Acid-sensing ion channels (ASICs) are H⁺-gated members of the degenerin/epithelial Na⁺ channel (DEG/ENaC) family in vertebrate neurons. Several ASICs are expressed in sensory neurons, where they play a role in responses to nociceptive, taste, and mechanical stimuli; others are expressed in central neurons, where they participate in synaptic plasticity and some forms of learning. Stomatin is an integral membrane protein found in lipid/protein-rich microdomains, and it is believed to regulate the function of ion channels and transporters. In Caenorhabditis elegans, stomatin homologs interact with DEG/ENaC channels, which together are necessary for normal mechanosensation in the worm. Therefore, we asked whether stomatin interacts with and modulates the function of ASICs. We found that stomatin co-immunoprecipitated and co-localized with ASIC proteins in heterologous cells. Moreover, stomatin altered the function of ASIC channels. Stomatin potently reduced acid-evoked currents generated by ASIC3 without changing steady state protein levels or the amount of ASIC3 expressed at the cell surface. In contrast, stomatin accelerated the desensitization rate of ASIC2 and heteromeric ASICs, whereas current amplitude was unaffected. These data suggest that stomatin binds to and alters the gating of ASICs. Our findings indicate that modulation of DEG/ENaC channels by stomatin-like proteins is evolutionarily conserved and may have important implications for mammalian nociception and mechanosensation.

Acid-sensing ion channels (ASICs) are non-voltage gated Na⁺ channels found in the central and peripheral nervous systems of mammals (1–7) and other vertebrates (8, 9). The expression of ASIC1, -2, and -3 genes generates H⁺-gated channels as either homomultimers when expressed individually or as heteromultimers when co-expressed in combination. In the central nervous system, ASIC1 and -2 are expressed in regions of synaptic activity where they play a role in synaptic plasticity and learning (10–13). Other ASICs are expressed in peripheral sensory neurons, including specialized sensory nerve terminals (14–16), where they may function as transducers of sensory stimuli. Because ASICs are activated by protons in pH ranges that occur during tissue ischemia or injury, they are candidates to sense acidosis associated with painful conditions such as cardiac ischemia (17, 18), skin (16, 19–21) and muscle inflammation (22), and malignancy (23). In other sensory neurons they may function as mechanotransducers; targeted deletion of ASIC2 or ASIC3 results in mice with altered mechanosensitivity in the skin (14, 16, 20).

ASICs are members of the larger degenerin/epithelial Na⁺ channel (DEG/ENaC) family of ion channels. Two members in the nematode Caenorhabditis elegans, MEC-4 and MEC-10, are required for normal touch sensation (24–26) and generate small constitutive currents when expressed in Xenopus oocytes (27). Additional mec genes that encode intracellular or extracellular proteins are also necessary for touch sensation in the worm. Genetic and direct interaction studies suggest a model of mechanotransduction whereby these accessory proteins function to link MEC-4 and MEC-10 channels to the intracellular cytoskeleton and the extracellular matrix; deformation of these components by mechanical stimuli is thought to open the channel (28, 29). One of these accessory proteins in C. elegans, MEC-2, is an integral membrane protein with a short cytoplasmic NH₂ terminus, a hydrophobic midportion that forms a hairpin turn within the cell membrane, and a longer cytoplasmic COOH terminus (30). mec-2 mutations abolish touch sensation in the worm (31, 32), and genetic studies suggest that it interacts with the channel subunits (MEC-4 and MEC-10) and cytoplasmic microtubules (MEC-7 and MEC-12), forming a link between the channel and the underlying cytoskeleton (30, 33). Heterologous expression studies confirmed that MEC-2 interacts with MEC-4 and MEC-10 and found that MEC-2 potentiates current (27).

Stomatin is the mammalian homolog of MEC-2, sharing 65% identity and 85% similarity. In a rare autosomal dominant hemolytic anemia called stomatocytosis, stomatin is absent in red blood cells, which have increased cationic leak current and swell and lyse prematurely (34–38). However, the role of stomatin in the pathogenesis of the disease is unclear (39, 40). Similar to MEC-2, stomatin interacts with the cytoskeleton in red blood cells (38) and co-localizes with actin in epithelial cells (41). Stomatin has also been shown to bind to the glucose transporter GLUT-1 and decrease the rate of glucose uptake (42). In addition, stomatin is also expressed in sensory neurons (43), and it co-localizes with ASIC-related DEG/ENaC subunits in rat mechanosensory neurons of whisker follicles (44). We therefore tested the hypothesis that stomatin modulates the activity of ASICs.


