Supporting Information

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SI Text

Constructs and Reagents. The WT mouse ASIC1a construct was described earlier (1). HA-tagged ASIC1a was made by adding the influenza hemagglutinin epitope (YPYDVPDYAGV) to the N terminus of ASIC1a. ASIC1a point mutations were generated using a Quick-Change Mutagenesis Kit II (Stratagem) following the manufacturer's instructions. An ASIC1a-ASIC1a fusion was made using standard procedures; the encoded protein contained 2 full-length copies of ASIC1a with no linker sequence. ASIC1a-GFP was made by adding eGFP to N terminus of ASIC1a.

H_2O_2, DTT, and NEM were purchased from Sigma or CalBiochem. DTSSP, Sulfo-EGS, NHS-biotin, and NeutrAvidin were from Pierce. Culture media were from Gibco. Rabbit anti-ASIC1a MTY antibody was described earlier (2). Other antibodies used were goat anti-ASIC1a antibody (Santa Cruz Biotechnology), mouse and rabbit anti-HA (Abcam, Santa Cruz Biotechnology, or Sigma), monoclonal anti-tubulin (University of Iowa Developmental Hybridoma Bank), monoclonal anti-actin and anti-biotin (Sigma), HRP-conjugated secondary antibodies (Sigma, Amersham, and Santa Cruz Biotechnology), and Alexa-680 or -800-conjugated secondary antibodies (Li-cor and Invitrogen). All other reagents were from Sigma, Calbiochem, Pierce, or BD Biosciences.

Transfection, Surface Biotinylation, Cross-Linking, and Cell Lysis. Transfection was done with Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Biotinylation was performed 2 to 3 days after transfection. Cells were washed 3 times with ice-cold PBS+/+ solution, and 3 mL of 0.5 mg/mL NHS-biotin in PBS+/+ solution was added to each 10-cm dish, followed by incubation at 4 °C for 30 min with gentle rocking. Cells were washed once with PBS+/+ solution, and 0.1 M glycine in PBS+/+ solution was added to quench the reaction, followed by 2 washes with PBS+/+ solution.

When needed, cross-linking of surface proteins was performed 2 days after transfection. Cells were washed 3 times with ice-cold PBS+/+ solution, and cross-linking reagents (Sulfo-EGS or DTSSP) were added in 3 mL to each 10-cm dish, followed by incubation at 4 °C for 30 min with gentle rocking. Cells were washed twice with PBS+/+ solution, and 50 mM Tris, pH 7.4, was used to quench the reaction. Biotinylation of surface proteins was then performed.

For precipitation of surface proteins, cells were lysed in NEM lysis buffer [30 mM NEM in PBS+/+ solution (Invitrogen)] with 1% Nonidet P-40, 0.5% deoxycholate, 0.5% SDS, and freshly added proteinase inhibitors (Roche). For immunoprecipitation, SDS was not included in the lysis buffer. Cell lysate was sonicated briefly and cleared by centrifugation. Protein concentrations were quantified using a modified Bradford assay kit (Bio-Rad).

NeutrAvidin Pull-Down and Immunoprecipitation. For NeutrAvidin pull-down, 60 μL of a 50% slurry of NeutrAvidin beads were added to 200 μL of cell lysate and incubated at 4 °C overnight with gentle rotation. Beads were washed 3 times with wash buffer (Tris 50 mM, pH 7.4, 1% Triton X-100). Beads were then boiled in 80 μL of SDS sample buffer with or without reducing agent. Thus, when loaded in equal volume, the amount of total lysate loaded was approximately 20% that of the surface fraction.

For immunoprecipitation, Tris was added to cell lysate to a final concentration of 50 mM to quench excess NEM reagents. Two microliters of anti-HA was added to 200 μL of cell lysate and incubated at 4 °C overnight with gentle rotation. Antibody complex was then captured by 50 μL of protein A or protein G beads. Beads were washed 3 times with wash buffer. Beads were then boiled in 80 μL of SDS sample buffer with or without reducing agent.

Brain Lysate. Whole mouse brain or different brain regions were isolated rapidly on ice following decapitation. Brain tissues were homogenized in NEM lysis buffer by brief sonication. Lysates were cleared by centrifugation and quantified using a modified Bradford assay kit (Bio-Rad). Mice were WT or ASIC1a$^{-/-}$ on a congenic C57/BL6 background and were studied at P6 or as adults (5–8 months old). All animal protocols complied with the regulations and guidelines of the University of Iowa Animal Care and Use Committee.

SDS PAGE, Western Blot, and Quantification. Non-reducing and reducing SDS PAGE gels were run separately. After gel electrophoresis, transfer to PVDF membrane was done with a modified transfer buffer (1×TBS + 40 g glycine per liter of transfer buffer, no methanol) to facilitate the transfer of high molecular weight proteins. Transfer was done at 4 °C overnight. Standard Western blots were performed. Antibody dilutions were: anti-MTY, 1:4,000–5,000, goat anti-ASIC1a (Santa Cruz Biotechnology), 1:1,000; anti-HA antibodies, 1:1,000; monoclonal anti-biotin (Sigma), 1:1,000; and secondary antibodies, 1:10,000. Chemiluminescence was done with a West Pico kit (Pierce), following manufacturer's instructions. Alternatively, we used Alexa-conjugated secondary antibodies and detected with a laser scanning imager (Odyssey; Li-cor) following manufacturer's instructions. Quantification was performed in ImageJ as described earlier (1). In some cases the monomers migrated as doublet or triplet bands, and we quantified all of them together as band M. Error bars represent SE.

