observed with matriptase, but no ASIC2-GFP cleavage fragments specific to matriptase could be detected even when cells were treated with MG132 (Fig. 6D). In the presence of MG132, a fragment at 37 kDa appeared. This fragment was present in all the ASIC2-GFP lanes with MG132, including the control with ASIC2-GFP alone and the ASIC2-GFP with the inactive matriptase S805A. Because this fragment was not specific to matriptase (and its intensity actually decreased with matriptase compared to control or inactive matriptase) it was ignored and was not included in the densitometry. The absence of ASIC2-GFP fragments with matriptase, suggests that matriptase does not cleave ASIC2. However, because we do detect a decrease in ASIC2-GFP protein with matriptase (in both untreated and MG132 treated, as compared to the control/inactive matriptase) we cannot exclude the possibility that in CHO cells, matriptase could cleave ASIC2 and we were unable to observe ASIC2 fragments. Another possibility is that the decrease in ASIC2-GFP expression with matriptase is an indirect effect of matriptase on protein expression rather than cleavage of ASIC2 by matriptase. In all of these experiments, the catalytically
inactive S805A matriptase was able to prevent the effect of matriptase on ASIC1 or ASIC2 (Fig.6).

It is important to note some differences between expression in oocytes and mammalian CHO cells: several ASIC1 fragments are observed with the ASIC1-GFP construct in oocytes, compared to only two fragments in CHO cells; two fragments are observed with the GFP-ASIC1 construct in oocytes, compared to one fragment in CHO cells. Matriptase did not result in ASIC2 fragments either in oocytes or in CHO cells, and it did not dramatically decrease ASIC2 expression in oocytes; however, matriptase decreased ASIC2 expression in CHO cells. Despite these differences in the two expression systems, matriptase-specific fragments were detected for ASIC1, but not for ASIC2, in both oocytes and CHO cells; however, the sites of cleavage, downstream signaling events, or downstream cleavage events caused by matriptase may be different in the two expression systems.

The identification of matriptase cleavage sites on ASIC1.

Because matriptase decreased the ASIC1 current and cleaved ASIC1, but had no effect on the function of ASIC2, we searched for matriptase cleavage sites on the ASIC1 and the ASIC2 amino acid sequences. The preferred cleavage sequence of matriptase and the activation sites of known matriptase substrates were obtained from Uhland et al. (20). The four preferred residues proximal to the cleavage site (P4, P3, P2, P1) and P1’, which is the position distal to the cleavage site, were entered in the FindPatterns program of GCG Software. Arginine, lysine, and non-basic amino acids were entered for both P4 and P3; serine, alanine, phenylalanine, leucine, glycine and arginine were entered for P2, and
arginine/lysine for P1; finally, alanine, valine, isoleucine, serine, and glycine were entered for P1’. The search resulted in the identification of three extracellular cleavage sites on ASIC1 (Fig. 7A). Because the matriptase catalytic domain is extracellular and matriptase is activated after it reaches the cell surface(20), we ignored the intracellular N- and C- termini of ASIC1 and ASIC2. The software recognized one site (K174) as a matriptase cleavage site on the extracellular loop of ASIC2. However, the electrophysiological and biochemical data showed no effect of matriptase on ASIC2 function and no matriptase-specific ASIC2 fragments were detected, suggesting this site may not be accessible to cleavage.

Alignment of the ASIC1 and ASIC2 amino acid sequences with ClustalW shows the locations of the three matriptase cleavage sites on ASIC1, the equivalent sites on ASIC2, and that the matriptase recognition sequences on ASIC1 are not conserved on ASIC2 (Fig. 7A1). A schematic of the ASIC1-GFP, GFP-ASIC1 and ASIC1-HA proteins indicating the three cleavage sites, the fragments, and their sizes is shown in Fig. 7A2. The size estimates take into account the weight added by potential glycosylations at N368 and N395(28). For two glycosylations, ~5kDa were added to the calculated size of a peptide fragment (29).

We mutated the matriptase sites on the ASIC1-GFP construct with site-directed mutagenesis, measured whole-cell peak pH4.0 currents of the mutants with or without matriptase, and detected the proteins with a GFP antibody. We chose the oocyte expression system instead of CHO cells for the mutagenesis experiments, because in oocytes we observed cleavage of ASIC1 but not ASIC2, without the substantial decrease in the expression of ASIC2 protein, which was caused by matriptase in CHO cells.
Mutation of the first matriptase site to A (R145A) (referred to as ASIC1 AKK) prevented the decrease in current observed when matriptase was co-injected with the wild type ASIC1 (Fig. 7B). ASIC1-GFP AKK was not cleaved by matriptase to the same extent as wild type ASIC1; however, some fragments of ASIC1 AKK-GFP were detected (Fig. 7C). The fragments correspond to the same ones observed in wild type ASIC1-GFP, with the exception that the biggest cleavage product (the one observed right above 75kDa, corresponding to cleavage at position 145) was missing (Fig. 7C). The densitometry of the 100 kDa band of ASIC1 AKK-GFP showed that this mutation prevented the decrease in full-length ASIC1-GFP, which was observed for the wild type ASIC1-GFP shown in Fig. 4B (Fig. 7C). Because the GFP tag is located in the C terminus of ASIC1, and the size of the fragment from R145 to the C-terminus of GFP would be approximately 72kDa, it was not surprising to not detect this fragment, when R145 is changed to the non-cleavable alanine residue (A) (Fig. 7A1, and Fig. 7C). R145 is the main trypsin cleavage site on ASIC1, although some cleavage of ASIC1 R145A by trypsin was also observed (20).

