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Neuroprotection in Ischemia: Blocking Calcium-Permeable Acid-Sensing Ion Channels

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Summary

Ca^{2+} toxicity remains the central focus of ischemic brain injury. The mechanism by which toxic Ca^{2+} loading of cells occurs in the ischemic brain has become less clear as multiple human trials of glutamate antagonists have failed to show effective neuroprotection in stroke. Acidosis is a common feature of ischemia and is assumed to play a critical role in brain injury; however, the mechanism(s) remain ill defined. Here, we show that acidosis activates Ca^{2+}-permeable acid-sensing ion channels (ASICs), inducing glutamate receptor-independent, Ca^{2+}-dependent, neuronal injury inhibited by ASIC blockers. Cells lacking endogenous ASICs are resistant to acid injury, while transfection of Ca^{2+}-permeable ASIC1a establishes sensitivity. In focal ischemia, intracerebroventricular injection of ASIC1a blockers or knockout of the ASIC1a gene protects the brain from ischemic injury and does so more potently than glutamate antagonism. Thus, acidosis injures the brain via membrane receptor-based mechanisms with resultant toxicity of $[Ca^{2+}]_i$, disclosing new potential therapeutic targets for stroke.

Introduction

Intracellular Ca^{2+} overload is essential for neuronal injury associated with neuropathological syndromes, including brain ischemia (Choi, 1995, 1988a). Excessive Ca^{2+} in the cell activates a cascade of cytotoxic events leading to activation of enzymes that break down proteins, lipids, and nucleic acids. NMDA receptors, the most important excitatory neurotransmitter receptors in the central nervous system (McLennan, 1983; Dingledine et al., 1999), have long been considered the main target responsible for Ca^{2+} overload in the ischemic brain (Simon et al., 1984; Rothman and Olney, 1986; Choi, 1988b; Meldrum, 1995). However, recent clinical efforts to prevent brain injury through the therapeutic use of NMDA receptor antagonists have been disappointing (Lee et al., 1999; Wahlgren and Ahmed, 2004). Although multiple factors, including difficulty in early initiation of treatment, may have contributed to trial failures, it is likely that glutamate receptor-independent Ca^{2+} toxicity might also be responsible for ischemic brain injury.

The normal brain requires complete oxidation of glucose to fulfill its energy requirements. During ischemia, oxygen depletion forces the brain to switch to anaerobic glycolysis. Accumulation of lactic acid as a byproduct of glycolysis and protons produced by ATP hydrolysis causes pH to fall in the ischemic brain (Rehncrona, 1985; Siesjo et al., 1996). Consequently, tissue pH typically falls to 6.5–6.0 during ischemia under normoglycemic conditions and can fall below 6.0 during severe ischemia or under hyperglycemic conditions (Nedergaard et al., 1991; Rehncrona, 1985; Siesjo et al., 1996). Nearly all in vivo studies indicate that acidosis aggravates ischemic brain injury (Tombaugh and Sapolsky, 1993; Siesjo et al., 1996). However, the mechanisms of this process remain unclear, although a host of possibilities has been suggested (Siesjo et al., 1996; McDonald et al., 1998; Swanson et al., 1995; Ying et al., 1999).

Acid-sensing ion channels (ASICs), a newly described class of ligand-gated channels (Waldmann et al., 1997a; Krishtal, 2003), have been shown to be expressed throughout neurons of mammalian central and peripheral nervous systems (Waldmann et al., 1997a, 1999; Waldmann and Lazdunski, 1998; Krishtal, 2003; Alvarez de la Rosa et al., 2002, 2003). They are members of the degenerin/epithelial sodium channel (Deg/ENaC) superfamily (Benos and Stanton, 1999; Bianchi and Driscoll, 2002; Krishtal, 2003). Pertinent to ischemia, ASICs may also flux Ca^{2+} (Waldmann et al., 1997a; Chu et al., 2002; Yermolaieva et al., 2004).

To date, six ASIC subunits have been cloned. Four of these subunits can form functional homomultimeric channels that are activated by acidic pH to conduct a sodium-selective, amiloride-sensitive, cation current. The pH of half-maximal activation ($pH_{0.5}$) of these channels differs: ASIC1a (or ASIC1), $pH_{0.5} = 6.2$ (Waldmann et al., 1997a); ASIC1β, a splice variant of ASIC1a with a unique N-terminal, $pH_{0.5} = 5.9$ (Chen et al., 1998); ASIC2a, $pH_{0.5} = 4.4$ (Waldmann et al., 1999); and ASIC3, $pH_{0.5} = 6.5$ (Waldmann et al., 1997b). Neither ASIC2b nor ASIC4 can form functional homomeric channel (Akopian et al., 2000; Grunder et al., 2000; Lingueglia et al., 1997), but ASIC2b has been shown to associate with other subunits and modulate their activity (Lingueglia et al., 1997). In addition to Na^+ permeability, homomeric ASIC1a can flux Ca^{2+} (Waldmann et al., 1997a; Chu et al., 2002; Yermolaieva et al., 2004). Although the exact

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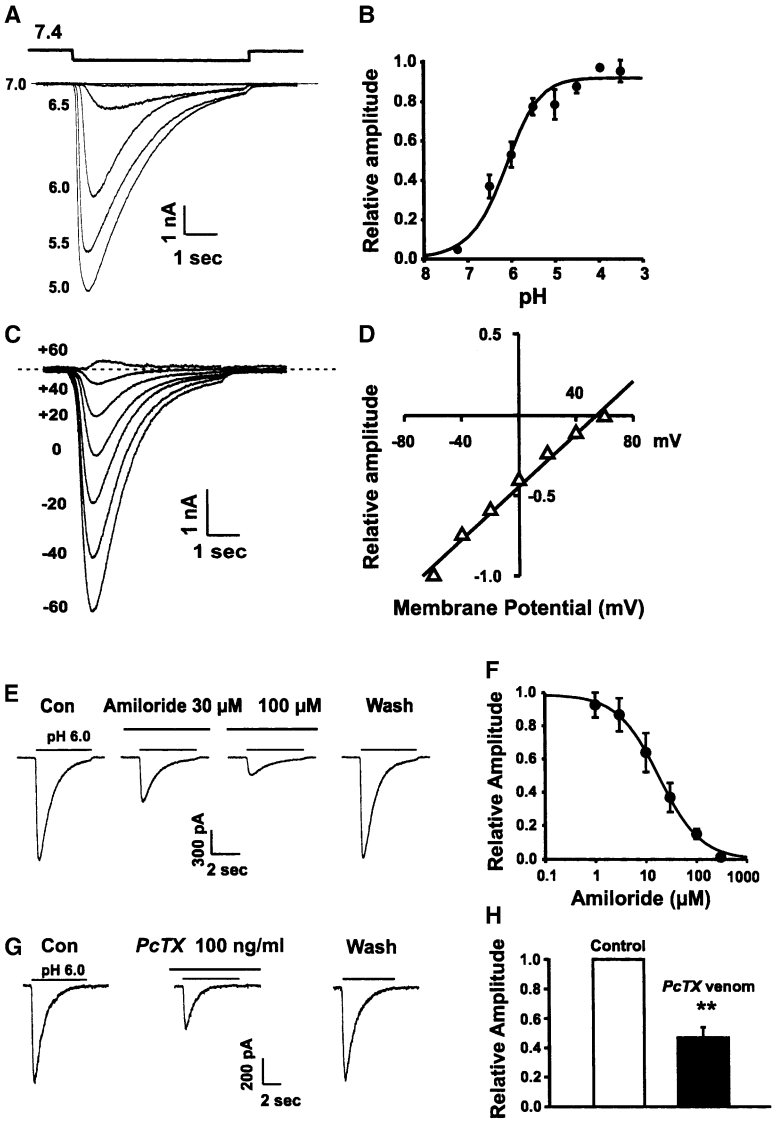

