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Characterization of Acid-Sensing Ion Channel Expression in Oligodendrocyte-Lineage Cells

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Abstract

Acid-sensing ion channels (ASICs) are widely expressed in neurons, where they serve in pain and mechanical sensation, and contribute to learning and memory. Six ASIC subunit proteins form homo- or heteromeric channel complexes with distinct physiological properties. Of such complexes, only monomeric ASIC1a channels are Ca²⁺ permeable. Prior pharmacologic and genetic studies have shown that ASIC1a channel inactivation markedly diminishes CNS susceptibility to ischemic damage. Here, we characterize ASIC expression in oligodendrocyte lineage cells (OLC) by molecular, electrophysiological, calcium imaging, and immunofluorescence techniques. ASIC1a, ASIC2a, and ASIC4 mRNAs were expressed in cultured rat OLC, with steady-state levels of each of these mRNAs several-fold higher in oligodendroglial progenitors than in mature oligodendroglia. ASIC transcripts were also detected in brain white matter, and ASIC1a protein expression was detected in white matter oligodendroglia. Inactivating, proton-gated, amiloride-sensitive OLC currents were detected by whole-cell voltage clamp. These currents showed profound tachyphylaxis with slow recovery, and were predominantly blocked by psalmotoxin, indicating that homomeric ASIC1a comprised a large fraction of functional ASIC in the cultured OLC. ASIC activation substantially depolarized OLC plasma membrane in current clamp studies, and elicited transient elevations in intracellular Ca²⁺ in imaging studies. Thus, OLC ASIC1a channels provide a means by which an acid shift in CNS extracellular pH, by diminishing plasma membrane potential and increasing Ca²⁺ permeability, can activate OLC signaling pathways, and may contribute to OLC vulnerability to CNS ischemia.

Keywords

ischemia; inflammation; intracellular calcium

INTRODUCTION

Acid-sensing ion channels (ASICs) are receptors that respond to extracellular pH < 7.0 by generating desensitizing inward currents. ASICs have been described in many types of neurons

throughout the central and peripheral nervous systems (reviewed in Krishtal, 2003). ASIC complementary DNAs (cDNAs) have been isolated from several neural tissues. The genes are related to the amiloride-sensitive epithelial sodium channel family in vertebrates and the degenerin family of *C. elegans*. Four different genes encode ASICs 1–4, and splice variants of ASICs 1 and 2 are known, giving rise to at least six ASIC proteins. These proteins, which vary in tissue distribution, have intracellular N- and C-termini and two hydrophobic transmembrane domains flanking a large, cysteine-rich extracellular domain. The channels are believed to be multimeric structures, either homomers or heteromers. Alternate splicing, together with assembly of heteromers creates channels with diverse functional properties that are regionally and developmentally regulated. Whereas ASIC1a, ASIC1b, and ASIC2a expressed alone can form functional acid-responsive channels, ASIC2b does not form functional channels alone but can alter the properties of other functional ASICs when co-expressed. ASICs 1a, 2a, and 2b are expressed widely in the both CNS and PNS; ASIC1b and ASIC3 are found mainly in sensory ganglia (reviewed in Krishtal, 2003; Lingueglia et al., 2006; Waldmann et al., 1998).

Although all ASICs are blocked by amiloride and its derivatives, only homomeric ASIC1a channels are blocked by the tarantula venom toxin psalmotoxin (Diochot et al., 2007; Escoubas et al., 2000). Additionally, other functional properties including ionic selectivity, pH sensitivity, and kinetics of activation and desensitization, vary depending on the subtype. All functional ASIC channels conduct Na⁺ ions predominantly, but some subtypes show appreciable K⁺ or Ca²⁺ permeability. Homomeric ASIC1a is uniquely calcium permeant (Gunthorpe et al., 2001; Waldmann et al., 1997). It was recently shown that mice lacking ASIC1a show reduced neuronal cell loss under ischemic, acidotic conditions in the brain (Xiong et al., 2004, 2006), presumably due to reduced calcium influx. Furthermore, intracerebroventricular or intranasal administration of psalmotoxin has been shown to be neuroprotective under these conditions (Pignataro et al., 2007).

Oligodendrocyte lineage cells (OLC), particularly at early developmental stages, are vulnerable to excessive calcium overload, which leads to death and myelin loss (Deng et al., 2003; Follett et al., 2004; Itoh et al., 2002; Karadottir et al., 2005; McDonald et al., 1998; Salter and Fern, 2005; Sanchez-Gomez and Matute, 1999). In light of the role of ASICs in neuronal damage, it would be of interest to know whether OLC express ASIC channels and, if so, whether they play a role in OLC pathology. There is one early report that OLCs show responses to acidic extracellular medium with the properties of ASICs (Sontheimer et al., 1989), but the molecular basis of these acid-induced currents have not been previously addressed. We have therefore studied the expression of ASICs and extended the functional characterization of OLC using a combination of molecular biology, electrophysiology, and calcium imaging.

MATERIALS AND METHODS

Oligodendrocyte Lineage Cultures

Primary OLC cultures were prepared from 1-day-old postnatal Sprague–Dawley rats by a panning method as previously described (Itoh et al., 2002). Briefly, mixed glial cultures prepared from enzymatically and mechanically dissociated, subcortical, rat brain were grown for 5 days in the presence of growth medium (GM) supplemented with 30% B104 neuroblastoma conditioned medium, L-glutamine (6 mM), biotin (10 ng/mL), insulin (5 µg/mL), transferrin (50 µL/mL), sodium selenite (30 nM), progesterone (20 nM), and putrescine (100 µM). Confluent mixed glial cells were resuspended and purified by two cycles of negative panning with a RAN-2 antibody and one cycle of positive panning with an A2B5 antibody. As previously described (see Itoh et al., 2002), the resulting oligodendrocyte progenitor (OP) cultures were 95% A2B5 positive, and O4- and glial fibrillary acidic protein-negative (see Fig. 1). A2B5 (Eisenbarth et al., 1979) and O4 (Sommer and Schachner, 1982) primary antibodies were collected as supernatants from hybridomas. Anti-myelin basic protein was obtained from

Novus (Littleton, CO.) Progenitor cells were expanded by proliferation in GM further supplemented with 30% B104 neuroblastoma conditioned medium, PDGF (1 ng/mL), and FGF2 (5 ng/mL) up to three passages. Immature oligodendrocytes were produced by substitution of GM with unsupplemented differentiation medium (DM) for 2 days, and mature oligodendrocytes (MOs) by substitution with DM for 4 days.