Because the mutation of R145 to A completely prevented the effect of matriptase on ASIC1, we hypothesized that this is the main site of ASIC1 cleavage by matriptase, and that the other two sites (K185 and K384) were not as important. To test this hypothesis, we made an ASIC1-GFP construct with K185 and K384 mutated to A (ASIC1 RAA). If R145 is the only site of matriptase cleavage, we would expect matriptase to decrease the current of the ASIC1 RAA-GFP construct similarly to the wild type ASIC1, and also to detect similar cleavage products on western blots. However, although matriptase decreased the current of ASIC1 RAA-GFP by about 30%, this
decrease was not statistically significant or comparable to the much larger decrease of the wild type ASIC1 current caused by matriptase (by about 70-90%) (Fig.7C). Some cleavage of ASIC1 RAA-GFP was observed with cleavage products at approximately 75kDa, in agreement with the location of the R145 site. Another fainter smaller fragment is also observed and it could be further degradation of the fragment from the R145 to the C-terminus of the ASIC1-GFP, or cleavage at another non-specific site. The smaller cleavage products, which could result from cleavage at the other two sites, (Fig. 7A2) are not present in the RAA construct, where the K185 and K384 are changed to non-cleavable amino acids (Fig.7C). The densitometry shows that unlike the wild type ASIC1-GFP, there is no decrease in the full-length ASIC1 RAA-GFP. These results suggest that R145 is a matriptase cleavage site and that K185 and K384 are also important.

Mutation of all three matriptase cleavage sites to alanines (R145A, K185A, and K384A) (referred to as ASIC1 AAA) prevented the effect of matriptase on ASIC1 current and also prevented ASIC1 protein cleavage (Fig. 7C). Compared to the effect of matriptase on the wild type ASIC1, the densitometry shows that there is no decrease in the full-length ASIC1 AAA-GFP protein. Some cleavage of ASIC1 AAA-GFP was observed with matriptase in overexposed films (not shown). Not unlike trypsin (16), matriptase might cleave at non-specific sites (there are many additional R or K residues in the extracellular loop near the matriptase sites). Alternatively, the minimal cleavage observed for ASIC1 AAA-GFP could be the result of some other unknown endogenous proteases that get activated by matriptase in the oocyte expression system.
DISCUSSION

Matriptase is an epithelial cell surface serine protease that is expressed in a variety of carcinomas and has been implicated in malignant progression of cancers (20). Although matriptase is expressed in many different epithelial tissues and organs, matriptase mRNA has not been detected in normal brain tissue (17,27). In this study we show that matriptase is expressed in glioblastoma multiforme (GBM) cells. The data show that matriptase mRNA is expressed in glioma cell lines and in freshly-excised tissues from GBM and grade III glioma (anaplastic astrocytoma, AA). Matriptase RNA cannot be detected in primary human normal astrocytes from astrogliosis, or in two different freshly-excised astrogliosis tissues. Unlike the mRNA, matriptase protein is present in human primary normal astrocytes and in glioma cell lines, but its expression in normal astrocytes is much lower compared to glioma cells. It is possible that the levels of matriptase RNA in the normal astrocytes are too low to detect in our conditions.

The presence of matriptase protein suggests a role for this protease in glioma. Some of the physiological matriptase substrates (namely, urokinase plasminogen activator (uPA), hepatocyte growth factor/scatter factor (HGF/SF), and protease activated receptor-2 (PAR-2)) are associated with increased proliferation and invasiveness of glioma (30). In addition, overexpression of HAI-1, the cognate matriptase inhibitor, suppressed the in vitro invasiveness of the U251 glioblastoma cell line (24).

As a cell-surface protease, matriptase is in an ideal position to cleave the extracellular loops of ion channels. In the Xenopus oocyte expression system, matriptase increased the activity of αβγENaC (17,18). ASIC modulation by proteases has been less studied, but most proteases that have been tested have an effect on ASIC1 or ASIC1-
containing heteromeric ASICs, and no effect on ASIC2 or ASIC3 (15). The goal of this study was to test the hypothesis that matriptase can modulate ASIC1 function through proteolytic cleavage. We used heterologous expression systems, and electrophysiological and biochemical approaches to determine the effect of matriptase on the function of ASIC1, and also to determine if/where in the extracellular loop of ASIC1 matriptase cleavage occurs.

Our data show that ASIC1 is a matriptase substrate in two heterologous expression systems, *Xenopus* oocytes and CHO cells, and that unlike its effect on ENaC, matriptase decreases the function of ASIC1. This effect requires the catalytic activity of matriptase and is associated with the presence of ASIC1 cleavage products. The putative matriptase cleavage sites on ASIC1 are R145, K185, and K384. The crystal structure of chicken ASIC1 and alignments of ASICs from different species enabled us to locate these sites in the extracellular loop of ASIC1, which resembles a clenched hand and contains a palm (made of β strands), knuckle, finger, thumb (all made of α helices), and a β-ball. The thumb, β-ball and finger, along with the palm of an adjacent subunit form the acidic pocket or proton-binding site of the channel (4). R145 is located in the finger domain, K185 in the β-ball, and K384 between the palm and knuckle domains. Because all three sites are located at or close to the pH sensing region (4), channels cleaved at these sites are likely not functional, suggesting that the small current measured in ASIC1+matriptase is probably due to a few non-cleaved channels. In agreement with this idea, the data show that the pH activation and PcTX-1 sensitivity of this current are not different from that of non-cleaved ASIC1.
The first matriptase cleavage site, R145, was previously characterized as a trypsin site (16). Similarly to our results with matriptase, the application of extracellular trypsin decreased the peak acid-activated current of ASIC1, but had no effect on ASIC2 (16). The mutagenesis experiments strongly suggest that the effect of matriptase is direct, involving cleavage of ASIC1 in several locations in the extracellular loop. However, because some cleavage was observed in the ASIC1 AAA mutant, our results do not rule out the possibility that matriptase cleaves at adjacent arginines or lysines, or that it activates another endogenous protease, which could cleave at other nearby sites. One such protease is prostasin. Matriptase co-localizes with and activates prostasin, which has been shown to activate αβγENaC (17,18,31). Therefore, the substrate recognition motif for prostasin, which is [R,K]-[H,K,R]-X-[R,K] (where X is a basic or hydrophobic amino acid) (32), and the ASIC1 amino acid sequence were entered in the FindPatterns program in SeqWeb v3.1.2 in GCG (UAB), to look for putative prostasin sites on ASIC1. However, the search resulted in no prostasin sites on ASIC1, suggesting that cleavage of ASIC1 by matriptase-activated prostasin is unlikely.

The observed ASIC1 cleavage fragments due to matriptase correlate well with what would be expected from cleavage at those three sites. Because many fragments are observed, it is possible that not all the channels are cleaved at all three sites. If that were the case, then the only size observed, for example, in the ASIC1-GFP construct, would be the smallest fragment still attached to GFP. This is the fragment from K384 of ASIC1 to the C-terminus of GFP with a calculated size of approximately 40kDa (Fig. 7A2). We do not know if the ASIC1 fragments remain associated once cleaved by matriptase. Because
cleaved fragments of αENaC and γENaC remain associated within the channel, it is possible that this is the case for ASIC1 as well (11).