Figure 1. Electrophysiology and Pharmacology of ASICs in Cultured Mouse Cortical Neurons

(A and B) pH dependence of ASIC currents activated by pH drop from 7.4 to values indicated. Dose-response curves were fit to Hill equation with an average $pH_{0.5}$ of 6.18 ± 0.06 (n = 10).

(C and D) Current-voltage relationship of ASICs (n = 5). The amplitudes of ASIC current at various voltages were normalized to that recorded at −60 mV.

(E and F) Dose-dependent blockade of ASIC currents by amiloride. IC_{50} = 16.4 ± 4.1 µM, n = 8.

(G and H) Blockade of ASIC currents by *PcTX* venom. **p < 0.01.

subunit composition of ASICs in native neurons has not been determined, both ASIC1a and ASIC2a subunits have been shown to be abundant in the brain (Price et al., 1996; Bassilana et al., 1997; Wemmie et al., 2002; Alvarez de la Rosa et al., 2003).

Detailed functions of ASICs in both peripheral and central nervous systems remain to be determined. In peripheral sensory neurons, ASICs have been implicated in mechanosensation (Price et al., 2000, 2001) and perception of pain during tissue acidosis (Bevan and Yeats, 1991; Krishtal and Pidoplichko, 1981; Ugawa et al., 2002; Sluka et al., 2003; Chen et al., 2002), particularly in ischemic myocardium where ASICs likely transduce anginal pain (Benson et al., 1999). The presence of ASICs in the brain, which lacks nociceptors, suggests that these channels have functions beyond nociception. Indeed, recent studies have indicated that ASIC1a is involved in synaptic plasticity, learning/memory, and fear conditioning (Wemmie et al., 2002, 2003). Here, using a combination of patch-clamp recording, Ca^{2+} imaging, receptor subunit transfection, in vitro cell toxicity assays, and in vivo ischemia models combined with gene knockout, we demonstrate activation of Ca^{2+}-permeable ASIC1a as largely responsible for glutamate-independent, acidosis-mediated, and ischemic brain injury.

Results

Acidosis Activates ASICs in Mouse Cortical Neurons

We first recorded ASIC currents in cultured mouse cortical neurons, a preparation commonly used for cell toxicity studies (Koh and Choi, 1987; Sattler et al., 1999). At a holding potential of −60 mV, a rapid reduction of extracellular pH (pH_e) to below 7.0 evoked large transient inward currents with a small steady-state component in the majority of neurons (Figure 1A). The amplitude of inward current increased in a sigmoidal fashion as pH_e decreased, yielding a $pH_{0.5}$ of 6.18 ± 0.06 (n = 10, Figure 1B). A linear I-V relationship and a reversal close to the Na^+ equilibrium potential were obtained (n = 6, Figures

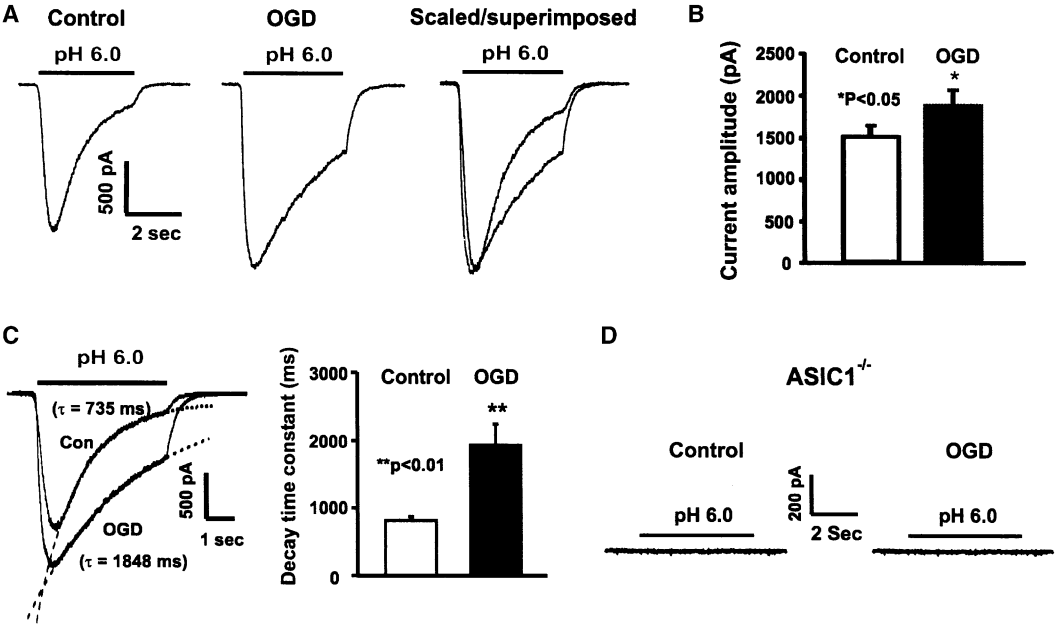

Figure 2. Modeled Ischemia Enhances Activity of ASICs

(A) Representative traces showing increase in amplitude and decrease in desensitization of ASIC currents following 1 hr OGD.

(B) Summary data of increase of ASIC current amplitude in OGD neurons. n = 40 and 44, *p < 0.05.

(C) Representative traces and summary data showing decreased ASIC current desensitization in OGD neurons. n = 6, **p < 0.01.

(D) Representative traces showing lack of acid-activated current at pH 6.0 in ASIC1$^{-/-}$ neurons, in control condition, and following 1 hr OGD (n = 12 and 13).

1C and 1D). These data demonstrate that lowering pH_e activates typical ASICs in mouse cortical neurons.