**EXPERIMENTAL PROCEDURES**

**cDNA Constructs**—Mouse (m) ASIC1α, ASIC1β, and ASIC2b (BNC1) constructs were cloned as described (45, 46). ASIC1 and -2 have alternative splice forms involving the amino termini. Human (h) and m-stomatin cDNAs were cloned by reverse transcription-polymerase chain reaction (PCR) using the following primers: h-stomatin, 5'-ATG GCC GAG AAG CCG CAC ACA-3' and 5'-CTA GCC TAG ATG GCT GTG TTT-3'; m-stomatin, 5'-ATG TCT GTC AAA CCG CAG TCC-3' and 5'-TCA GTG ATG AAC ACC CAT GAT-3'. The constructs were cloned into unique EcoRI and XhoI sites of the mammalian expression vector pMT3 with a FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) at the carboxyl terminus (stomatin-FLAG). Intact protein did not inhibit stomatin interaction with the ASIC3 current (data not shown).

**Absorti** Antisera—Anti-m/hASIC1α, m/hASIC2b, and mASIC3 rabbit polyclonal antibodies have been described previously (10, 16, 47). Anti-FLAG M2 monoclonal antibody was purchased from Sigma. Anti-cystic fibrosis transmembrane conductance regulator (CFTR) antibody (13-1) was purchased from Genzyme (Cambridge, MA).

**Heterologous Expression of cDNA**—For co-immunoprecipitation and co-localization studies, COS-7 cells were transfected by electroporation using 10^7 cells and 20 μg of plasmid DNA. mASIC1α, -2α, or -3 (10 μg) were transfected alone or with m-stomatin-FLAG or empty vector (10 μg). COS-7 cells were maintained in culture with Dulbecco's modified Eagle's medium with 10% fetal calf serum in a humidified atmosphere of 5% CO2 and 95% air at 37 °C. Cells were studied 24 to 72 h after transfection.

For electrophysiological experiments, cDNAs were expressed using 6 μg/ml lentivirus (Dojo Labs, Ithaca, NY) containing the protease inhibitors (10 μg/ml) and 1 μg/ml polybrene. Virus was added to the cells after 24 h and further cultured for 48 h. immortalized CHO cells stably expressing and expressing mASIC subunits were expressed alone or with m-stomatin-FLAG or empty vector (10 μg) and incubated for 2 h followed by incubation with protein A-Sepharose and then washed two times with TBS containing 0.05% Tween 20. Bound anti-ASIC1, anti-ASIC3, or anti-FLAG antibodies were detected by enhanced chemiluminescence (Pierce) in CHO cells grown in 35-mm dishes. mASIC subunits were expressed alone or with m/h-stomatin at the described concentrations. DrRed was co-transfected to keep the total amount of cDNA constant. cDNA for green fluorescent protein (0.33 μg/0.4 ml) was also expressed to facilitate detection of transfected cells by epifluorescence. We have found that >90% of green cells and <5% of green cells have an acid-activated current. Cells were cultured in F12 medium with 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C and were studied 48-72 h after transfection.

**Immunoprecipitation and Immunoblotting**—24 h after transfection, COS-7 cells were solubilized using a glass/Teflon motor-driven homogenizer in radioimmune precipitation assay lysis buffer (1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 10 mM Tris-Cl, pH 7.4, 150 mM NaCl) containing the protease inhibitors (10 μg/ml peptatin A, 20 μg/ml leupeptin, 20 μg/ml aprotinin, and 0.4 mM phenylmethylsulfonyl fluoride). Following ultracentrifugation (100,000 g, 20 min, 4 °C), 500 μg of detergent-soluble protein was precleared and immunoprecipitated using anti-ASIC1 6.4 (10), anti-ASIC2 5.3 (47), anti-ASIC3 3.2 (16), or anti-FLAG antibodies and protein A-Sepharose and then washed two times with TBS containing 0.05% Tween 20. Bound proteins were detected by enhanced chemiluminescence using the related ion channel CFTR. Together, the co-immunoprecipitation and co-localization results suggest that stomatin associates with ASICs using two strategies. First, we asked whether ASICs and stomatin co-immunoprecipitate. ASIC1α, ASIC2a, or ASIC3 was co-expressed with stomatin (containing a FLAG epitope on the carboxyl terminus) in COS-7 cells. When stomatin was immunoprecipitated, all three ASICs were precipitated alone. ASIC3 currents desensitized rapidly in the continued presence of acidic solution (see current returning to base level 100 mV) when expressed without stomatin. Thus, m-stomatin co-expressing 1:0.125 expression ratio (1:8 ratio) caused identical inhibition of the current (data not shown). Thus, m-stomatin was used for the other co-expression experiments.