The cleavage of ASIC1 by matriptase has implications for the pathologies in which ASIC1 is involved. ASIC1 is a mainly neuronal channel, and matriptase expression in neurons has not been reported; however, matriptase expressed by astrocytes could possibly modulate ASIC1 in neurons. Matriptase could modulate ASIC1 activity acting to decrease ASIC1 function through proteolytic cleavage.

So far the regulation of ASIC1 by proteases has been explored using heterologous expression systems (15,16). We report matriptase expression in glioblastoma cells, where ASIC1 is also expressed (5-8). Although nothing is known about the proteolytic cleavage of ASIC1 in glioma, the expression of both matriptase and ASIC1 in the same cell type makes proteolytic cleavage of ASIC1 by matriptase in a non-heterologous system a realistic possibility.
REFERENCES


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Figure 1. RT-PCR and Western Blot detection of matriptase in glioma cell lines and fresh GBM tissues. **A.** Agarose gels of RT-PCR products detect matriptase RNA in SKMG and U251MG cell lines, freshly-resected glioblastoma multiforme (GBM), and anaplastic astrocytoma (AA). Matriptase message is not detected in the primary human astrocytes or the fresh astroglial tissue. No RNA in the RT-PCR reaction is used as a negative control. **B.** Western blot of whole-cell lysates with a matriptase antibody shows that expression of matriptase in glioma cell lines (D54MG, U251MG, and U87MG) and two different human primary GBM is higher than its expression in two different primary human astrocytes. The blot was probed for actin as a loading control.
Figure 2. Matriptase decreases the peak acid-activated current of ASIC1, but not that of ASIC2 in Xenopus oocytes. A. Peak $I_{pH4.0}$ were measured by two-electrode voltage clamp in oocytes injected with 12 ng ASIC1 cRNA alone, ASIC1 with matriptase (8 or 12ng), or ASIC1 with the catalytically inactive matriptase S805A (8 ng). Representative traces are shown above each graph. Graph values are means + SD. Numbers of oocytes measured are from 2-3 experiments and are shown in parentheses. The non-parametric Kruskal-Wallis test with the Dunn’s post-hoc was used to determine the p-value. B. Dose-response of matriptase effect on the peak $I_{pH4.0}$ of ASIC1. Oocytes were injected with 12ng ASIC1 and increasing amounts of matriptase cRNA. Values are means + SD. Numbers of oocytes measured are shown in parentheses and are from 5
experiments. The non-parametric Kruskal-Wallis test with Dunn’s post-hoc test was used to determine the p values. C. Normalized $I_{pH4.0}$ of oocytes injected with 12ng ASIC2 cRNA alone or ASIC2 with 12ng matriptase. Unlike the effect on ASIC1, matriptase does not have an effect on the peak $I_{pH4.0}$ of ASIC2. Representative traces are shown. Values are means ± SD, and the numbers of oocytes measured are shown in parentheses. p value was determined with a two-tailed, unpaired t-test. D. Oocytes were injected with ASIC1 or ASIC2 cRNA. After the peak $I_{pH4.0}$ current was measured, the catalytically active purified matriptase was added to the bath for 5 min at 50ng/ml, and the peak $I_{pH4.0}$ was measured again. The graph shows normalized values: for each oocyte, the peak $I_{pH4.0}$ measured “before” and “after” matriptase addition, was normalized to the “before” $I_{pH4.0}$. The peak $I_{pH4.0}$ of ASIC2 did not decrease after addition of active matriptase to the bath. Representative traces for ASIC1 and ASIC2 before and after matriptase are shown. p values were obtained with a two-tailed paired student’s t-test of the raw current values before and after matriptase. Values are means ± SD. Numbers of oocytes measured are shown in parentheses.
Figure 3. Matriptase does not change the pH activation curve or inhibition by PcTX-1 of ASIC1. A. pH activation curve of currents measured in oocytes injected
with 12 ng ASIC1 alone, ASIC1 and 8 or 12ng matriptase, or ASIC1 and 12ng matriptase S805A. Oocytes were exposed to decreasing activation pHs from 7 to 4.0. The peak current at each activation pH was normalized to the peak $I_{pH4.0}$ for each oocyte. Representative pH activation curves and the pH protocol used are shown. The graphed values are means ± SD of 10 oocytes (ASIC1), 10 oocytes (ASIC1+ matriptase) and 7 oocytes (ASIC1+ matriptase S805A) from 2-3 different batches. **B.** Peak $I_{pH4.0}$ was measured in oocytes expressing ASIC1 alone or ASIC1 with 12 ng matriptase. 100nM PcTX-1 in the pH 7.4 solution abolished the $I_{pH4.0}$ of control ASIC1-injected oocytes, as well as of those co-injected with matriptase. Traces shown are representative of 3 oocytes for ASIC1 and 3 oocytes for ASIC1 and matriptase.
A

![Graph showing normalized I pH4.0 for ASIC1-GFP and ASIC1-GFP + Matriptase. The p-value is 0.021.](image)

B

![Blot showing GFP expression with control, Matriptase, and Uninjected conditions.](image)
Figure 4. Detection of matriptase-specific cleavage fragments of GFP-tagged ASIC1 but not of tagged ASIC2 in *Xenopus* oocytes. A, C. Normalized $I_{pH4.0}$ of oocytes injected with C-terminal GFP-tagged ASIC1 (A) or with N-terminal GFP-tagged ASIC1 (C), with or without 12 ng cRNA for matriptase shows that matriptase significantly
decreases the current of ASIC1-GFP (A) and GFP-ASIC1 (C). The values are means ± SD. Numbers of oocytes measured are shown in parentheses, and p values were determined with unpaired two-tailed t-tests. B. D. Western blot of oocyte lysates with GFP antibody, which detects ASIC1 fragments in the matriptase lane for both the ASIC1-GFP (B) and GFP-ASIC1 (D) samples. The arrows point to GFP-tagged ASIC1 cleavage products. The schematics of ASIC1-GFP (below A and B) and of GFP-ASIC1 (below C and D) show the locations of possible cleavage sites in each construct and their sizes. The black boxes represent the two transmembrane domains. E. Western blot of oocyte whole cell lysates from oocytes injected with ASIC2-YFP, with or without 12ng matriptase cRNA or 12ng matriptase S805A cRNA. The GFP antibody does not detect any fragments for ASIC2 in the ASIC2-YFP + matriptase samples, suggesting that matriptase does not cleave ASIC2.
Figure 5. Detection of cleavage fragments of HA-tagged ASIC1 but not of HA-tagged ASIC2 in *Xenopus* oocytes.  