We then tested the effect of amiloride, a nonspecific blocker of ASICs (Waldmann et al., 1997a), on the acid-activated currents. Similar to previous studies, mainly in sensory neurons (Waldmann et al., 1997a; Benson et al., 1999; Chen et al., 1998; Varming, 1999), amiloride dose-dependently blocked ASIC currents in cortical neurons with an IC_{50} of 16.4 ± 4.1 μM (n = 8, Figures 1E and 1F). Psalmotoxin 1 (or PcTX1) from venom of the tarantula *Psalmopoeus cambridgei* (*PcTX* venom) has been shown to be a specific ASIC1a blocker (Escoubas et al., 2000). Our studies show that, at a protein concentration of 25 ng/ml, *PcTX* venom itself also blocks the current mediated by homomeric ASIC1a expressed in COS-7 cells by ~70% (n = 4, see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/118/6/687/DC1). However, it does not affect currents mediated by heteromeric ASIC1a/2a, homomeric ASIC2a, or ASIC3 channels at 500 ng/ml (n = 4–6). In addition, at 500 ng/ml, *PcTX* venom does not affect the currents through known voltage- and ligand-gated channels, further indicating its specificity for homomeric ASIC1a (n = 4–5, Supplemental Figure S2, and Supplemental Data).

We then tested the effect of *PcTX* venom on acid-activated current in cortical neurons. At 100 ng/ml, *PcTX* venom reversibly blocked the peak amplitude of ASIC current by 47% ± 7% (n = 15, Figures 1G and 1H), indicating significant contributions of homomeric ASIC1a to total acid-activated currents. Increasing *PcTX* concentration did not induce further reduction in the amplitude of ASIC current in the majority of cortical neurons (n = 8, data not shown), indicating coexistence of *PcTX*-insensitive ASICs (e.g., heteromeric ASIC1a/2a) in these neurons.

ASIC Response Is Potentiated by Modeled Ischemia

Since acidosis is a central feature of brain ischemia, we determined whether ASICs are activated in ischemic conditions and whether ischemia modifies the properties of these channels. We recorded ASIC currents in neurons following 1 hr oxygen glucose deprivation (OGD), a common model of in vitro ischemia (Goldberg and Choi, 1993). One set of cultures was washed three times with glucose-free extracellular fluid (ECF) and subjected to OGD, while control cultures were subjected to washes with glucose containing ECF and incubation in a conventional cell culture incubator. OGD was terminated after 1 hr by replacing glucose-free ECF with Neurobasal medium and incubating cultures in the conventional incubator. ASIC current was then recorded 1 hr following the OGD when there was no morphological alteration of neurons. OGD treatment induced a moderate increase of the amplitude of ASIC currents (1520 ± 138 pA in control group, n = 44; 1886 ± 185 pA in neurons following 1 hr OGD, n = 40, p < 0.05, Figures 2A and 2B). More importantly, OGD induced a dramatic decrease in ASIC desensitization as demonstrated by an increase in time constant of the current decay (814.7 ± 58.9 ms in control neurons, n = 6; 1928.9 ± 315.7 ms in neurons following OGD, n = 6, p < 0.01, Figures 2A and 2C). In cortical neurons cultured from ASIC1$^{-/-}$ mice, reduction of pH from 7.4 to 6.0 did not activate any inward current (n = 52), similar to a previous study in hippocampal neurons (Wemmie et al., 2002). In

these neurons, 1 hr OGD did not activate or potentiate acid-induced responses (Figure 2D, n = 12 and 13).

Acidosis Induces Glutamate-Independent Ca^{2+} Entry via ASIC1a

Using a standard ion-substitution protocol (Jia et al., 1996) and the fura-2 fluorescent Ca^{2+}-imaging technique (Chu et al., 2002), we determined whether ASICs in cortical neurons are Ca^{2+} permeable. With bath solutions containing 10 mM Ca^{2+} (Na^+ and K^+-free) as the only charge carrier and at a holding potential of −60 mV, we recorded inward currents larger than 50 pA in 15 out of 18 neurons, indicating significant Ca^{2+} permeability of ASICs in the majority of cortical neurons (Figure 3A). Consistent with activation of homomeric ASIC1a channels, currents carried by 10 mM Ca^{2+} were largely blocked by both the nonspecific ASIC blocker amiloride and the ASIC1a-specific blocker *PcTX* venom (Figure 3B). The peak amplitude of Ca^{2+}-mediated current was decreased to 26% ± 2% of control by 100 μM amiloride (n = 6, $p < 0.01$) and to 22% ± 0.9% by 100 ng/ml *PcTX* venom (n = 5, $p < 0.01$). Ca^{2+} imaging, in the presence of blockers of other major Ca^{2+} entry pathways (MK801 10 μM and CNQX 20 μM for glutamate receptors; nimodipine 5 μM and ω-conotoxin MVIIC 1 μM for voltage-gated Ca^{2+} channels), demonstrated that 18 out of 20 neurons responded to a pH drop with detectable increases in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Figure 3C). In general, $[Ca^{2+}]_i$ remains elevated during prolonged perfusion of low pH solutions. In some cells, the $[Ca^{2+}]_i$ increase lasted even longer than the duration of acid perfusion (Figure 3C). Long-lasting Ca^{2+} responses suggest that ASIC response in intact neurons is less desensitized than in whole-cell recordings or that Ca^{2+} entry through ASICs induces subsequent Ca^{2+} release from intracellular stores. Preincubation of neurons with 1 μM thapsigargin partially inhibited the sustained component of Ca^{2+} increase, suggesting that Ca^{2+} release from intracellular stores may also contribute to acid-induced intracellular Ca^{2+} accumulation (n = 6, data not shown). Similar to the current carried by Ca^{2+} ions (Figure 3B), both peak and sustained increases in $[Ca^{2+}]_i$ were largely inhibited by amiloride and *PcTX* venom (Figures 3C and 3D, n = 6–8), consistent with involvement of homomeric ASIC1a in acid-induced $[Ca^{2+}]_i$ increase. Knockout of the ASIC1 gene eliminated the acid-induced $[Ca^{2+}]_i$ increase in all neurons without affecting NMDA receptor-mediated Ca^{2+} response (Figure 3E, n = 8). Patch-clamp recordings demonstrated lack of acid-activated currents at pH 6.0 in 52 out of 52 of the $ASIC1^{-/-}$ neurons, consistent with absence of ASIC1a subunits. Lowering pH to 5.0 or 4.0, however, activated detectable current in 24 out of 52 $ASIC1^{-/-}$ neurons, indicating the presence of ASIC2a subunits in these neurons (Figure 3F). Further electrophysiological studies demonstrated that $ASIC1^{-/-}$ neurons have normal responses for various voltage-gated channels and NMDA, GABA receptor-gated channels (data not shown).