Reverse Transcriptase Polymerase Chain Reaction and Quantitative PCR

Total RNA was isolated from OLC cultures by a standard Trizol method, followed by repurification with RNeasy kits (Qiagen, Valencia, CA). Passage 3 OP cultures (as earlier) were grown in T-75 flasks with GM supplemented with 30% B104 neuroblastoma conditioned medium, PDGF (1 ng/mL), and FGF2 (5 ng/mL). Substitution of GM with DM, lacking conditioned medium and growth factors, produced IO cultures after 2 days and MO after 4 days. Rat forebrain total RNA was similarly prepared from neonatal whole forebrain samples. White matter (optic nerve and corpus callosum) and cortical grey matter tissues were dissected from adult rat brains, and RNA was extracted from these tissues with RNeasy Lipid Tissue kits (Qiagen). RNA was diluted to 1 µg/mL total RNA, estimated by A₂₆₀. Reverse transcription reactions for conventional RT-PCR were performed with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) primed with oligo-dT. Conventional PCR was performed with Platinum TAQ hot-start polymerase (Invitrogen) in 50 µL reactions, using 2 µL (10%) of RT reaction as template. The primers employed are shown in Table 1. The cycling parameters used were as follows: 2 min, 94°; 35 cycles of [30 s, 94°; 30 s, 55°; 1 min, 72°]; 10 min, 72°. Agarose (2%) minigels were loaded with 10 µL PCR product, and stained with SYBR Gold (Invitrogen) and imaged with a CCD camera-based gel documentation system (Kodak, Rochester, NY).

Quantitative RT-PCR was performed using Taqman hydrolysis probes (ABI, Foster City, CA), as specified in Table 1. Random primed cDNA templates were prepared with Stratascript or Accuscript First Strand Synthesis kits (Stratagene, La Jolla, CA). Amplitaq Gold-based chemistry (ABI) was used, together with 0.5 µL cDNA reaction as substrate, in 25 µL reactions, and run on a Stratagene MPC3005 thermocycler. Relative levels of gene expression were estimated by a delta C_T method, where the threshold cycle (C_T) for each ASIC probe, from each tissue/developmental stage cDNA template, was normalized to the C_T for the GAPDH housekeeping probe determined for the same template. Levels were expressed by the formula:

$$2^{-(C_{\text{TASIC gene}} - C_{\text{T GAPDH}})}$$

Immunofluorescence

Mice expressing an enhanced green fluorescent protein (EGFP) transgene driven by the 2,3-cyclic nucleotide-3-phosphohydrolase (CNPase) promoter in oligodendroglial progenitors and oligodendroglia (CNPase-EGFP mice) (Yuan et al., 2002) were backcrossed to a C57BL/6 background for at least 10 generations prior to use. Two-month-old CNPase-EGFP mice were deeply anesthetized with ketamine/xylazine, then perfused with saline, followed by phosphate-buffered 4% (w/v) paraformaldehyde. Brains were removed then post-fixed at 25°C with phosphate-buffered 4% paraformaldehyde for 1 h. Brains were transected coronally at the level of the corpus callosum and cryoprotected in 30% sucrose for 2 days prior to embedding in OCT. Ten-micrometer frozen sections were cut and subjected to immunohistochemical analysis as follows. Sections were incubated with affinity purified rabbit anti-ASIC1 polyclonal antibodies (Alpha Diagnostic International, San Antonio, TX, Cat. no. ASIC11-A used at 20 µg/mL; Alomone Labs, Jerusalem, Israel, Cat. no. ASC-014) or purified rabbit immunoglobulins (control) overnight at 4°C. The Alpha Diagnostic antibody is selective for ASIC1a, and the Alomone antibody recognizes either ASIC1a or ASIC1b. Following incubation with biotin conjugated donkey anti-rabbit secondary antibody (species specific,

Jackson Immunoresearch), the sections were treated with rhodamine conjugated streptavidin (Jackson, diluted 1:500) for 20 min. The sections were post-fixed with -20°C methanol and counterstained with Hoechst 33258. Between all steps the sections were washed $5\times$ with phosphate buffered saline. Fluorescent images were captured with a $40\times$ objective by confocal microscopy (Nikon C1). Figures represent projections of 15 serial 300-nm optical sections.