A. C. Normalized $I_{pH4.0}$ of oocytes injected either with ASIC1-HA, ASIC1-HA + Matriptase, or ASIC2-HA, ASIC2-HA + Matriptase (9, 10).  

B. Blot: HA

D. Blot: HA
with ASIC1-HA (A) or ASIC2-HA(C) with or without 8 or 12ng cRNA of matriptase. Matriptase decreases the peak $I_{pH4.0}$ of ASIC1-HA, but not that of ASIC2-HA. Data are mean + SD. Numbers of oocytes measured are shown in parentheses. $p$ values were determined with unpaired two-tailed t-tests. **B, D.** Consistently with the effect of matriptase on the function of the channels, western blotting of whole oocyte lysates with an HA antibody detects fragments of ASIC1-HA (B), but no of ASIC2-HA (D), suggesting that matriptase does not cleave ASIC2. The schematic of ASIC1-HA (below A and B) shows the locations of possible cleavage sites in the construct and the predicted sizes. The black boxes represent the two transmembrane domains.
Figure 6. Matriptase cleaves ASIC1-GFP and GFP-ASIC1, but not ASIC2-GFP in transfected CHO cells.  
A. Dose-response of increasing amounts of matriptase DNA co-transfected in CHO cells with 2 µg of ASIC1-GFP DNA. Transfected CHO cells were lysed 2 days post-transfection and whole-cell lysates were subjected to western blot with a GFP antibody. The densitometry of the full length ASIC1-GFP band shows a linear relationship between increasing amounts of matriptase DNA and decreasing amounts of full-length ASIC1-GFP, and no effect of the catalytically inactive matriptase S805A. Data in the densitometry graph are means + SE. 

B. CHO cells transfected with 2µg ASIC1-GFP alone (control lanes) or plus 2µg matriptase or 2µg S805A were lysed after being left untreated, or after treatment with the proteasome inhibitor MG132 (5 µg/ml, 5h or overnight). The whole cell lysates were subjected to western blot with GFP antibody. The amount of full length ASIC1-GFP decreases when it is co-transfected with matriptase, but not with the catalytically inactive matriptase S805A. Two small fragments are barely detected in the untreated samples, matriptase lane. In the samples treated with MG132, the signal of the fragments in the matriptase lane (F1 and F2) is increased. There are no ASIC1-GFP fragments in the ASIC1-GFP or ASIC1-GFP + matriptase S805A in either the untreated or the MG132 treated samples. Data in the densitometry graph are means + SE. 

C. The same conditions as in (B) apply with the exception that GFP-ASIC1 (ASIC1 with an N-terminal GFP tag) was used instead. One fragment of GFP-ASIC1 is detected only in the matriptase lanes, and its intensity increases with MG132 treatment. The densitometry data are means + SE. 

D. The same conditions as in (B) and (C) apply, with the exception that ASIC2-GFP was used instead, and MG132 treatment was overnight. Even though matriptase decreases the full-length ASIC2-GFP, no ASIC2-GFP fragments appear in the matriptase lane of untreated or MG-132 treated samples, suggesting that matriptase does not cleave ASIC2-GFP. The densitometry data are means + SE of 3 separate experiments.
A1

\[
\text{hASIC1} b \quad \text{RYEIPDTQMADEKQLEILQDKANFRSFKPKPFNMREFYDRAHGHDIRMLLLCSCHFRGVE} \quad 180
\]

\[
\text{hASIC2} b \quad \text{NLQIPDPHELPSLEALRQKANFKHYKPKQFSMLEFLR} \quad 174
\]

\[
\text{hASIC1} b \quad \text{AEDFKVVFTRYGKYTFNSGRDGRPLKTMKGGGNGLEIMLDIQQDEYLPVWGETDETS} \quad 240
\]

\[
\text{hASIC2} b \quad \text{HQDFTTVFTRYGKYCYMFGNSGDKPLLTVKGOTNGGNGLEIMLDIQQDEYLPWGETTEE} \quad 239
\]

\[
\text{hASIC1} b \quad \text{CVCEMPCNLTRYGKSMVPIPSKA} \quad 420
\]

\[
\text{hASIC2} b \quad \text{CLCRTPCNLTRYKSMVPIPSKT} \quad 417
\]

A2

![Diagram of ASIC-GFP and GFP-ASIC1 constructs with molecular weights](image)
B

Control Matriptase

Normalized I pH4.0

p = 0.74

A145/K/K

(26)

(29)

p = 0.054

R/A185/A384

(14)

(16)

p = 0.77

A145/A185/A384

(12)

(15)
Figure 7. Identification and confirmation of the matriptase sites on ASIC1. A1. Alignment of the human ASIC1b (NM_001095) and human ASIC2b (NM_001094) proteins showing the locations of the three matriptase cut sites on ASIC1, and the equivalent sites on ASIC2. All three locations are in the extracellular loop, and N- and C- termini of ASIC1 and ASIC2 were ignored, because matriptase catalytic domain is extracellular. The matriptase cut sites were identified with Genetics Computer Group (University of Alabama at Birmingham) software in which the information for positions P4, P3, P2, P1, and P1’, with P1 being the cut site, was manually entered (20). One matriptase site was identified by the software in the extracellular loop of ASIC2 at K174, but because no ASIC2 fragments were detected, it may not be accessible to cleavage. A2.
The schematics of ASIC1-GFP, GFP-ASIC1, and ASIC1-HA are shown, indicating the three matriptase sites in each protein, and the sizes of the fragments expected from cleavage at these sites. The sizes take into account weight added by potential glycosylation(s). Only the fragments that are attached to tags, and therefore could be detected by western blots are shown. *, potential glycosylation sites (N368 and N395)(28).