ASIC Blockade Protects against Acidosis-Induced, Glutamate-Independent Neuronal Injury

Acid-induced injury was studied on neurons grown on 24-well plates incubated in either pH 7.4 or 6.0 ECF containing MK801, CNQX, and nimodipine. Cell injury was assayed by the measurement of lactate dehydrogenase (LDH) release (Koh and Choi, 1987) at various time points (Figures 4A and 4B) and by fluorescent staining of alive/dead cells (Figure 4C). Compared to neurons treated at pH 7.4, 1 hr acid incubation (pH 6.0) induced a time-dependent increase in LDH release (Figure 4A). After 24 hr, 45.7% ± 5.4% of maximal LDH release was induced (n = 25 wells). Continuous treatment at pH 6.0 induced greater cell injury (Figure 4B, n = 20). Consistent with the LDH assay, alive/dead staining with fluorescein diacetate (FDA, blue) and propidium iodide (PI, red) showed similar increases in cell death by 1 hr acid treatment (Figure 4C, and Supplemental Figure S3 on the *Cell* web site). One hour incubation with pH 6.5 ECF also induced significant but less LDH release than with pH 6.0 ECF (n = 8 wells, data not shown).

To determine whether activation of ASICs is involved in acid-induced glutamate receptor-independent neuronal injury, we tested the effect of amiloride and *PcTX* venom on acid-induced LDH release. Addition of either 100 μM amiloride or 100 ng/ml *PcTX* venom 10 min before and during the 1 hr acid incubation significantly reduced LDH release (Figure 4D). At 24 hr, LDH release was decreased from 45.3% ± 3.8% to 31.1% ± 2.5% by amiloride and to 27.9% ± 2.6% by *PcTX* venom (n = 20–27, $p < 0.01$). Addition of amiloride or *PcTX* venom in pH 7.4 ECF for 1 hr did not affect baseline LDH release, although prolonged incubation (e.g., 5 hr) with amiloride alone increased LDH release (n = 8, data not shown).

Activation of Homomeric ASIC1a Is Responsible for Acidosis-Induced Injury

To determine whether Ca^{2+} entry plays a role in acid-induced injury, we treated neurons with pH 6.0 ECF in the presence of normal or reduced $[Ca^{2+}]_e$. Reducing Ca^{2+} from 1.3 to 0.2 mM inhibited acid-induced LDH release (from 40.0% ± 4.1% to 21.9% ± 2.5%), as did ASIC1a blockade with *PcTX* venom (n = 11–12, $p < 0.01$; Figure 5A). Ca^{2+}-free solution was not tested, as a complete removal of $[Ca^{2+}]_e$ activates large inward currents through a Ca^{2+}-sensing cation channel, which may otherwise complicate data interpretation (Xiong et al., 1997). Inhibition of acid injury by both amiloride and *PcTX*, nonspecific and specific ASIC1a blockers, and by reducing $[Ca^{2+}]_e$ strongly suggests that activation of Ca^{2+}-permeable ASIC1a is involved in acid-induced neuronal injury.

To provide additional evidence that activation of ASIC1a is involved in acid injury, we studied acid injury of nontransfected and ASIC1a transfected COS-7 cells, a cell line commonly used for expression of ASICs due to its lack of endogenous channels (Chen et al., 1998; Immke and McCleskey, 2001; Escoubas et al., 2000). Following confluence (36–48 hr after plating), cells were treated with either pH 7.4 or 6.0 ECF for 1 hr. LDH release was measured 24 hr after acid incubation. Treatment of nontransfected COS-7 cells with pH 6.0 ECF did not induce increased LDH release when compared with pH 7.4-treated cells (10.3% ± 0.8% for pH 7.4, and 9.4% ± 0.7% for pH 6.0, n = 19 and 20 wells; $p > 0.05$, Figure 5B). However, in COS-7 cells stably transfected with ASIC1a, 1 hr incubation at pH 6.0 significantly increased