Electrophysiology

Whole-cell patch clamp recordings were performed on OLC cultures grown on poly-L-lysine-coated glass coverslips, under observation on an upright Nikon E660FN microscope equipped with a $40\times$ water-dipping objective, and differential interference contrast optics. The coverslip was placed in a perfusion chamber with a coverslip bottom (Warner Instrument, Hamden, CT), which was bulk-perfused ($\sim 0.4\text{ mL min}^{-1}$) with physiological external solution of the following composition (in mM): NaCl, 130; KCl, 5.4; CaCl_2 , 1.8; MgCl_2 , 1; HEPES, 25; D-glucose, 20; pH = 7.4, adjusted with NaOH. Internal pipette solutions were either Cs^+ or K^+ based, and had the following composition (in mM): CsCl or KCl, 130; MgCl_2 , 2; EGTA, 10; HEPES, 10; pH = 7.4, adjusted with either CsOH or KOH. The local external solution surrounding recorded cells was rapidly changed to alter the external pH, or apply drugs/toxins, with the aid of a multireservoir, pressurized, solenoid-valve driven microperfusion apparatus (Automate Scientific, Berkeley, CA). A flexible glass-polyimide tip, $100\text{ }\mu\text{m}$ ID, was mounted on the supplied perfusion pencil. The tip of this delivery system was brought to within $\sim 20\text{ }\mu\text{m}$ of the cell under study, producing a local laminar stream 10–20 times the diameter of the cells. A continuous stream of pH = 7.4 HEPES-buffered solution was locally applied for control conditions. This solution was of similar composition to the extracellular solution given earlier, except that the CaCl_2 concentration was reduced to 1 mM to minimize the Ca^{2+} block of ASICs (Immke and McCleskey, 2003), and glucose was replaced by D-mannitol. Acid-shifted solutions had identical composition, except that for solutions with pH < 7, MES buffer (25 mM) was used instead of HEPES. Our standard ASIC activating solution was buffered at pH 5.5, but other pH (5.2–6.0) was occasionally employed when appropriate, as described in the text. Qualitatively similar results were obtained regardless of the specific acidic pH employed. Amiloride was dissolved in these extracellular solutions from a 100 mM stock in DMSO. *Psalmopoeus* venom, reconstituted from a lyophilized stock (Spider Pharm, Yarnell, AZ) in 0.05% bovine serum albumin, was diluted 1:20,000–1,000,000 in the pH 7.4 buffer for application. Synthetic psalmotoxin peptide (PcTx1) was obtained from either Peptides International (Louisville, KY) or Phoenix Pharmaceuticals (Burlingame, CA) and was prepared as aqueous stock solutions from the lyophilized peptide. PcTx1 was used at 10 or 20 nM in the pH 7.4 application buffer, without added carrier protein. The osmolality of all solutions was measured with an osmometer (Wescor, Logan, UT) and adjusted to $\sim 315\text{ mosm/kg}$ with D-mannitol. The local perfusion system could exchange the extracellular solution within $\sim 50\text{ ms}$.

Recordings were made with fire-polished, Sylgardcoated capillaries (Clark no. 8520, Warner Instrument) that had resistances of 1.2–2.5 M Ω . The signal was measured with an Axopatch 200B; voltage stimuli, including software control of the Automate microperfusion system, were generated by, and signals digitized, with pClamp 9.2 software (Molecular Devices, Sunnyvale, CA). Membrane capacitances were measured with the membrane test routine of pClamp. When that measurement was unavailable (due to occasional nonconvergence of the routine) capacitance was estimated from the telegraphed readout of the whole-cell capacity compensation dial of the Axopatch 200B. Cells were held nominally at -60 mV in voltage clamp experiments, except for brief clamp to -90 mV during applications of acid-shifted solution. Signals were not adjusted for liquid junction potentials, which were calculated to be 2.5 mV with KCl-based pipette solution, and 3.1 mV with CsCl-based solution, such that true transmembrane potentials would be shifted negative to the nominal command

or measured values. Data were analyzed with pClamp, Excel (Microsoft, Redmond, WA) and Origin (OriginLab, Northampton, MA).

Calcium Imaging

OP cultures on poly-L-lysine coated glass coverslips were incubated 40–50 min at 23°C in glucose-containing, pH = 7.4, physiological saline (composition as described earlier) with fluo-4-AM (Invitrogen) at 2 µg/mL, supplemented with Pluronic-F127 (Invitrogen), 0.02%. Cells were mounted in the same chamber as used for electrophysiology, perfused with pH = 7.4 buffered saline for at least 30 min prior to initiating imaging to remove excess fluo-4 and allow for intracellular hydrolysis. Cells were locally perfused with the 100-µm tip, under conditions and with the same solutions as described earlier for electrophysiology experiments. Cells were imaged with the 40× water dipping objective (NA = 0.8) on the Nikon E600FN microscope, and illuminated by a xenon arc lamp (Sutter LS; Novato, CA) through an epifluorescence filter set excited at 500 nm and emitting at 542 nm (filter set YFP-2427A, Semrock, Rochester, NY). Images were detected by a Coolsnap ES (Photometrics, Tucson, AZ) interline CCD camera, and acquired with SimplePCI software (Hamamatsu, Bridgewater, NJ). Images were acquired at ~0.5–1 s⁻¹ with exposure times of 0.2 s, with 3 × 3 binning. The fluorescence intensities of regions of interest (ROI; typically entire cell bodies, or proximal regions of processes) were analyzed with the Dynamic Intensity Analysis module of SimplePCI. Averaged intensity values for each ROI on each image frame so obtained were exported to Excel and/or Origin for further analysis. To normalize for variations in dye loading or cell thickness, intensity data are expressed as $\Delta F/F_o$, where F is the ROI average from each frame and F_o is the “resting” baseline intensity for the same ROI, averaged over five frames immediately prior to the application of lowered extracellular pH. Background fluorescence (from a region within the same field lacking cells or processes) was subtracted from ROI averages.

Materials

Chemicals whose sources were not otherwise mentioned were from Sigma (St. Louis, MO) or Fisher (Pittsburg, PA.).

RESULTS

RT-PCR in OLC

Reverse transcription was performed on total RNA extracted from OP, IO, and MO. Thirty-five cycles of PCR were performed with each cDNA using the primer pairs listed in Table 1. The products of these PCR reactions are shown in Fig. 2A. The appearance of the expected fragments in PCR reactions indicated the presence of ASICs 1a, 2, and 4 at all three developmental stages of cultured OLC. ASIC3 template was weakly detectable with the highly-sensitive dye SYBR Gold only in cDNA prepared from IO and MO; the amount of product produced after 35 cycles of PCR suggested that the ASIC3 substrate was present at low copy number. ASIC4 product appeared most abundant in OP.