**B.** Matriptase does not decrease the current of ASIC1 with R145 mutated to A (R145A/K/K) or of the triple mutant R145A/K185A/K384A (AAA). Matriptase only slightly decreases the current of the R/K185A/K384A mutant. Representative traces from each experiment are shown. The normalized \( I_{pH4.0} \) are means + SD. Numbers of oocytes are shown in parentheses and are from 2-3 different experiments.

**C.** Oocytes were injected with 12 ng ASIC1 AKK-GFP, ASIC1 RAA-GFP, or ASIC1 AAA-GFP with or without 12ng matriptase cRNA. The whole-cell lysates were subjected to 12% SDS-PAGE and western blot with a GFP antibody. Some cleavage products were detected for the ASIC1 AKK and ASIC1 RAA. For the ASIC1 AAA, in which all three matriptase sites are mutated to alanines, no fragments were detected. The normalized densitometry of the full-length bands for ASIC1 AKK, ASIC1 RAA, and ASIC1 AAA, with and without matriptase shows that despite the presence of some fragments, there is no dramatic decrease in the full-length protein (in contrast to the wild type ASIC1-GFP protein). The densitometry values are means + SE.
DISCUSSION

ASIC1, PKC, AND MATRIPTASE: IMPLICATIONS FOR GLIOMA BIOLOGY

Acid Sensing Ion Channel 1 (ASIC1) is a member of the Epithelial Na⁺ Channel/ Degenerin (ENaC/ Deg) family of amiloride-sensitive ion channels. It is gated by protons and activated by a decrease in extracellular pH (10,14,163,164). The Benos laboratory has proposed a role for ASIC1 in glioblastoma multiforme (GBM), a World Health Organization (WHO) Grade IV primary brain tumor of astrocytic origin (146). We have previously described a constitutively active and amiloride-sensitive current in high-grade glioma cells (53-55,124,165). These cells express ASIC1 and ENaC. The hypothesis is that the amiloride-sensitive channel complex is composed of a mixture of ASIC1 and ENaC subunits (54,124,165); however the exact composition and stoichiometry of the subunits in this channel complex are not known. ASIC1 is a component of this complex because: a) amiloride blocks this current (with an IC₅₀ of 29 µM), suggesting involvement of ENaC/Deg family subunits (53-56), b) PcTX-1, a specific blocker of ASIC1 blocks this current, suggesting ASIC1 is part of the complex (55), c) ASIC1 is expressed at the plasma membrane in these cells (124), d) ASIC1 and ENaCs are capable of forming heteromeric complexes when co-expressed in CHO cells (52), and they co-immunoprecipitate in glioma cells (56), and e) knockdown of ASIC1 (or αENaC, and γENaC, but not of δENaC) in D54-MG glioma cells inhibits the amiloride-sensitive current (56). It is physiologically important for glioma cells to express this amiloride-sensitive current because amiloride as well as psalmotoxin1 (PcTX-1) inhibit regulatory volume increase following a hyperosmotic challenge (166),
and glioma cell migration and proliferation (124). Knockdown of ASIC1, \( \alpha \)ENaC, or \( \gamma \)ENaC also inhibits migration of D54-MG glioma cells (56).

Normal astrocytes and low-grade glioma express ASIC1 as well, but they do not express an amiloride-sensitive current (53,54). The molecular mechanisms that lead to the expression of this current in gliomas, and to its suppression in normal astrocytes are not clear, but some data point to a role for PKC and syntaxin 1A. PKC regulates this channel complex because addition of PKC abolishes the amiloride-sensitive current of a glioma cell, and PKC inhibition is necessary in order for normal astrocytes to exhibit an amiloride-sensitive current when syntaxin1A function is blocked with Munc-18 (102). For syntaxin 1A to inhibit the channel, ASIC2 must be present at the plasma membrane with ASIC1 and \( \alpha \)ENaC or \( \gamma \)ENaC (54). ASIC2 is present at the plasma membrane of normal astrocytes, but it is absent from the plasma membrane of glioma cells (124). Therefore, the reason why this conductance is turned on in glioma cells, and turned off in normal astrocytes could be a differential regulation of ASIC1/ENaC subunits. For the first project of my thesis I focused on the role of PKC in the regulation of ASIC1 channels.

PKC

The presence of a PDZ binding domain in the C-terminus of ASIC1 and the fact that ASIC1 interacts with PICK-1 (95) also suggested that phosphorylation by PKC might regulate ASIC1. In planar lipid bilayers, addition of PKC to the intracellular side of ASIC1 inhibited its open probability (102), and queries with databases searching for motifs in the amino acid sequence of a protein revealed three consensus PKC
phosphorylation sites (T26, S40, and S499) in the cytoplasmic N- and C- termini of ASIC1.

Activation or inhibition of PKC can both decrease the whole-cell hASIC1b current. The S40 and S499 sites mediate the effect of PKC activation and PKC inhibition on ASIC1, respectively. These results suggest the possibility of a basal phosphorylation of ASIC1 at S499. This basal phosphorylation may be necessary to obtain a maximal ASIC1 current. Either lack of phosphorylation at this site, or phosphorylation of S40 can decrease ASIC1 current amplitude. The effects of PKC activators and inhibitors on the ASIC1 current are not due to a decreased surface expression of the channel and are likely an effect on the gating of the channel. This modulation of ASIC1 by PKC may be relevant for physiological and pathophysiological situations involving ASIC1 function.

ASIC1 is highly expressed in the brain, which is also the highest source for PKC in terms of catalytic activity and levels of expression. An exciting aspect of PKC is its involvement in the learning and memory phenomena (167), in which ASIC1 has also been implicated. There is a role for ASIC1 in synaptic plasticity (109), which is positively influenced by PKC activation (167). I did not study the effects of prolonged PKC activation on ASIC1 because prolonged PKC activation depletes the cell of PKC. The time course of PKC activation in my studies was five min, and some data show that the maximum PKC activity is obtained 5 min after DAG stimulation (168). Other than PKC affecting ASIC1 function, it is possible that ASIC1 can regulate PKC. ASIC1 homomers are permeable to Ca$^{2+}$, and extracellular acidosis results in an increase in intracellular [Ca$^{2+}$]. Rapid rises in intracellular Ca$^{2+}$ drive rapid activation of
conventional PKC isozymes at the plasma membrane (140), where PKC could in turn phosphorylate ASIC1 and/or other substrates.