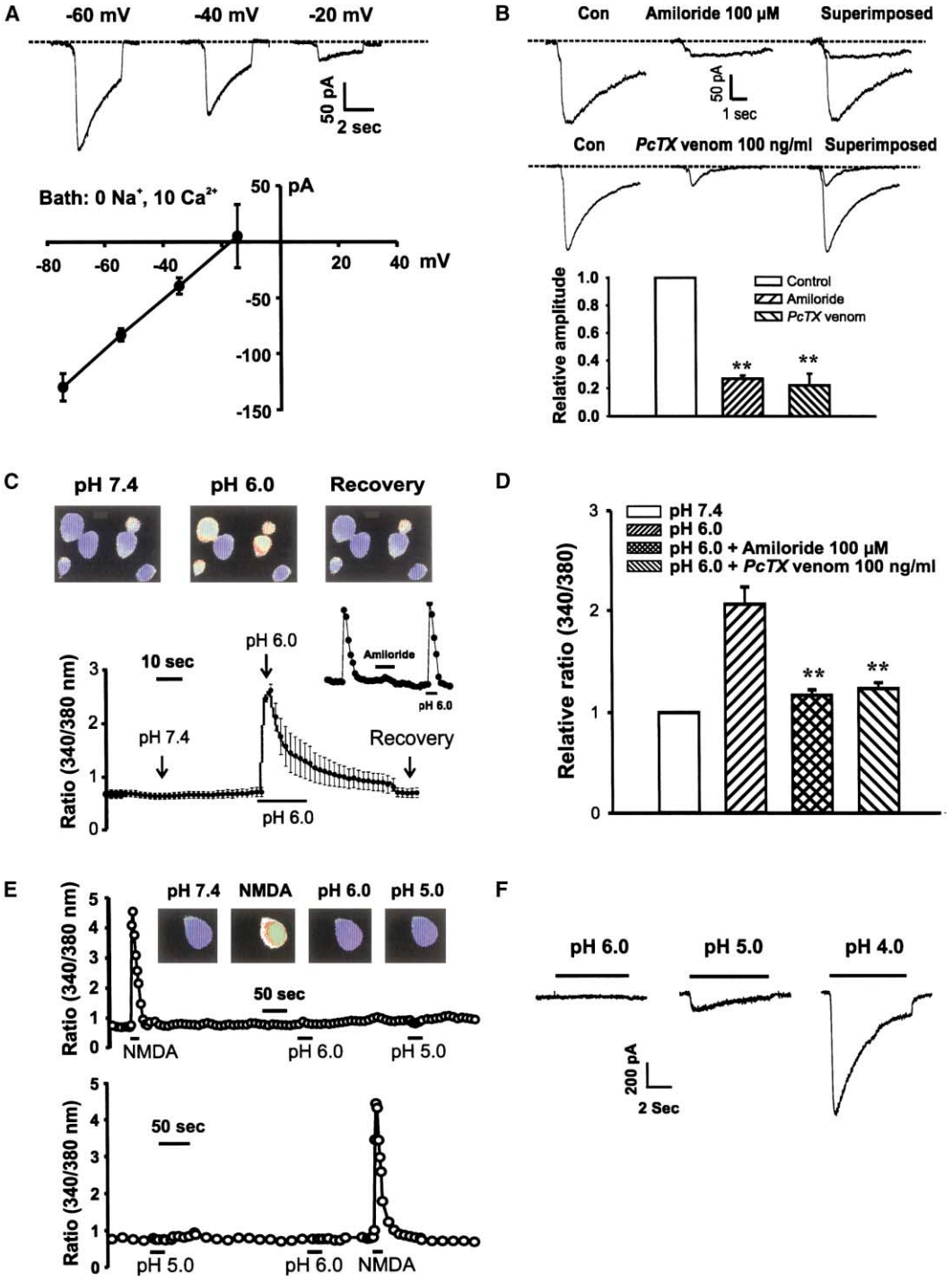

Figure 3. ASICs in Cortical Neurons Are Ca^{2+} Permeable, and Ca^{2+} Permeability Is ASIC1a Dependent

(A) With Na^{+}-free ECF containing 10 mM Ca^{2+} as the only charge carrier, inward currents were recorded at pH 6.0. The average reversal potential is ~ -17 mV after correction of liquid junction potential ($n = 5$).

(B) Representative traces and summary data showing blockade of Ca^{2+}-mediated current by amiloride and *PcTX* venom. The peak amplitude of Ca^{2+}-mediated current decreased to 26% $\pm$ 2% of control value by 100 μM amiloride ($n = 6$, $p < 0.01$) and to 22% $\pm$ 0.9% by 100 ng/ml *PcTX* venom ($n = 5$, $p < 0.01$).

(C) Representative images and 340/380 ratios showing increase of $[Ca^{2+}]_i$ by pH drop to 6.0. Neurons were bathed in normal ECF containing 1.3 mM $CaCl_2$ with blockers for voltage-gated Ca^{2+} channels (5 μM nimodipine and 1 μM ω-conotoxin MVIIC) and glutamate receptors (10 μM MK801 and 20 μM CNQX). (Inset) Inhibition of acid-induced increase of $[Ca^{2+}]_i$ by 100 μM amiloride.

(D) Summary data showing inhibition of acid-induced increase of $[Ca^{2+}]_i$ by amiloride and *PcTX* venom. n = 6–8, **$p < 0.01$ compared with pH 6.0 group.

(E) Representative image and 340/380 ratio demonstrating lack of acid-induced increase of $[Ca^{2+}]_i$ in ASIC1$^{-/-}$ neurons; neurons had a normal response to NMDA ($n = 8$).

(F) Representative traces showing lack of acid-activated current at pH 6.0 in ASIC1$^{-/-}$ neurons.

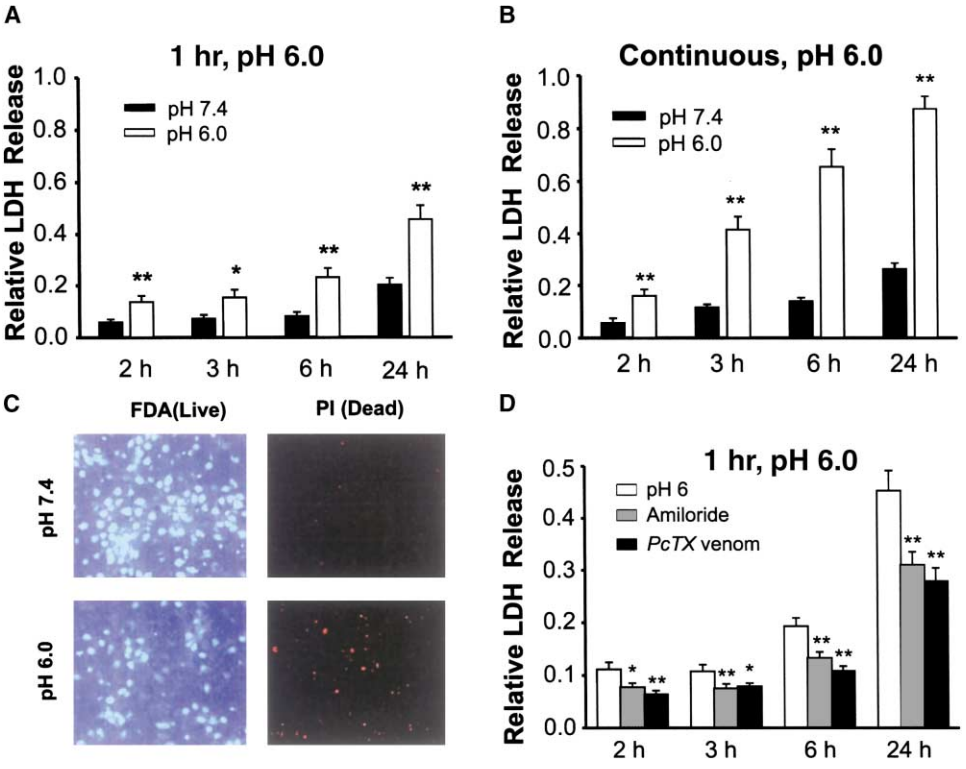

Figure 4. Acid Incubation Induces Glutamate Receptor-Independent Neuronal Injury Protected by ASIC Blockade

Time-dependent LDH release induced by 1 hr (A) or 24 hr incubation (B) of cortical neurons in pH 7.4 (solid bars) or 6.0 ECF (open bars). n = 20–25 wells, *p < 0.05, and **p < 0.01, compared to pH 7.4 group at same time points. (C) Analysis of acid-induced neuronal injury with fluorescein diacetate (FDA) staining of cell bodies of alive neurons and propidium iodide (PI) staining of nuclei of dead neurons. (D) Inhibition of acid-induced LDH release by 100 μM amiloride or 100 ng/ml *PcTX* venom (n = 20–27, *p < 0.05, and **p < 0.01). MK801, CNQX, and nimodipine were present in ECF for all experiments (A–D).

LDH release from 15.5% ± 2.4% to 24.0% ± 2.9% (n = 8 wells, $p < 0.05$). Addition of amiloride (100 μM) inhibited acid-induced LDH release in these cells (Figure 5B).

We also studied acid injury of CHO cells transiently transfected with cDNAs encoding GFP alone or GFP plus ASIC1a. After the transfection (24–36 hr), cells were incubated with acidic solution (pH 6.0) for 1 hr, and cell injury was assayed 24 hr following the acid incubation. As shown in Supplemental Figure S4, 1 hr acid incubation largely reduced surviving GFP-positive cells in GFP/ASIC1a group but not in the group transfected with GFP alone (n = 3 dishes in each group).