Quantitative PCR

To establish mRNA expression levels more definitively, quantitative, real-time RT-PCR (qPCR) was performed on cDNA derived from OP, IO, and MO. The results are summarized in Fig. 2B. Taqman probes were selected to identify ASIC1a, ASIC1b, ASIC2 (nonselective), ASIC3, and ASIC4. A probe for GAPDH was used as a house-keeping probe to normalize for mRNA/cDNA template. qPCR experiments with newborn rat forebrain cDNA were also performed with the same probes, to compare expression levels in OP to a neuron-rich mRNA source. In agreement with standard PCR experiments, ASIC1a, ASIC2, and ASIC4 were all

detected at each developmental stage, with maximal levels in OP, and decreased levels in IO and MO. At all stages, ASIC1a and ASIC2 were detected at similar levels and ASIC4 at levels 2–4-fold lower. ASIC 3 was detectable only in late cycles (≥ 35), if at all. ASIC1b was not detected. In general, the expression levels for each of these genes observed in OP were similar (within 1–2 C_T) to those seen in whole forebrain.

RT-PCR in White Matter

To verify that ASIC transcripts are expressed in white matter tissue *in situ*, RT-PCR was also performed on RNA isolated from adult rat corpus callosum and optic nerve (see Fig. 3). Adult rat cortical grey matter was used as a positive control. In these experiments additional primer pairs (Table 1) were used to distinguish between ASICs 2a and 2b. ASICs 1a and 2a mRNAs were detected strongly in RNA preparations from each of these sources. Lesser abundances of mRNAs encoding ASICs 3 and 4 were observed, with both being weakly detectable in optic nerve and cortical grey, but only ASIC4 being detected in corpus callosum. ASIC 2b was not detected, suggesting that ASIC2 expression in cultured cells is likely to represent the ASIC2a splice variant. The relative expression levels of ASIC4 in these white matter tissues is apparently lower than in OP, possibly indicating an upregulation of ASIC4 expression in our cultured cells. These experiments demonstrate the expression of several ASIC transcripts in white matter, and show that expression of these transcripts in white matter is by and large similar to that in grey matter.

Immunofluorescent Localization of ASIC1a in White Matter

To verify that ASIC proteins are expressed in cells of the oligodendroglial lineage *in situ*, we labeled cells in the corpus callosum from a 2-month-old CNPase-EGFP mouse with antibodies against ASIC1a (see Fig. 4). In this transgenic mouse strain, oligodendroglial progenitors and immature and mature oligodendroglia are intrinsically labeled with EGFP driven by the CNPase promoter (Bannerman et al., 2007; Yuan et al., 2002). Our attempts to label cells in white matter of perinatal or adult rats were unsuccessful with available antibodies (four commercial anti-ASIC1 antibodies raised against different immunogenic peptides in all). Two different antibodies, one selective for an epitope in the extracellular loop of ASIC1a, and one that recognizes the C-terminus of both ASIC1a and ASIC1b, successfully labeled mouse white matter and gave similar staining patterns. The results shown in Fig. 4 are with the ASIC1a selective antibody.

As shown in Fig. 4, many, but not all of the EGFP expressing cells in the corpus callosum are co-labeled with the anti-ASIC1a antibody. The cluster of small anti-ASIC1a labeled cells in the upper left corner of Fig. 4A, that are not positive for EGFP, are presumptively neurons of the cingulate cortex. Widespread labeling of neurons in the CNS by anti-ASIC1 antibodies has been previously reported (Alvarez de la Rosa et al., 2003).

Electrophysiological Characterization

Whole-cell voltage clamp recordings from OP ($n = 45$) detected inward currents representing functional ASIC channels (I_{ASIC}). Cells were held at -60 mV, and then switched to -90 mV during acidic solution application. In response to brief (1.5–3 s) applications of acidic (pH 5.2–6.0) extracellular solution delivered at 30-s intervals, rapidly desensitizing inward currents in the range of -7 to -325 pA were observed. Qualitatively similar results were obtained regardless of the specific acidic pH applied, and the following values are pooled results from all experiments. Thirty-six of 45 OP cells (80%) responded to acid with detectable inward currents. For those OP cells with non-zero responses, the mean initial current magnitude was -63.5 ± 72 pA, and the mean current density was -7 ± 7 pA/pF. Current densities ranged from -0.7 to -22.6 pA/pF, indicating a highly variable level of ASIC expression across the population.

A small number of high-quality recordings were made from IO cells. Currents were detected in only one of five IO cells; that cell had a current magnitude of -37 pA and a current density of -2.2 pA/pF. Although the number of recorded IO cells is too small to draw statistical conclusions, these results suggest that while IO cells may express functional ASIC currents, they are apparently of lower density than those of OP cells.

The currents were virtually completely blocked ($n = 10$) by amiloride (100 μ M), as shown in Fig. 5. Amiloride block was quickly reversed upon washout, but currents generally did not recover to their original magnitude due to long-lasting desensitization or tachyphylaxis (Chen and Grunder, 2007) which was commonly observed in these cells. Typically, after even one application (3 s, pH = 5.5) of low pH solution, the response magnitude dropped 40–50% on subsequent trials delivered at 30 s or longer intervals. These results are in agreement with the observations of Sontheimer et al. (1989) in a prior study of OLC responses to lowered pH. Tachyphylaxis lasted many minutes (up to 1 h) and was not reversed by application of high calcium (10 mM) pH = 7.4 solution. Similar long-lasting tachyphylaxis was described in oocytes expressing cloned ASIC1a (Chen and Grunder, 2007), but was not observed with other ASIC variants expressed alone or in combination with ASIC1a. This result therefore suggests that homomeric ASIC1a contributes predominantly to the I_{ASIC} in OP.