**RESULTS**

**Stomatin Interacts with ASICs**—We tested whether stomatin associates with ASICs using two strategies. First, we asked whether ASICs and stomatin co-immunoprecipitate. ASIC1α, ASIC2a, or ASIC3 was co-expressed with stomatin (containing a FLAG epitope on the carboxyl terminus) in COS-7 cells. When stomatin was immunoprecipitated, all three ASICs were precipitated alone. ASIC3 currents were desensitized rapidly in the continued presence of acidic solution (see current returning to base level 100 mV) when expressed without stomatin. Thus, stomatin co-immunoprecipitates with each of the ASIC subunits.

Second, we tested whether stomatin and ASICs proteins co-localize by examining their immunostaining patterns. When transfected alone, stomatin localized in a punctate pattern around the nucleus and at the cell membrane (Fig. 2A) in agreement with previous studies (43, 48). ASIC1α, ASIC2α, and ASIC3 expressed alone showed a predominantly reticular pattern of staining. Co-expressing stomatin with individual ASIC proteins altered its cellular localization so it co-localized with the ASIC1α, showing a less punctate and more reticular pattern of staining (Fig. 2B). On the other hand, stomatin did not change its expression pattern or co-localize when co-expressed with the unrelated ion channel CFTR. Together, the co-immunoprecipitation and co-localization results suggest that socket associates with all three ASIC subunits.

**Stomatin Modulates ASIC Currents**—The association of ASIC proteins with stomatin suggested that stomatin might alter ASIC channel function. To test this, we measured acid-evoked currents in CHO cells co-expressing stomatin with ASIC1α, ASIC2a, or ASIC3. When co-expressed with ASIC3, stomatin potent decreased current amplitude (Fig. 3, A and B). A 1:0.125 expression ratio (1 μg of ASIC3:0.125 μg of stomatin cDNA) eliminated the current, which was tested by applying pH 5 and 4 solutions. At a 1:0.0625 ratio (1 μg of ASIC3:0.0625 μg of stomatin), most cells produced small currents with properties that were otherwise similar to those from ASIC3 expressed alone. ASIC3 currents desensitized rapidly in the continued presence of acidic solution (see current returning to base level 100 mV) when expressed without stomatin. Thus, m-stomatin co-immunoprecipitates with each of the ASIC subunits.
This rate was unchanged by co-expression with stomatin at a 1:0.0625 ratio (Fig. 3C), and sensitivity to pH activation was not altered (Fig. 3D). However, given this low stomatin to ASIC3 cDNA expression ratio (1:16), these residual currents may reflect ASIC3 channels not bound to stomatin. As a negative control, we tested protein interacting with C kinase-1 (PICK1), a PDZ protein that binds to ASIC1a and ASIC2a but not ASIC3 (47, 49). At a 1:1 cDNA ratio, PICK1 had no effect on ASIC3 current amplitude (Fig. 3B). Thus, the data indicate that stomatin potently inhibits the ASIC3 acid-evoked current.

Stomatin had a different effect upon ASIC2a. When co-expressed at a 1:1 ratio, stomatin did not decrease current amplitude; in fact, there was an insignificant trend toward increased current amplitude (Fig. 4, A and B). However, stomatin increased the rate of ASIC2a desensitization, which decreased the time constant (Fig. 4, A and C). Stomatin had little effect on pH sensitivity (Fig. 4D).
In contrast to ASIC2a and ASIC3, stomatin did not alter the ASIC1a current. At a 1:1 or 1:10 cDNA ratio (ASIC1a:stomatin), stomatin did not change current amplitude (Fig. 5, A and B), desensitization kinetics (Fig. 5, C), or pH sensitivity (Fig. 5, D). Thus, although stomatin interacted with each of the ASIC subunits, it had differential effects on ASIC channel function, depending on the subunit composition.

ASIC subunits can function as homomultimers or they can heteromultimerize with one another (5, 46, 50–53). Therefore, we asked whether stomatin alters currents generated by channels composed of more than one ASIC subunit. Because stomatin produced the most dramatic effect on ASIC3, we focused on combinations that included this subunit. The fast desensitization kinetics (Fig. 6, A and C) confirm that co-expression of ASIC subunits resulted in the formation of heteromultimeric channels because heteromeric channels desensitize at a faster rate than homomeric channels (46, 53, 54). When we co-expressed ASIC3 with ASIC1a or ASIC2a, stomatin did not alter current amplitude (Fig. 6, A and B) or pH sensitivity (Fig. 6, D). However, stomatin produced small but significant increases in the desensitization rates of both heteromultimeric channels (Fig. 6, C).

**Stomatin Does Not Alter ASIC Cell Surface Expression**—Stomatin could decrease ASIC3 current amplitude by altering its expression at the cell surface or altering its gating. To distinguish between these possibilities, we quantitated ASIC3 expressed at the cell surface by biotinylating cell surface proteins. Following biotinylation, we precipitated proteins with avidin and detected ASIC3 by Western blot analysis. Stomatin did not alter the amount of biotinylated ASIC3, indicating that stomatin did not change ASIC3 surface expression (Fig. 7). Moreover, stomatin co-expression did not alter the total amount of ASIC3 protein (Fig. 1). These results suggest that stomatin reduced ASIC3 current by altering its gating rather than its expression at the cell surface.