To further demonstrate an involvement of ASIC1a in acidosis-induced neuronal injury, we performed cell toxicity experiments on cortical neurons cultured from $ASIC^{+/+}$ and $ASIC1^{-/-}$ mice (Wemmie et al., 2002). Again, 1 hr acid incubation of $ASIC^{+/+}$ neurons at 6.0 induced substantial LDH release that was reduced by amiloride and *PcTX* venom (n = 8–12). One hour acid treatment of $ASIC1^{-/-}$ neurons, however, did not induce significant increase in LDH release at 24 hr (13.8% ± 0.9% for pH 7.4 and 14.2% ± 1.3% for pH 6.0, n = 8, $p > 0.05$), indicating resistance of these neurons to acid injury (Figure 5C). In addition, knockout of the ASIC1 gene also eliminated the effect of amiloride and *PcTX* venom on acid-induced LDH release (Figure 5C, n = 8 each), further suggesting that the inhibition of acid-induced injury of cortical neurons by amiloride and *PcTX* venom (Figure 4D) was due to blockade of ASIC1 subunits. In contrast to acid incubation, 1 hr treatment of $ASIC1^{-/-}$ neurons with 1 mM NMDA + 10 μM glycine (in Mg^{2+}-free [pH 7.4] ECF) induced 84.8% ± 1.4% of maximal LDH release at 24 hr (n = 4, Figure 5C), indicating normal response to other cell injury processes.

Modeled Ischemia Enhances Acidosis-Induced Glutamate-Independent Neuronal Injury via ASICs

As the magnitude of ASIC currents is potentiated by cellular and neurochemical components of brain ischemia—cell swelling, arachidonic acid, and lactate (Allen and Attwell, 2002; Immke and McCleskey, 2001)—and, more importantly, the desensitization of ASIC currents is dramatically reduced by modeled ischemia (see Figures 2A and 2C), we expected that activation of ASICs in ischemic conditions should produce greater neuronal injury. To test this hypothesis, we subjected neurons to 1 hr acid treatment under oxygen and glucose deprivation (OGD). MK801, CNQX, and nimodipine were added to all solutions to inhibit voltage-gated Ca^{2+} channels and glutamate receptor-mediated cell injury associated with OGD (Kaku et al., 1991). One hour incubation with pH 7.4 ECF under OGD conditions induced only 27.1% ± 3.5% of maximal LDH release at 24 hr (n = 5, Figure 5D). This finding is in agreement with a previous report that 1 hr OGD does not induce substantial cell injury with the blockade of glutamate receptors and voltage-

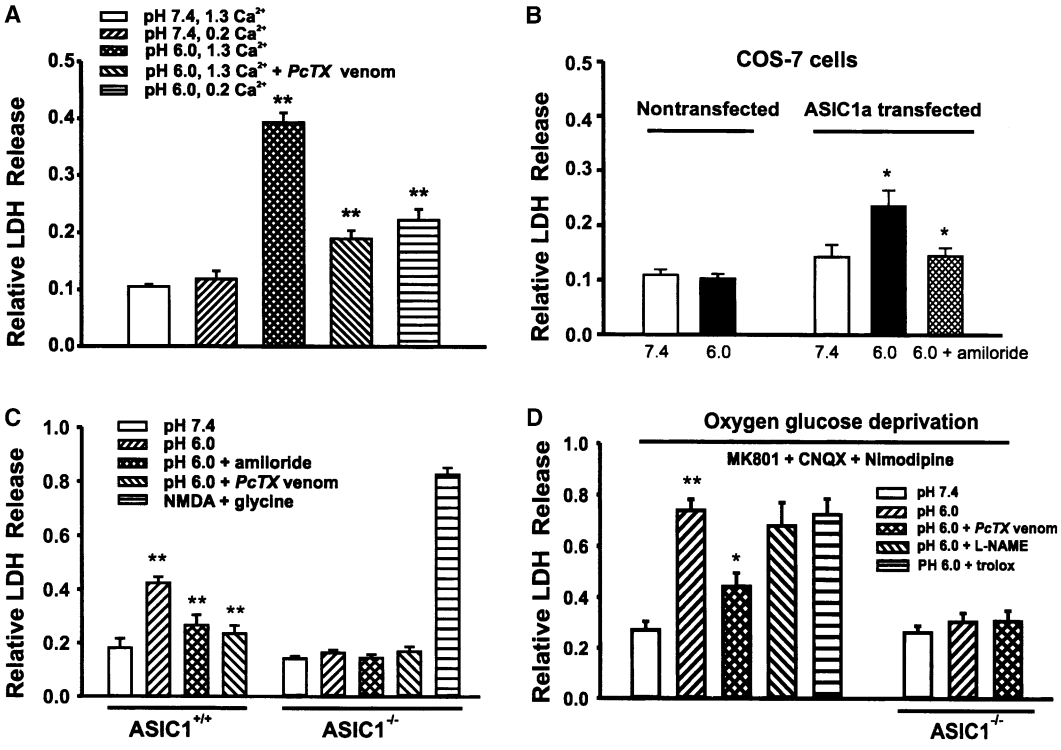

Figure 5. Involvement of ASIC1a in Acid-Induced Injury In Vitro

(A) Inhibition of acid-induced LDH release by reducing $[Ca^{2+}]_e$ (n = 11–12, **p < 0.01 compared with pH 6.0, 1.3 Ca^{2+}).

(B) Acid incubation induced increase of LDH release in ASIC1a-transfected but not nontransfected COS-7 cells (n = 8–20). Amiloride (100 μM) inhibited acid-induced LDH release in ASIC1a-transfected cells. *p < 0.05 for 7.4 versus 6.0 and 6.0 versus 6.0 + amiloride.

(C) Lack of acid-induced injury and protection by amiloride and *PcTX* venom in ASIC1$^{-/-}$ neurons (n = 8 in each group, p > 0.05).

(D) Acid-induced increase of LDH release in cultured cortical neurons under OGD (n = 5). LDH release induced by combined 1 hr OGD/acidosis was not inhibited by trolox and L-NAME (n = 8–11). OGD did not potentiate acid-induced LDH release in ASIC1$^{-/-}$ neurons. **p < 0.01 for pH 7.4 versus pH 6.0 and *p < 0.05 for pH 6.0 versus 6.0 + *PcTX* venom. MK801, CNQX, and nimodipine were present in ECF for all experiments (A–D).

gated Ca^{2+} channels (Aarts et al., 2003). However, 1 hr OGD, combined with acidosis (pH 6.0), induced 73.9% ± 4.3% of maximal LDH release (n = 5, Figure 5D, p < 0.01), significantly larger than acid-induced LDH release in the absence of OGD (see Figure 4A, p < 0.05). Addition of the ASIC1a blocker *PcTX* venom (100 ng/ml) significantly reduced acid/OGD-induced LDH release to 44.3% ± 5.3% (n = 5, p < 0.05, Figure 5D).