A further property that distinguishes homomeric ASIC1a from other functional ASIC variants is the sensitivity of ASIC1a to a toxin (PcTx1) from the venom of the tarantula spider *Psalmopoeus christei* (Chen et al., 2005; Escoubas et al., 2000). As shown in Fig. 6, I_{ASIC} was virtually completely blocked ($n = 7$) by *Psalmopoeus christei* venom (1:20,000), indicating that the functional I_{ASIC} was carried predominantly through homomeric ASIC1a (Diochot et al., 2007; Escoubas et al., 2000). The action of the toxin was rapid; with complete block within 30 s. Block was partially reversible with a time course of the order of minutes. The lack of complete recovery was likely due to a combination of slow dissociation/washout of the toxin and tachyphylaxis, as described earlier. Although block by *Psalmopoeus* venom has only been reported for ASIC1a homomeric channels (Diochot et al., 2007; Escoubas et al., 2000), we cannot rule out the possibility that at 20,000-fold dilution non-specific blocking of other ASIC variants might occur. Therefore, we performed similar experiments with synthetic PcTx1 peptide (10–20 nM) and observed block of most or all current in three OP cells. In one cell current inhibition by the peptide was virtually complete, while in two others, the current was inhibited by nearly 80% (see Fig. 7), suggesting that in at least some cells a fraction of the current may be carried through channels that are not homomeric ASIC1a, which might be expected since transcripts for ASICs 2 and 4 were detected in OP.

Application of acidic extracellular solution causes a rapid depolarization of OP, detectable under whole-cell current clamp conditions (see Fig. 8). The magnitude of depolarization and the resting membrane potential was dependent on the quality of the recording seal, as expected, but in cells with high input resistance (1–2 G Ω) and strongly negative resting potentials (near -60 mV,) the membrane depolarized reversibly to levels of -30 to -20 mV even when the magnitude of I_{ASIC} was small (10–20 pA). The mean resting potential for four OP cells was -55 mV (± 7.6) and mean depolarization was 33.4 mV (± 14).

Calcium Imaging

In contrast to the high selectivity for sodium permeability shown by ASICs including 1b or 2a subunits, homomeric ASIC1a channels are reported to have considerable calcium permeability (Gunthorpe et al., 2001; Waldmann et al., 1997). To determine whether intracellular calcium levels rise detectably during ASIC activation by acidic extracellular medium application, we imaged cells loaded with the calcium-sensing dye Fura-4-AM. Image frames were collected every 0.2–0.5 s. After a baseline period for several frames in standard pH 7.4 extracellular solution, pH 5.5 solution was locally applied with the rapid exchange system for 1.5 s before

returning to pH 7.4. In some experiments amiloride was co-applied at pH 5.5. In experiments with dilute *Psalmopoeus* venom (1:100,000; $n = 3$ different fields) or PcTx1 synthetic peptide (20 nM; three different fields) brief pH drops were delivered after 1–2 min preapplication of toxin, and again after 8–10 min of perfusion with toxin free solution. In some experiments kainic acid (300 μ M) and cyclothiazide (50 μ M) were applied for 1.5 s as a positive control for calcium perturbation.

Figure 9 shows results of a typical experiment in which dilute *Psalmopoeus* venom (1:100,000) was used to block the response to low pH. Figure 10 shows an experiment that employed synthetic PcTx1 to inhibit the response. As predicted for homomeric ASIC1a channels, upon application of pH 5.5 extracellular solution (1.5 s application) the fluorescence intensity began to rise within a fraction of 1 s (Figs. 9 and 10) and peaked within 1–4 s. Little or no change in fluorescence was observed when amiloride (100 μ M) was co-applied with the pH 5.5 ($n = 8$; not shown.) Recovery to 1/e of peak value occurred within 10–20 s. Calcium elevation was detected both in the cell body and processes of OP in >20 different experiments. As shown in Figs. 9 and 10, cells varied in the degree of calcium response to an ~1.5 s application of acidic solution. Twenty percent to 40% of ROIs in a given field showed no detectable response to application of pH 5.5. In experiments in which kainic acid was used as a positive control, virtually every imaged cell responded to kainic acid, including the 20–40% that lacked responses to pH. For most cells the kainic acid response was stronger than that for low pH (see Fig. 10). Both *Psalmopoeus* venom (see Fig. 9) and synthetic PcTx1 (see Fig. 10) completely eliminated the responses to low pH, indicating that calcium elevation requires activation of homomeric ASIC1a channels. In the experiment depicted in Fig. 10 kainic acid elicited strong responses in the presence of PcTx1.

We also examined whether acid application could initiate calcium responses in differentiating OLC. In contrast to the readily observed responses in the majority of OP, calcium responses in IO were very rare. Of more than 200 IO cells, distributed in 12 different fields, only one cell responded unequivocally to application of pH 5.5. Results from that cell are shown in Fig. 11. The cell showed a dramatic increase in fluorescence in both the cell body and in most of its network of processes, starting within 0.5 s after the switch to pH 5.5 for 1.5 s. After a return to pH 7.4 for ~30 s, a second application of pH 5.5 elicited a weaker fluorescence increase. The relative fluorescence increase observed in that IO cell was greater than responses typically observed in seen in OP cells, and took longer to reach a peak. A possible explanation for these differences is that secondary amplification of calcium signaling, perhaps including release from intracellular calcium stores, occurred subsequent to initiation by ASIC1a activation. Considering the rarity of acid-induced responses in IO, it will be challenging to clarify the nature of the response and compare it to the responses of OP. The observation of this single response suggests that although differentiating OLC may occasionally express levels of homomeric ASIC1a sufficient to produce measurable calcium responses, apparently such acid-induced calcium responses are largely restricted to OP cells.