Electrophysiology. Expression of ASIC channels in *Xenopus* oocytes was performed by injecting cDNA into the nucleus of the de-folliculated albino *Xenopus* leaves oocyte as described earlier (3). Each oocyte received 0.2–0.3 ng of plasmid and was incubated in ND-96 solution for 2 to 3 days before recording. Whole-cell currents were recorded with 2-electrode voltage clamps using an Oocyte Clamp OC-725C amplifier (Warner Instrument), digitized with DigiData 1200 series (Axon Instrument), and analyzed with pClamp 8 (Axon Instrument). Oocytes were bathed in frog Ringer solution containing (in mM) 116 NaCl, 2 KCl, 0.4 $CaCl_2$, 1 $MgCl_2$, 5 Hepes, and 5 MES, pH 7.4. pH was adjusted with HCl. Each experiment was repeated with at least 3 batches of oocytes from different frogs.

For intracellular application of H_2O_2, oocytes were impaled with glass microelectrodes filled with H_2O or concentrated H_2O_2. Small volumes of solution were delivered by applying 20-psi pulses to the electrodes with a pneumatic PicoPump (PV820 World Precision Instruments) for 50 msec. Pulses were applied before impaling, after impaling, during recording, and after recording to visually confirm proper solution delivery. Peak transient currents in response to extracellular pH 6.5 were recorded before and after H_2O_2 delivery.

Data are reported as means ± SEM. Statistical significance was assessed with the unpaired Student *t* test.

1. Zha X-M, Wemmie JA, Green SH, Welsh MJ (2006) ASIC1a is a postsynaptic proton receptor that influences the density of dendritic spines. *Proc Natl Acad Sci USA* 103:16556–16561.
2. Wemmie JA, *et al.* (2002) The acid-activated ion channel ASIC contributes to synaptic plasticity, learning, and memory. *Neuron* 34:463–477.
3. Snyder PM, Cheng C, Prince LS, Rogers JC, Welsh MJ (1998) Electrophysiological and biochemical evidence that DEG/ENaC cation channels are composed of nine subunits. *J Biol Chem* 273:681–684.

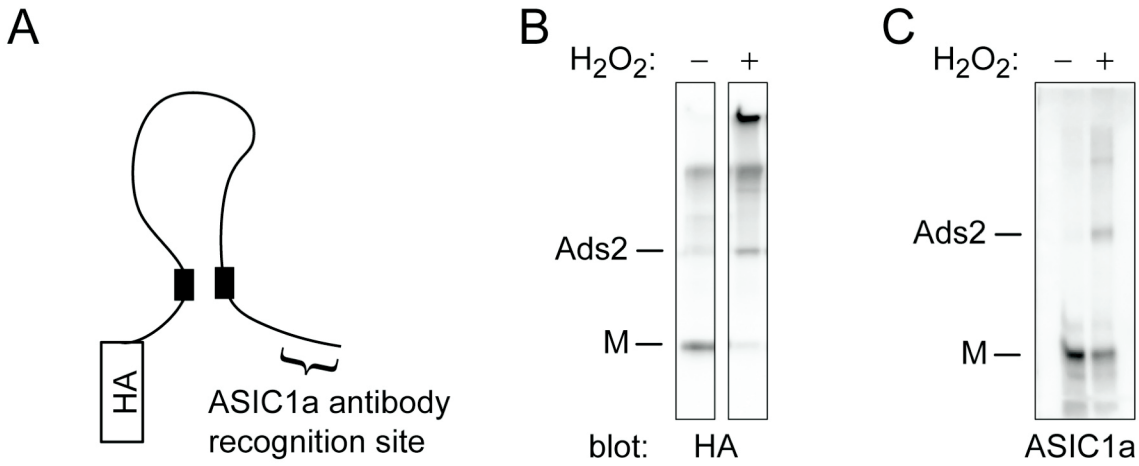

Fig. S1. An antibody to the ASIC1a C terminus weakly detects inter-subunit disulfide-linked subunits. (*A*) Diagram showing the HA-ASIC1a construct and the relative position of the epitopes recognized by antibodies against ASIC1a and HA tag. (*B* and *C*) CHO cells were transfected with HA-ASIC1a and treated with H_2O_2. Total lysates were separated using non-reducing condition and blotted with anti-HA (*B*) or anti-ASIC1a (*C*) antibody that recognizes the C terminus of ASIC1a. Note that the anti-HA antibody detected both the Ads2 and monomeric bands and revealed a significant H_2O_2-dependent shift. In contrast, the anti-ASIC1a antibody preferentially detected the monomeric band.

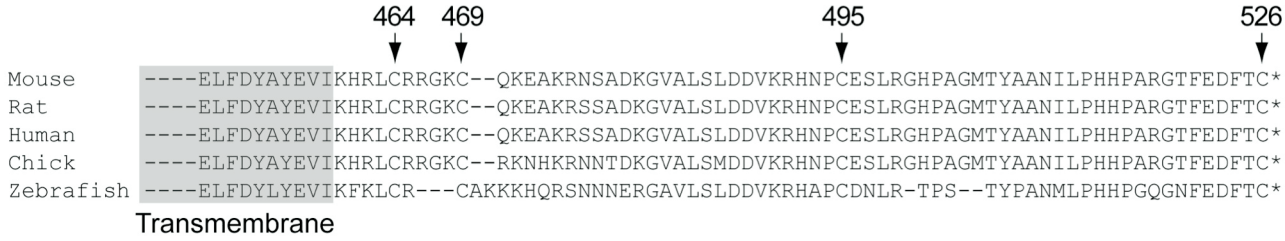

Fig. S2. Sequence of C-terminal portion of ASIC1a from several species. The second transmembrane domain is in gray and C-terminal cysteines are indicated.