PKC activity levels are much higher in neoplastic astrocytes when compared with normal glia, and there is evidence for PKC involvement in glioma cell proliferation and invasion (146). Reduction of PKC activity in cultured glioma cells using the PKC inhibitors staurosporine, tamoxifen, CGP 41251, calphostin C, or specific anti-PKC antisense oligonucleotides, decreased their growth rate, while stimulation of cells with PMA, mitogens, or serum had the opposite effects (146). Moreover, exposure of normal or transformed astrocytes or glioma cells to PKC-activating agents such as PMA, led to a more pronounced invasive phenotype, including an increased production of matrix-metalloprotease-2 (MMP-2), and increased invasion of an artificial basement membrane. On the contrary, inhibition of PKC activity decreased glioma invasion (146).

The roles of specific PKC isoforms in gliomas can be confusing. For example, PKCα activity has been especially implicated as a positive regulator of glioma proliferation and invasiveness (146). However, a mutation of PKCα in the first Pro of the PXXP motif was identified as a cancer-driven mutation in glioblastoma. The PXXP motif binds to HSP90, which is required for the activity and processing of conventional and novel PKCs. Dysregulation of this interaction results in PKC that is not phosphorylated and is degraded (140). This goes against the above idea that gliomas express overactive PKCα (146).
Matriptase

Matriptase, a recently identified membrane-associated serine protease with an extracellular catalytic domain is overexpressed in several human cancers (154). It has been shown that matriptase can activate other subunits of the same family, the ENaCs, when co-expressed in Xenopus oocytes (158). Moreover, serine proteases can regulate ASIC1 function (46). Some preliminary data in the lab on matriptase expression in glioma led me to pursue the question of whether matriptase can regulate ASIC1.

In the second paper of my thesis, I have shown that: 1) matriptase is expressed in both normal astrocytes and high-grade glioma cells, and its expression in glioma cells is higher than in the normal astrocytes, and 2) matriptase cleaves ASIC1 protein in Xenopus oocytes, resulting in an inhibition of the ASIC1 current. Three matriptase cleavage sites were identified on ASIC1: R145, K185, and R384. The effect of matriptase on ASIC1 is specific because matriptase does not cleave ASIC2 or have an effect on the ASIC2 current in oocytes. The above findings suggest that matriptase could cleave one of the glioma channel complex subunits. Because the overall hypothesis is that the glioma channel is heteromeric composed of a mixture of ASIC1, αENaC, and γENaC, and because matriptase has opposite effects on αβγENaC and ASIC1, the question of the effect of matriptase on the glioma channel complex would be difficult to predict. However, because this heteromeric channel is constitutively active in glioma, matriptase could play a role in its activation.

There could be a role for matriptase in glioma, encouraging further work to determine its exact cellular localization and to confirm that it is an active protease at the cell surface of these cells. Many physiological matriptase substrates such as uPA,
HGF/SF, and PAR-2 are associated with increased proliferation and invasiveness of glioma. Highly malignant gliomas express increased levels of the receptor for uPA (uPAR). Moreover, uPAR is consistently present at the invasive edges of malignant glioma, where it could aid in dissolution of the extracellular matrix during invasion (169). Furthermore, down-regulation of uPA and uPAR effectively inhibits glioma angiogenesis (170). The expression of HGF/SF and its tyrosine kinase receptor c-Met has been reported in GBM. HGF/SF expression is very low in low-grade astrocytoma and is up-regulated in the transition from low-grade to high-grade malignant glioma, correlating with tumor grade and poor prognosis. HGF/SF induces migration/invasion of GBM, and promotes angiogenesis (171-173). PAR-2 inhibition also decreases proliferation, migration, and invasion of malignant glioma (174). All of the above molecules have been considered as targets for glioma therapy. Because matriptase is upstream and activates all of the above malignant-promoting pathways, it would be a good target for therapy of these highly invasive and lethal tumors.

Matriptase could have a role in glioma not only through activating these receptors and cytokines, but also through direct proteolysis of extracellular matrix components to facilitate invasion. A role for matriptase in glioma is supported by Miyata et.al, showing that overexpression of HAI-1, the cognate matriptase inhibitor, suppressed the in vitro invasiveness of the U251 glioblastoma cell line (155).

ENaC/Deg Channel and its regulation by PKC or matriptase in glioma migration

Ion channels are also major players in cell migration and invasion. In the case of glioblastoma, their highly invasive nature makes complete surgical resection
impossible. Migrating cells are highly polarized with a front and a rear end (175). They have polarized distribution of the actin cytoskeleton, ion channels, vesicular transport, and signaling molecules (176,177). When cells move, they cycle through four steps beginning with protruding a leading edge, adhering it to the substrate, translocating the cell body, and finishing with retracting the trailing edge (178). The first step - formation of leading edge protrusions like lamellipodia, filopodia, or invadopodia (176,179) towards a chemoattractant - is caused by spatial and temporal regulation of phospholipids and Rho GTPases Rac and Cdc42 (178). Lamellipodia are broad flat sheet-like protrusions 1-5 µm wide (180) and 300 nm thin (177) containing an actin meshwork. Filopodia are thin round finger-shaped protrusions containing actin bundles, and invadopodia are short actin-filled protrusions with a core of actin filaments. Invadopodia extend vertically from the ventral cell membrane (179) and are able to degrade ECM (180,181). Formation of these protrusions depends on actin polymerization into the direction of movement. This requires the presence of free barbed ends for extension, which are caused by a protein called cofilin (176,179-181).