To explore the requirement for extracellular calcium influx via ASICs, we performed experiments comparing responses in oligodendroglial progenitors in reduced calcium (i.e. no calcium added to extracellular solutions, but with no calcium chelators present) to those in our standard 1 mM calcium-containing solutions. Because of the intimate role of calcium ions in ASIC gating and inactivation mechanisms (Chen and Grunder, 2007; Immke and McCleskey, 2003; Paukert et al., 2004), coupled with the uncontrolled release of protons and resulting acidification that would occur upon exchange of calcium containing solutions with chelator-containing solutions, we did not employ chelators to assure very low calcium concentrations. As a result, under our conditions with zero-added calcium solutions, we have by no means completely removed calcium. Nevertheless, despite these concerns, responses to pH 6.0 in zero-added calcium (after a pre-incubation in zero-added calcium, pH 7.4 for 10 s) were highly

variable, but on average 30–50% smaller than subsequent responses in the same cells after return to 1 mM calcium (not shown). Although not ideal, these results support the conclusion that acid-induced calcium elevation does involve influx of extracellular calcium.

DISCUSSION

Expression and Molecular Identity of OLC ASICs

We have shown that OLC, as well as adult CNS white matter tissues, express three of the four genes that comprise the ASIC family, and that the genes expressed and their relative levels resemble those found in total brain RNA. The ASIC1 and ASIC2 genes were expressed at similar levels, and were more abundant than ASIC4. The ASIC1a splice variant was detected, but not the ASIC1b variant, consistent with prior studies localizing ASIC1b exclusively to peripheral neurons (Bassler et al., 2001). Similarly, in standard RT-PCR experiments with white matter- derived cDNA templates, ASIC2a, but not 2b, was detected. ASIC3, which is also largely restricted to peripheral neurons (Chen et al., 1998), was weakly detected in differentiating OLC cDNAs and in adult optic nerve, but not at all in OP cDNA or in corpus callosum. We have additionally detected the presence of immunoreactive ASIC1a protein in mouse oligodendroglial cells. The appearance of ASIC3 transcript only in differentiating OLC, albeit at a very low copy number, suggests a pattern of developmental regulation opposite that of each of the other ASICs genes. ASICs 1a, 2, and 4 all were detected several fold higher in OP than in differentiating OLC. This developmental regulation may reflect some yet unknown role(s) played by ASICs in early OLC development.

Functional Properties of OP ASICs

Although mRNAs for three different ASIC genes are expressed at readily detectable levels in OLC, the ASIC1a subtype appears to contribute a majority of functional ASICs detectable under our whole-cell recording conditions, as shown by our observation that the tarantula toxin blocks most or all of the detected current. The pronounced ASIC tachyphylaxis observed in OP further supports the conclusion that homomeric ASIC1a comprises a substantial fraction of functional channels in OP. In addition, the complete inhibition of detectable acid-induced calcium elevation by psalmotoxin PcTx1 lends further support to the conclusion that toxin-insensitive ASICs are likely present at low density, since depolarization and sodium influx resulting from their activation would be expected to activate voltage gated calcium channels and/or reverse cycling of sodium/calcium exchangers (see discussion below). The apparent low level of functional PcTx1-insensitive ASICs is a surprising finding since coexpression of multiple cloned ASIC subtypes in *Xenopus* oocytes or transfected cells generally results in the expression of ASICs with altered properties compared with ASIC1a expressed alone, indicating that multiple subtypes readily co-assemble.

Implications for Cellular Signaling

ASIC1a has garnered considerable interest due to its low, yet significant calcium permeability. The many signaling pathways mediated by calcium raise the possibility that activity of ASICs may trigger important physiological processes and may contribute to pathophysiology of OLC. It was recently demonstrated with a mouse ASIC1a knockout model that ASIC1a activity under ischemic conditions accompanied by lowered extracellular pH in the CNS can cause the loss of neurons through a calcium-dependent mechanism analogous to excitotoxic cell death (Xiong et al., 2004, 2006); a neuroprotective effect of psalmotoxin has also been described in a stroke model (Pignataro et al., 2007); those studies considered only neuronal damage and did not discuss white matter damage that accompanies neuronal injury in these models. As we and others have shown, OLC are vulnerable to calcium-mediated excitotoxicity under conditions of inflammation or ischemia due to activation of calciumpermeable AMPA (Deng et al., 2003; Follett et al., 2004; Itoh et al., 2002; McDonald et al., 1998; Sanchez-Gomez and Matute,

1999) or NMDA (Karadottir et al., 2005; Salter and Fern, 2005) receptors. Our observation that ASIC activation can produce elevation of intracellular calcium supports a new independent pathway through which calcium can enter OLC under pathological conditions accompanied by lowered extracellular pH. This calcium-permeable pathway may operate independently from, or in concert with, glutamate-operated channels. A synergy between the actions of NMDA receptors and ASIC1a has been demonstrated in neurons in an ischemia model, involving phosphorylation of the ASIC1a protein through a Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) mediated pathway (Gao et al., 2005), which leads to enhancement of ASIC-mediated currents.

The expression of many types of ligand- and voltage gated channels in OLC at various developmental stages provides a tableau rich with possibilities for crosstalk that could work in synergy with ASICs. In addition to direct calcium influx through ASIC1a, ASIC activation may also affect OLC by membrane depolarization, which as we have shown here, can be considerable (>50 mV depolarization from resting potential). NMDA receptors, which are expressed by OLC *in vivo* (Karadottir et al., 2005; Salter and Fern, 2005), could be activated by relief from Mg^{2+} -block due to depolarization initiated by I_{ASIC} . Such a mechanism would provide a reciprocally synergistic network, together with the CaMKII-mediated activation of ASIC1a described earlier that could act when glutamate and low pH are present simultaneously, as would be expected under ischemic or inflammatory conditions. The net result would be a stimulation of Ca^{2+} influx greater than either pathway would produce on its own.

