Supplement Material for:

Hattori T, et al. ASIC2a and ASIC3 heteromultimerize to form pH-sensitive channels in mouse cardiac DRG neurons

EXPANDED MATERIALS AND METHODS

Labeling of mouse cardiac afferents

8 – 12 week old C57BL/6 or ASIC null mice were anesthetized with pentobarbital sodium (60 mg/Kg intraperintoneal injection), intubated and ventilated (Hugo Sachs Elektronik, Type 845), and the heart was exposed through a left lateral thoracotomy. We initially attempted to mimic our previous protocol in rat by injecting 5 μl of a suspension of 1,1'-di-octtadecyl-3,3',3'-tetramethyl indocarbocyanine perchlorate (DiI; Molecular Probes) in sterile saline solution into the pericardial space.[1] Two weeks after injection, the mice were euthanized, and the heart and lungs were sectioned and viewed under a fluorescence microscope. Supplementary Figure 1A shows the surface of the heart stained with dye, however the surface of the lungs were also stained indicating that dye had leaked from the pericardial sac. This probably occurred because the pericardial sac of mouse is substantially more porous than that of rat.[2] To overcome this problem, we injected a mixture of DiI-coated polystyrene microbeads into the pericardial space. 0.4 ml of DiI solution (5mg/ml in dimethylsulphoxide) was sonicated with 10 mg of beads (diameter 7.32 μm; Bang Laboratories) for 5 minutes. The beads were washed with water 3-4 times until the supernatant was clear, and then resuspended in 20 μl of sterile saline. Two weeks after injection of 10 μl of this bead suspension into the pericardial space, we saw adherence of DiI-stained beads to the outside of the heart, and no staining of the lungs (supplementary Figure 1B). All animal procedures were followed in accordance with institutional guidelines.

Culture of mouse NG and DRG Neurons

2 to 3 weeks after the injection of DiI-beads suspension, the mice were euthanized, and the right and left DRG (C_8-T_3) and/or NG were collected, and dissociated with papain, and collagenase/dispase solutions successively as previously discribed.[3] After trituration, the cells were plated on poly (D-lysine)- and laminin-coated plastic, and stored at 37°C in F12 medium supplemented with nerve growth factor. Cardiac sensory neurons were identified by fluorescence microscopy (supplementary Figure 1C). Cells were studied 18–48 h after plating.

Generation of ASIC knockout mice

The generation of mice missing individual ASIC1, -2, and -3 subunits has been reported.[4-6] Subsequently, these mice were produced on a C57BL/6J congenic background, and crossed to generate mice with the simultaneous disruption of multiple ASIC subunits. Mice missing ASIC2 and -3 (ASIC2/3 -/-) were easier to produce and maintain if they were heterozygous for ASIC1, thus the ASIC2/3 -/- mice used in this study are ASIC1 +/- and ASIC2/3 -/-.

Heterologous Expression of cDNA in CHO Cells

Mouse ASIC1a, ASIC2a, and ASIC3 in pMT3 were cloned as previously described.[3, 7] Chinese hamster ovarian (CHO) cells plated at ~ 10% confluence were transfected with ASIC cDNAs (2 mg/1.5ml) using Transfast transfection reagent (Promega, Madison, WI) in 35 mm dishes according to the manufacturer's recommendations. GFP cDNA (0.33 mg/1.5ml) was cotransfected to facilitate detection of expressing cells by epifluorescence. Coexpressed ASIC cDNAs were transfected at equal concentrations for a total of 2 mg/1.5ml. Cells were cultured in F12 nutrient medium (GIBCO) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C, 5% CO_2 and were studied 48-72 hours after transfection.

Analysis of DRG mRNAs by qPCR
Total cellular mRNA was isolated using the RNeasy mini kit (Qiagen), according to the manufacturer's protocol. cDNA synthesis was performed using the AffinityScript qPCR cDNA Synthesis kit (Stratagene) followed by qPCR using the Brilliant II SYBR Green qPCR Master Mix (Stratagene). The qPCRs were run in triplicate with cycle threshold values averaged, the data were analyzed using the ΔΔCt method. mRNA encoding 36B4 was used as the invariant control. qPCR primer sequences are listed in Table 1 of Online Data Supplement.

Electrophysiology
Whole-cell patch-clamp recordings (at -70 mV) from NG and DRG neurons, and CHO cells were performed at room temperature with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) and were acquired and analyzed with PULSE/PULSEFIT 8.70 (HEKA Electronics, Lambrecht, Germany) and IGOR PRO 6.01 (WaveMetrics, Lake Oswego, OR) software. Currents were filtered at 5 kHz and sampled at 2 or 0.2 kHz. Micropipettes (2–4 MW) were filled with internal solution (mmol/L): 100 KCl, 10 EGTA, 40 HEPES, and 5 $MgCl_2$, pH 7.4 with KOH. External solution contained (mmol/L): 120 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 MES; pH was adjusted with tetramethylammonium hydroxide, and osmolarity adjusted with tetramethylammonium chloride. Extracellular solutions were changed within 20 msec by using a computer-driven solenoid valve system.[3] Kinetics of desensitization were fit to with single exponential equations and time constants (τ) reported. pH activation curves were fit using the Hill equation: Fraction of open channels = $1/\{1+(pH^{10}/K_{0.5}{}^{10})^n\}$, where $K_{0.5}$ is the pH at which half of the channels are opened. Data are means ±SEM. Statistical significance was assessed using unpaired Student's *t*-test.

ONLINE TABLE AND FIGURE LEGENDS

Online Table 1. Primers used in quantitative real-time PCR.

Online Figure 1. Flourescent labeling of mouse cardiac afferents. Corresponding phase (left) and fluorescence (right) micrographs are shown. A, Heart (top) and left lung (bottom) after injection of DiI suspension (without microbeads) into the pericardial sac. Both the epicardium of the heart and visceral pleura of the lung are stained with dye (red), indicating DiI leakage from the pericardial sac. B, Heart (top) and left lung (bottom) and C, a DiI-labeled cardiac DRG neuron in primary dissociated culture two weeks after intra-pericardial injection of DiI-beads suspension (see methods). Scale bars represent 0.5 mm for A and B, and 50 μm for C.

SUPPLEMENTAL REFERENCES

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7. Askwith CC, Cheng C, Ikuma M, Benson C, Price MP, Welsh MJ. Neuropeptide FF and FMRFamide potentiate acid-evoked currents from sensory neurons and proton-gated DEG/ENaC channels. *Neuron*. 2000;26:133-141.

Online Table 1. Primers used in quantitative real-time PCR.

Subunit	NCBI Accession #	QPCR Primers	Product Length
ASIC1a	BC067025	5' CCTGCTCAACAACAGGTATG 3' 5' GAACTCACGCATGTTGAAGG 3'	124 bp
ASIC1b	AB208022	5' CCTGTGGTCCCCACAACTTC 3' 5' GTTGCCAGTCCCACCTTTCA 3'	117 bp
ASIC2a	AF348465	5' GGGCATCAAGACTTCACCACAGTGTT 3' 5' TAACTCAGGCGAGGATGGCAA 3'	74 bp
ASIC3	NM_183000	5' ATGTTGCTGGACTGCCGATA 3' 5' GCACCAGAGTTGAAGGTGTA 3'	101 bp
36B4	NM_007475	5' CACTGGTCTAGGACCCGAGAAG 3' 5' GGTGCCTCTGGAGATTTTCG 3'	73 bp

Online Figure 1