Migration of cells depends critically on the integrity of the actin cytoskeleton, which can be regulated by cell volume. Cell swelling causes actin depolymerization, while cell shrinkage causes actin polymerization (182). The cell volume itself is regulated by the activity of ion channels, and cytoskeletal components themselves can regulate ion channels (177,183). Although the long-term job of ion channels is to maintain a constant cell volume, during lamellipodia formation and the retraction of the leading edge, the cell volume may increase and decrease respectively, because the protrusion and retraction do not always occur simultaneously (177).
Intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) is another factor that regulates cell migration through regulating actin filament turnover, phosphorylation of myosin light chain (MLC), integrin recycling, and protease activity, when cells migrate through ECM (177). There is a gradient of [Ca\(^{2+}\)]\(_i\) in a migrating cell, with higher [Ca\(^{2+}\)]\(_i\) at the cell rear and lower [Ca\(^{2+}\)]\(_i\) at the cell front. This is consistent with the fact that high [Ca\(^{2+}\)]\(_i\) promotes actin depolymerization, and low [Ca\(^{2+}\)]\(_i\) promotes actin polymerization, as happens in the protrusion of the lamellipodium (177). Ion channels set the membrane potential of the cell, and changes in ion channel activity could change the membrane potential, affecting the gating of Ca\(^{2+}\)-permeable ion channels and/or the electrochemical gradient for Ca\(^{2+}\) (177).

Some of the channels that have been implicated in the migration of tumor cells include K\(^+\) channels, Cl\(^-\) channels, Ca\(^{2+}\) channels, Na\(^+\) channels and aquaporins. Almost all migrating cells express Ca\(^{2+}\)-sensitive K channels like IK1 (184). IK channels activate from a rise in [Ca\(^{2+}\)]\(_i\). Because [Ca\(^{2+}\)]\(_i\) is higher at the rear of the cell, IK channels are active only at the rear of the cell, as found in studies where the IK channel blocker charybdotoxin was applied to the rear or front of a migrating cell (177,182). IK channel inhibition slows down migration, and IK channel expression in cells that do not express it such as MDCK II cells, microglia, and fibroblasts increases migration (177). A voltage gated K\(^+\) channel, Kv1.3, interacts with β1 integrin and controls cell adhesion because blockage of K\(^+\) channel inhibited integrin-mediated adhesion in human lymphocytes and melanoma cells (177).

K\(^+\) channels can affect migration by hyperpolarizing the cell membrane, increasing the driving force for Ca\(^{2+}\) influx and therefore increasing [Ca\(^{2+}\)]\(_i\), which can
regulate several migration factors. K$^+$ ions themselves can affect cell volume, when they are accompanied by an anion like Cl$^-$ (177). Large conductance Ca$^{2+}$-activated K$^+$ channels (BK channels) and Cl$^-$ channels like ClC2 and ClC3 have been implicated in the migration of glioma cells (185,186). In a model of glioma cell migration being affected by Cl$^-$ and K$^+$ channels, cell shrinkage is necessary for glioma cells to squeeze through the tight extracellular spaces in the brain and invade surrounding areas in the brain. Cell shrinkage requires a loss of salt and water from the cytoplasm, which requires concerted opening of K$^+$ and Cl$^-$ channels. The electrochemical gradient for K$^+$ and Cl$^-$ to leave the cell in gliomas is set by the activity of the Na$^+$/K$^+$-ATPase, which maintains a high [K$^+$], and the Na$^+$/K$^+$/2Cl$^-$ cotransporter, which accumulates intracellular Cl$^-$ (186). BK channels and the ClC3 channels colocalize with the IP3 receptors within lipid rafts in the invadopodia of glioma cells. The hypothesis is that BK channels need [Ca$^{2+}$], of at least 500 nM to activate, and must be located close to regions where there is an increase in [Ca$^{2+}$], therefore close to IP3 receptors (186). The location of these channels at the leading edge of the cell (invadopodia) where there is lower [Ca$^{2+}$], during migration, and which is usually associated with increases in cell volume is a little puzzling. Perhaps the cell shrinkage mediated by them is even more localized than to a region like the whole front or whole rear of the cell. Cl$^-$ and K$^+$ channel blockers also inhibit glioma cell migration (187), while in another study it was shown that BK channel openers inhibit migration of glioma cells as well (188).

It is thought that protrusion of the lamellipodium or invadopodium is helped by uptake of solutes and water at the leading edge, which provides a force that extends the plasma membrane (177), and creates more room for actin polymerization. Indeed, water
channels Aquaporins (AQP) 1, 4, and 9 are located at the edge of lamellipodia in migrating astroglial cells, CHO cells, and neutrophils. The actin meshwork at the leading edge might serve as a valve, allowing local changes in cell volume by slowing down osmotic water flow (177). AQP4, the main water channel expressed in gliomas is localized to the same lipid rafts as the BK and Cl⁻ channels (186). Chloride influx, not efflux is necessary for cell swelling at the local front resulting in lamellipodium formation in microglial cells (189).

In glioma cells, the amiloride-sensitive Na⁺ current mediated by ASIC1 and ENaC subunits plays a very important role in migration (53-55,124). Blockers of this channel like amiloride and the ASIC1-specific blocker psalmotoxin (PcTX-1) also inhibit the migration of glioma cells as assessed with a Transwell migration assay or a scratch assay (124). Amiloride and PcTX-1 affect the volume regulation of glioma cells by inhibiting regulatory volume increase (RVI) following hyperosmotic shrinkage, but they have no effect on the regulatory volume decrease (RVD) following cell swelling (166). It is not known where this amiloride-sensitive complex is located in a migrating cell, but its function to mediate RVI suggests that it might be functional at the leading edge, mediating the local increase in cell volume required for protrusion of the lamellipodia in 2D in vitro assays (e.g., scratch assays) or invadopodia in the case of 3D migration (e.g., Matrigel Transwell assay) within an ECM.

This channel complex might also affect migration through changing/setting the resting membrane potential of glioma cells, which is depolarized at about -30 to -40 mV compared to other cells (186). A relationship between membrane potential and migration has been shown before. The migration of corneal epithelial cells decreases upon
inhibition of ENaC, which results in membrane hyperpolarization (190). The depolarized membrane potential of glioma cells might open voltage-sensitive Ca$^{2+}$ channels, increasing influx of Ca$^{2+}$, which is required for activation of MLC kinase, which phosphorylates MLC, increasing its contractility and providing the force necessary for cell body translocation and retraction of the trailing edge in migrating cells. In fact, basal [Ca$^{2+}$]$_i$ is higher in glioma cell lines compared to normal astrocytes (191). Glioma cells also show an increased intracellular pH (pH$_i$) compared to normal astrocytes. This is due to Na$^+$/H$^+$ exchanger (NHE) activity (191). pH affects the activity of cofilin. NHE can activate the severing activity of cofilin, by increases in intracellular pH (180). This causes increased number of barbed ends for polymerization of actin and protrusion of the cell membrane. NHE has been localized to the lamellipodia of migrating cells (192).