Alternatively, ASIC-mediated membrane depolarization could activate voltage-dependent channels, including sodium (Sontheimer et al., 1996; Sontheimer and Waxman, 1993), potassium (Barres et al., 1990; Bevan et al., 1987; Sontheimer and Waxman, 1993), or calcium (Berger et al., 1992; Blankenfeld Gv et al., 1992), all of which are expressed by developing OLC. Both low-voltage and high-voltage activated subtypes of voltage-dependent calcium channels are found in OLC, with a differential pattern of distribution such that low-voltage activated T-type channels are localized at the tips of processes, while high-voltage activated channels are distributed on the soma (Kirischuk et al., 1995). Both types of voltage-gated calcium channels could be activated by depolarizations of the magnitude we report here. Activation of voltage-dependent calcium channels by AMPA/KA receptor-mediated membrane depolarization has been previously reported (Borges et al., 1994; Liu et al., 1997) and therefore seems a likely consequence of ASIC-mediated depolarization. An additional means by which ASIC activation could lead to elevated intracellular calcium would be by reverse action of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, driven by intracellular Na^+ accumulation and depolarization. Reversed $\text{Na}^+/\text{Ca}^{2+}$ exchange has been reported to supplement AMPA receptor-mediated calcium accumulation in OLC (Liu et al., 1997). Furthermore, such a mechanism contributes to axonal damage in demyelinating conditions (Craner et al., 2004; Stys et al., 1992, 1993), and might be especially relevant in the fine processes of OLC where significant Na^+ accumulation likely occurs during receptor activation. Finally, calcium-induced calcium release from intracellular stores could serve to further amplify the calcium signal (reviewed in Bootman et al., 2002). Inositol triphosphate and/or ryanodine receptors expressed by OLC (Haak et al., 2001) could be triggered to release calcium from intracellular stores after an initial influx of calcium via ASICs. Despite the presence of these various potential pathways for calcium elevation, our finding that psalmotoxin blocks any detectable acid-induced calcium elevation suggests that the responses to acid must be *initiated* by homomeric ASIC1a activation. This discussion of candidate calcium pathways that could be triggered by, or interact with, ASIC activation is presently speculative. An important next step will be a determination of the relative contributions of calcium influx vs. mobilization of intracellular calcium stores that result from ASIC activation. Further study will be needed to dissect the contributions of the afore-mentioned candidate pathways to the calcium elevations we describe here.

We have shown that OLC ASICs can respond to acidshifted extracellular environment by both depolarizing the cells and elevating intracellular calcium. It is not yet known what physiological functions are served by ASICs in OLC. Since both normal and pathological physiological processes in the brain can lead to perturbations of pH, it seems likely that ASICs could play roles in oligodendrocyte development, pathology, and perhaps regeneration.

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Fig. 1. Morphological and immunofluorescent characterization of OLC cultures. **(A)** A representative primary culture of oligodendroglial progenitor cells (OP). OPs were stained with A2B5 monoclonal antibody (red), a surface marker of rat OP. **(B)** A representative primary culture of immature oligodendrocytes (IO) at day 2 in the differentiation medium (DM). IOs were stained with O4 antibody (red). **(C)** A representative primary culture of mature oligodendrocytes (MO) at day 4 in DM, stained with anti-myelin basic protein antibody (green), a marker of MO. Nuclei in all panels were counterstained with 4,6-diamidino-2-phenylindole (blue). Scale bar: 50 μ m.

Fig. 2.

Expression of ASIC transcripts in cultured OLC detected by RT-PCR. **(A)** Conventional RT-PCR products visualized after 35 cycles of PCR; total RNA templates from OP, IO, or MO cultures. The primer combinations used were as given in Table 1. The DNA size markers are at 100 base pair intervals. For each gel photograph the expected product size in base pairs, followed (in *italics*) by the size of the DNA marker indicated by the white arrowheads are: ASIC1a, 375 (*300*); ASIC2, 641 (*600*); ASIC3, 267 (*200*); ASIC4, 332 (*300*). **(B)** Summary of quantitative PCR results to compare relative expression of ASIC genes in OLC developmental stages and whole brain. Bars for each gene assay, for each tissue source, represent the mean relative value normalized to GAPDH level from the same tissue, as described in Methods. The error bars are standard errors, derived from three to six separate determinations for each gene/tissue combination. Whole brain (WB) RNA was used for comparison to RNA templates for each OLC developmental stage.

Fig. 3.

Expression of ASIC transcripts in adult rat white and grey matter detected by RT-PCR. Conventional RT-PCR products visualized after 35 cycles of PCR; total RNA templates from corpus callosum, optic nerve, and cortical grey matter. The primer combinations used were as given in Table 1. For each tissue the gels were stained identically and photographed at two different exposures. ASICs 1a and 2a lanes for each tissue are shown exposed for 1 s at f4. ASICs 3 and 4 are shown exposed for 1 s at f2 to visualize faint bands. In each gel the DNA size markers are at 100-bp intervals and the 300-bp band is marked by a white arrowhead. The expected product sizes in base pairs are: ASIC1a, 375; ASIC2a, 629; ASIC3, 267; ASIC4, 332.

Fig. 4. Immunofluorescence localization of ASIC1a on cells of the oligodendrocyte lineage in cerebral white matter of CNPase-EGFP transgenic mice. **(A)** Tissue section labeled by anti-ASIC1a (red). **(B)** Intrinsic fluorescence due to EGFP (green), driven by the 2,3-Cyclic Nucleotide-3-phosphohydrolase (CNPase) promoter, which marks oligodendroglial progenitors and both immature and myelinating oligodendrocytes. **(C)** Superimposed images from A and B. Note that some, but not all, EGFP1 OLC express immunoreactive ASIC1a.

Fig. 5.

Amiloride-sensitive inward currents evoked by low-pH. **(A)** Currents observed in an OP cell (holding potential -90 mV) upon shifting the extracellular bath from pH = 7.4 to 5.5 for 2 s, as indicated by the horizontal bar; **(B)** same, but during co-application of amiloride (100 μ M) at pH = 5.5; **(C)** as in (A), 30 s after washout of amiloride.