We also performed the same experiment with cultured neurons from the ASIC1$^{-/-}$ mice. Unlike in ASIC1 containing neurons, however, 1 hr treatment with combined OGD and acid only slightly increased LDH release in ASIC1$^{-/-}$ neurons (from 26.1% ± 2.7% to 30.4% ± 3.5%, n = 10–12, Figure 5D). This finding suggests that potentiation of acid-induced injury by OGD is largely due to OGD potentiation of ASIC1-mediated toxicity.

Aarts et al. (2003) have recently studied ischemia molded by prolonged OGD (2 hr) but without acidosis. In this model system, they demonstrated activation of a Ca^{2+}-permeable nonselective cation conductance activated by reactive oxygen/nitrogen species resulting in glutamate receptor-independent neuronal injury. The prolonged OGD-induced cell injury modeled by Aarts et al. is dramatically reduced by agents either scavenging free radicals directly (e.g., trolox) or reducing the production of free radicals (e.g., L-NAME) (Aarts et al., 2003). To determine whether combined short duration OGD and acidosis induced neuronal injury involves a similar mechanism, we tested the effect of trolox and L-NAME on OGD/acid-induced LDH release. As shown in Figure 5D, neither trolox (500 μM) nor L-NAME (300 μM) had significant effect on combined 1 hr OGD/acidosis-induced neuronal injury (n = 8–11). Additional experiments demonstrated that the ASIC blockers amiloride and *PcTX* venom had no effect on the conductance of TRPM7 channels reported to be responsible for prolonged OGD-induced neuronal injury by Aarts et al. (2003) (Supplemental Figure S5). Together, these findings strongly suggest that activation of ASICs but not TRPM7 channels is largely responsible for combined 1 hr OGD/acidosis-induced neuronal injury in our studies.

Activation of ASIC1a in Ischemic Brain Injury In Vivo

To provide evidence that activation of ASIC1a is involved in ischemic brain injury in vivo, we first tested the protective effect of amiloride and *PcTX* venom in a rat model of transient focal ischemia (Longa et al., 1989). Ischemia (100 min) was induced by transient middle cerebral artery occlusion (MCAO). A total of 6 μl artificial CSF (aCSF) alone, aCSF-containing amiloride (1 mM), or *PcTX* venom (500 ng/ml) was injected intracerebroven-

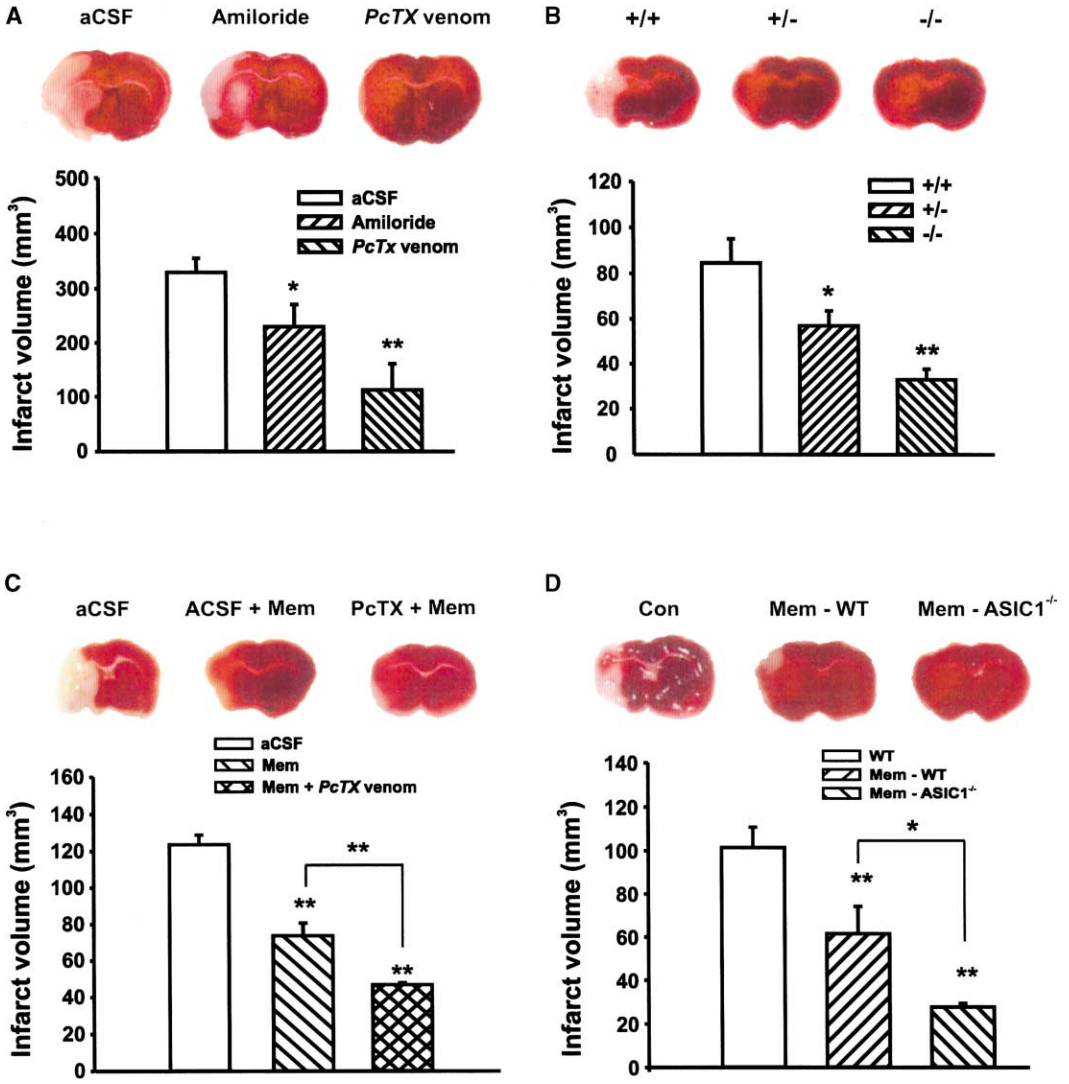

Figure 6. Neuroprotection by ASIC1 Blockade and ASIC1 Gene Knockout in Brain Ischemia In Vivo

(A) TTC-stained brain sections show infarct area (image) and volume (bar graph) in brains from aCSF (n = 7), amiloride (n =11), or *PcTX* venom (n = 5) injected rats. *$p < 0.05$ and **$p < 0.01$ compared with aCSF injected group.