In \textit{in vitro} Transwell migration and wound healing assays, the ECM is absent, and the effect of the amiloride-sensitive channel complex on migration is probably through affecting the membrane potential of the cell, [Ca$^{2+}$]$_i$, and actin cytoskeletal remodeling through either changes in cell volume or the [Ca$^{2+}$]$_i$. The ENaC/Deg glioma channel complex might interact with ECM components and integrins, regulating the formation of focal contacts, the turnover of which is important for a cell’s ability to move forward through adhesions at the leading edge and deadhesions at the trailing edge. NHE1 functions as a cytoskeletal anchor promoting focal complex assembly. De-assembly at the cell rear depends on the ion translocation function of NHE1, possibly because loss of NHE1 function decreases the activity of the protease calpain, which cleaves integrin-cytoskeletal linkages and thus regulates rear de-adhesion (192). It is possible that the glioma channel complex colocalizes with integrins. This has been
shown for the Kir4.2 channel, which concentrates at the leading edge of migrating CHO cells and colocalizes with integrin α9β1 in focal adhesions. Block or knockdown of Kir4.2 causes the cells to form numerous lamellipodial extensions instead of one dominant lamellipod and to lose directional locomotion (193,194).

Matriptase could locate to the invasion front of migrating cells with the ENaC/Deg channels as well. Matriptase co-localizes with pro-uPA at cell-extracellular matrix contacts, particularly at the invasion front of migrating cells (154). At the invasive edge of the cells, matriptase may serve to recruit and activate uPA, MMP-3, and HGF, which are important in extracellular matrix degradation, adhesion, and cellular motility (150). In normal breast epithelial cells, activated matriptase first appears at cell-cell contacts, and further matriptase activation propagates along cell-cell contacts, a process which depends on actin filaments and the assembly of adherens junctions. However, in breast cancer cells, localization of matriptase is altered. Activated matriptase is not detected only on cell-cell junctions. Both the latent and activated forms of matriptase are located at membrane ruffles, especially after EGF treatment (150).

The extracellular pH in tumors is more acidic than in normal cells (94). It has been recently shown that extracellular acidosis induces zymogen activation of matriptase (195). Some activated matriptase can be detected as early as 1 min after cells are exposed to pH 6.0 buffer, while the majority of latent matriptase is converted to activated matriptase within 20 min. Although generally matriptase activation occurs only at the cell surface, extracellular acidosis activates matriptase inside the cell, probably because of the parallel intracellular acidification. Following matriptase activation, the active enzyme is immediately inhibited by binding to HAI-1 (195).
PKCs are involved in cell migration because they regulate cytoskeletal dynamics, and the functions of surface proteins involved in cell-extracellular matrix interactions and migration. For example, PKCα and PKCε control the trafficking of β1 integrin. PKCε can positively influence MET signaling. MET signaling is activated through binding of HGF to the cMet receptor, after which the complex is internalized in order to cause subsequent activations in the signaling pathway (147). PKCε can recruit the MET signaling pathway members ERK1 and ERK2 to focal complexes, where they mediate HGF-induced migration. ERK1 and ERK2 regulate the dynamics of focal adhesion complexes. The atypical PKCs localize to the leading edge during normal rat kidney cell migration. There they trigger the activation of ERK1, ERK2, and Jun-N-terminal kinase 1 (JNK1), which control the dynamics of focal adhesion complexes (147). Focal adhesion complexes then predict the rates of migration. In migrating astrocytes, formation of the leading edge causes recruitment and activation of Cdc42, which then allows the recruitment and activation of PKCζ, activating downstream signaling events that result in polarization of the microtubule cytoskeleton, which is required for directed cell migration (147).

The glioma ion channel and therefore ASIC1, as a component of the channel, is likely located in a macromolecular complex in the membrane. It is unlikely that this channel complex exists in isolation. Interactions probably occur between this channel complex and the cytoskeleton (ENaC can bind to the actin cytoskeleton (183)), or other interacting proteins (the C terminus of ASIC1 can bind to PICK-1 (95)). Interactions and proximity with other membrane proteins, such as integrins, syntaxin1A, PKCs recruited to the membrane or near this channel complex with PICK-1, and proteases are all likely
to regulate not only many aspects of glioma cell migration, but also this particular channel. Because this channel complex is involved in glioma migration, and it is expressed on high-grade glioma cells but not normal astrocytes, it could make a good therapeutic target. Therefore, it is important to study the regulation of this channel or the component subunits by the many different modulatory proteins that could be in a complex with it, or in its proximity.

In the future, it would be important to continue to study the roles of PKC and matriptase and other possible modulators or interacting proteins in the context of glioma. As far as the role of matriptase in glioma, experiments showing the role of matriptase in the regulation of the amiloride-sensitive current would be very interesting. For example, amiloride-sensitive currents could be measured in glioma cells after knockdown of matriptase or overexpression of an inactive matriptase. Further experiments could be done to determine the role of phosphorylation of ASIC1 or ENaC subunits on the amiloride-sensitive current. For example, amiloride-sensitive currents could be measured after short treatment with a PKC inhibitor or activator, and the effect on expression of channel subunits or phosphorylation status determined by western blots. A better understanding of ASIC1 regulation by PKC and by matriptase would lead to a better understanding of this channel and also the aberrations that occur in glioma cells. The more we know about how these cells behave and use ion channels and their modulators like kinases and proteases to their advantage, the sooner one can design potentially beneficial therapies.
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APPENDIX I

IACUC APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

_Institutional Animal Care and Use Committee (IACUC)_

NOTICE OF APPROVAL

DATE: June 14, 2010

TO: Benos, Dale J.
    MCLM 704 0005
    934-6220

FROM: Judith A. Kapp, Ph.D., Chair
      _Institutional Animal Care and Use Committee_

SUBJECT: Title: Sodium Entry Into Amiloride-Sensitive Epithelia
         Sponsor: NIH
         Animal Project Number: 100609135

On June 14, 2010, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frogs</td>
<td>C</td>
<td>50</td>
</tr>
</tbody>
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Animal use is scheduled for review one year from June 2010. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 100609135 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

_Institutional Animal Care and Use Committee_
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