Fig. 6. Inhibition of ASIC current by psalmotoxin. **(A)** Currents in an OP cell (holding potential -90 mV) upon application of pH = 5.5 for 3 s. **(B)** After 1 min application of *Psalmopoeus* venom (1:20,000 dilution in normal pH = 7.4) the bath was switched to pH = 5.5 for 3 s, which failed to elicit a response. **(C)** After 2.5-min washout at pH = 7.4, pH was again shifted to 5.5 for 3 s, which elicited a response $\sim 18\%$ of the original pre-toxin magnitude. Similar results were obtained in seven different cells.

Fig. 7.

Inhibition of ASIC current by synthetic PcTx1. This experiment was similar to that shown in Fig. 6, except that pH 6.0 was applied for 1.5 s to reduce tachyphylaxis (Chen and Grunder, 2007), and synthetic PcTx1 was used to inhibit current. (A) Currents in an OP cell upon application of pH = 6.0 for 1.5 s. (B) After 1 min application of PcTx1 (20 nM) in normal pH = 7.4) the bath was switched to pH = 6.0 for 1.5 s, which elicited a response of ~23% of the control response. (C) After 11 min washout at pH = 7.4, pH was again shifted to 6.0 for 1.5 s, which elicited a response ~56% of the original pre-toxin magnitude.

Fig. 8.

Depolarization of OP membrane by ASIC activation. (A) Recording of membrane voltage in whole-cell current-clamp mode. A 2 s application of pH = 5.5 extracellular solution depolarizes the membrane by ~45 mV. (B) Voltage-clamp recording from the same cell (holding potential -90 mV) ~2 min after the recording shown in (A). The shift to pH = 5.5 for 3 s elicited a peak inward current of ~11 pA.

Fig. 9.

Intracellular calcium elevation initiated by ASIC activation and inhibition by psalmotoxin. Responses to acid before and after application of *Psalmopoeus* venom. Solid squares are averaged values of $\Delta F/F_0$ of all ROIs encompassing the cell bodies of cells ($n = 117$) in a series of image frames showing the control responses to application of pH 5.5 for 1.5 s, represented by the short horizontal bar. Before and after the acid application cells were bathed in pH 7.4 medium. The blue solid envelope surrounding the trace shows the standard deviations for each averaged value. Open squares are similarly derived averages of ROIs after incubation for 2 min with *Psalmopoeus* venom at a 1:100,000 dilution. The grey envelope represents the standard deviations.

Fig. 10.

Inhibition of calcium elevation by synthetic PcTx1. Traces in each panel represent the responses of individual cells whose response in (C) was a 10% or greater increase in $\Delta F/F_0$. The identical set of 22 ROIs (from a field of 70 ROIs measured) is represented in each panel. For clarity, cells whose responses were less than 10%, or those having highly unstable baselines are not shown. **(A)** Response to acid application after 2 min pre-incubation with PcTx1 (20 nM). To avoid any tachyphylaxis these cells had not been exposed to low pH prior to preincubation with PcTx1. **(B)** Response to brief application with kainic acid (KA; 0.3 mM) and cyclothiazide (CTZ; 0.05 mM) recorded in the presence of the PcTx1 30 s after the acid application shown in A. **(C)** Response to second application of pH 5.5 after 8 min of perfusion with pH 7.4 buffer to remove PcTx1.

Fig. 11.

Intracellular calcium elevation in an immature oligodendrocyte triggered by acid exposure. **(A)** An IO cell (2 days in differentiating medium) imaged prior to application (at 4 s on the scale indicated in part C. of this figure) of pH 5.5 solution ("pre"). Measurements were made from ROIs placed as indicated by white circles over the cell body (1) or over small segments of processes (2, 3). **(B)** The same cell imaged after application of pH 5.5 (at 12 s on the scale of part C. of this figure). **(C)** Relative fluorescence for each ROI 1–3. The numbered plots correspond to the fluorescence changes for each ROI, normalized to its prestimulus level. Values for ROI no. 1 (cell body) are shown by black squares; ROI nos. 2, 3 (processes) are shown by green triangles, or red circles, respectively. Application of pH 5.5 is marked by black bars (1.5 s each) at 4.5 and 30 s. The images corresponding to panels A and B of this figure are marked by black arrowheads beneath the data plots. The images in A and B were identically adjusted for black level and gamma ($\gamma = 1.5$) to illustrate the dimly fluorescent processes.

TABLE 1

Primers and Taqman Gene Assay Kits Used in This Study

Gene name	ASIC subtype	Forward primer	Reverse primer	ABI Taqman assay number
ACCN1	ASIC2 (nonselective)	GGCAACGGGCTGGAGATCAT	GCAGCAACTTCATACGCCTTCTTC	Rn00563654_m1
	ASIC2a	GCCAATACCTCCACTCTCCATGG	GGCAGGTACTCATCTTGCTGAATGTC	
	ASIC2b	TCCAAGGGGGACCTCTACTA	CCATCCTCGCCTGAGTTAAA	
ACCN2	ASIC1 (nonselective)	GACCATGAAAGGTGGGACTGGC	CTGCTCGATGGTCTCATAGTTGAGG	Rn00577292_m1 Rn01532749_m1
	ASIC1a	CTTGCCACATCTTCTCCTATGAGC	CATGTTGAAGGGCTTGGGCTTG	
	ASIC1b			
ACCN3	ASIC3	GCTCACGCCTCACACCAATGA	GTGTAGCATTGCCCCATTCGAGT	Rn00597579_m1
ACCN4	ASIC4	GCACTCAGCGATGCTGATATCTTCC	GGTAGGTATTCTCCTGCTGGATGTC	Rn00573723_m1
GAPDH	(NA)			Rn99999916_s1