**DISCUSSION**

The data indicate that stomatin binds to ASIC1a, -2a, and -3 subunits. Moreover, stomatin altered the function of specific ASIC channels. However, the functional effects of stomatin were strikingly different depending on the subunit. Stomatin had the most prominent effect on ASIC3, potently reducing acid-evoked current. In contrast, stomatin did not significantly decrease current amplitude generated by other ASIC channels; rather, it accelerated the rate of desensitization of ASIC2a homomeric channels as well as ASIC1a+3 and ASIC2a+3 heteromeric channels. Although stomatin interacted with ASIC1a protein, it did not alter ASIC1 current. Thus, binding alone is not sufficient to alter function.
Our data provide important clues for understanding how stomatin modulates the function of ASICs. Stomatin reduced ASIC3 current but did not alter ASIC3 protein level or surface expression. Thus, the data suggest that stomatin inhibits ASIC3 by altering its gating. Also consistent with this mechanism, stomatin increased the desensitization rate of ASIC2a homomeric channels and ASIC1a+3 and ASIC2a+3 heteromeric channels. Thus, stomatin is an accessory subunit that interacts with ASIC channels at the cell surface to modulate gating.

What is the mechanism whereby stomatin alters ASIC gating? The capacity of stomatin to bind to ASICs and accelerate channel desensitization is reminiscent of the role of accessory β subunits to facilitate inactivation of voltage-gated channels (55). In a similar manner, stomatin may function as an accessory subunit that directly modulates gating. Alternatively, stomatin could alter ASIC gating indirectly through the interaction of other proteins. Stomatin is highly expressed in mammalian lipid rafts (detergent-resistant, cholesterol- and sphingomyelin-rich regions of the membrane), which are important for sequestering proteins into complexes and localizing signal transduction processes (41, 56, 57). Recent experiments in C. elegans demonstrate that the stomatin-related proteins UNC-1 and UNC-24, along with the DEG/ENaC subunit UNC-8, are located in lipid rafts. Moreover, genetic disruption of UNC-1 abolished UNC-8 expression in lipid rafts (58). Stomatin might localize ASICs to specific membrane microdomains to facilitate interactions with other regulatory and signaling proteins.

ASIC activity can be modulated by several endogenous ligands and signaling molecules (45, 59–63). However, less is understood regarding intracellular proteins that directly bind to and affect ASIC function. Yeast two-hybrid screening recently identified two proteins that interact with ASICs. In contrast to stomatin, both proteins potentiate ASIC currents. PICK1 interacts with ASIC1 and ASIC2 (47, 49) and potentiates current via ASIC2a when stimulated by protein kinase C (64). Channel-interacting PDZ domain-containing protein (CIPP) binds to the COOH terminus of ASIC3 and increases current (65). In addition, the ASIC1a and -2a heteromeric current is potentiated by co-expression of CFTR (66). Earlier studies suggested that ASICs might share a conserved function with their homologs in C. elegans, the degenerins. Mutations of the genes encoding DEG channel subunits MEC-4 and MEC-10 disrupt light touch in specialized mechanosensory neurons (24–26). Similarly, targeted deletion of ASIC2 and ASIC3 genes produced altered single fiber sensory nerve responses to cutaneous mechanical stimuli in mice (14, 16). Our data identifies another conserved feature; both DEG and ASIC channels are modulated by stomatin-related proteins. Similar to our data, the stomatin homolog MEC-2 co-immunoprecipitated with DEG channels, modulated current, and did not alter channel surface expression when co-expressed in Xenopus oocytes (27). However, it is interesting that MEC-2 and stomatin produced opposite effects on current. MEC-2 (and to a lesser extent stomatin) increased constitutive current generated by DEG channels (27), whereas stomatin...
inhibited ASIC3 such that H\(^+\) no longer activated the channel. Given the purported role of DEG channels and MEC-2 in mechanosensations in \textit{C. elegans}, it is intriguing to speculate that the interaction of stomatin with ASIC3 changes the channel from a pH sensor to a mechanosensor.

Modulation of ASICs has important implications. As H\(^+\)-gated channels, they are poised to sense acidosis associated with such painful conditions as inflammation, ischemia, or malignancy; thus modulation of ASICs by stomatin might be an important means to regulate nociception. ASICs also function in sensation of mechanical stimuli. Thus, stomatin might play an important role in mammalian mechanosensation, analogous to the purported role of their homologs in \textit{C. elegans}.

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