(B) Reduction in infarct volume in brains from ASIC1$^{-/-}$ mice (n = 6 for each group). *$p < 0.05$ and **$p < 0.01$ compared with +/+ group.

(C) Reduction in infarct volume in brains from mice i.p. injected with 10 mg/kg memantine (Mem) or i.p. injection of memantine accompanied by i.c.v. injection of *PcTX* venom (500 ng/ml). **$p < 0.01$ compared with aCSF injection and between memantine and memantine plus *PcTX* venom (n = 5 in each group).

(D) Reduction in infarct volume in brains from either ASIC1$^{+/+}$ (wt) or ASIC1$^{-/-}$ mice i.p. injected with memantine (n = 5 in each group). *$p < 0.05$, and **$p < 0.01$.

tricularly 30 min before and after the ischemia. Based on the study by Westergaard (1969), the volume for cerebral ventricular and spinal cord fluid for 4-week-old rats is estimated to be ~60 μl. Assuming that the infused amiloride and *PcTX* were uniformly distributed in the CSF, we would expect a concentration of ~100 μM for amiloride and ~50 ng/ml for PcTX, which is a concentration found effective in our cell culture experiments. Infarct volume was determined by TTC staining (Bederson et al., 1986) at 24 hr following ischemia. Ischemia (100 min) produced an infarct volume of 329.5 ± 25.6 mm^3 in aCSF-injected rats (n = 7) but only 229.7 ± 41.1 mm^3 in amiloride-injected (n = 11, $p < 0.05$) and 130.4 ± 55.0 mm^3 (~60% reduction) in *PcTX* venom-injected rats (n = 5, $p < 0.01$) (Figure 6A).

We next used ASIC1$^{-/-}$ mice to further demonstrate the involvement of ASIC1a in ischemic brain injury in vivo. Male ASIC1$^{+/+}$, ASIC1$^{+/-}$, and ASIC1$^{-/-}$ mice (~25 g, with congenic C57Bl6 background) were subjected to 60 min MCAO as previously described (Stenzel-Poore et al., 2003). Consistent with the protection by pharmacological blockade of ASIC1a (above), −/− mice displayed significantly smaller (~61% reduction) infarct volumes (32.9 ± 4.7 mm^3, n = 6) as compared to +/+ mice (84.6 ± 10.6 mm^3, n = 6, $p < 0.01$). +/− mice also showed reduced infarct volume (56.9 ± 6.7 mm^3, n = 6, $p < 0.05$) (Figure 6B).

We then determined whether blockade of ASIC1a channels or knockout of the ASIC1 gene could provide additional protection in vivo in the setting of glutamate

receptor blockade. We selected the uncompetitive NMDA receptor antagonist memantine, as it has been recently used in successful clinical trials (Tariot et al., 2004). Memantine (10 mg/kg) was injected intraperitoneally (i.p.) into C57Bl6 mice immediately following 60 min MCAO and accompanied by intracerebroventricular injection (i.c.v.) of a total volume of 0.4 μl aCSF alone or aCSF containing *PcTX* venom (500 ng/ml) 15 min before and following ischemia. In control mice with i.p. injection of saline and i.c.v. injection of aCSF, 60 min MCAO induced an infarct volume of 123.6 ± 5.3 mm^3 ($n = 5$, Figure 6C). In mice with i.p. injection of memantine and i.c.v. injection of aCSF, the same duration of ischemia induced an infarct volume of 73.8 ± 6.9 mm^3 ($n = 5$, $p < 0.01$). However, in mice injected with memantine and *PcTX* venom, an infarct volume of only 47.0 ± 1.1 mm^3 was induced ($n = 5$, $p < 0.01$ compared with both control and memantine groups, Figure 6C). These data strongly suggest that blockade of homomeric ASIC1a can provide additional protection in in vivo ischemia in the setting of NMDA receptor blockade. Additional protection was also observed in $ASIC1^{-/-}$ mice treated with pharmacologic NMDA blockade (Figure 6D). In $ASIC^{+/+}$ mice i.p. injected with saline or 10 mg/kg memantine, 60 min MCAO induced an infarct volume of 101.4 ± 9.4 mm^3 or 61.6 ± 12.7 mm^3, respectively ($n = 5$ in each group, Figure 6D). However, in $ASIC1^{-/-}$ mice injected with memantine, the same ischemia duration induced an infarct volume of 27.7 ± 1.6 mm^3 ($n = 5$), significantly smaller than the infarct volume in $ASIC1^{+/+}$ mice injected with memantine ($p < 0.05$).

Discussion

Despite enormous recent progress defining cellular and molecular responses of the brain to ischemia, there is no effective treatment for stroke patients. Most notable are the failures of multicenter clinical trials of glutamate antagonists (Lee et al., 1999; Wahlgren and Ahmed, 2004). Here we demonstrate a new mechanism of ischemic brain injury and the role of ischemic acidosis in this biology. We show that ischemic injury in the setting of acidosis occurs via activation of Ca^{2+}-permeable ASICs, a newly described class of ligand-gated channels (Waldmann et al., 1997a; Waldmann and Lazdunski, 1998). This Ca^{2+} toxicity is independent of glutamate receptors or voltage-gated Ca^{2+} channels.

Using whole-cell patch-clamp recording in mixed cortical cultures, we demonstrate activation of ASIC currents in the range of pH_e that occurs commonly in ischemia. With Fura-2 fluorescent imaging and ion substitution protocols, we show ASICs flux Ca^{2+} in cortical neurons and do so in the presence of NMDA, AMPA, and voltage-gated Ca^{2+} channel blockade. Using in vitro cell toxicity models, we demonstrate that acidosis induces glutamate-independent neuronal injury, which is reduced by both nonspecific and specific ASIC1a antagonists, and by lowering $[Ca^{2+}]_e$. In addition, we show that neurons and COS-7 cells lacking ASIC1a are resistant to acid injury, while transfection of COS-7 cells with Ca^{2+}-permeable ASIC1a results in acid sensitivity. Using in vivo focal ischemia models, we demonstrate that pharmacologic blockade of ASIC1a channels and ASIC1a gene knockout both protected the brain from ischemic injury and do so in the presence of NMDA blockade.

Local $[H^+]$ is the agonist for ASICs functioning during normal synaptic transmission in the brain (Wemmie et al., 2002). This signaling is not injurious. However, ASICs respond also to the global, marked pH declines occurring in the ischemic brain. Within 1 min of global ischemia, pH_e falls from 7.2 to 6.5 (Simon et al., 1985), a level sufficient to activate ASIC1a channels, which have a $pH_{0.5}$ at 6.2. Remarkably, ischemia itself, modeled in vitro, markedly enhances the magnitude of ASIC response at a given level of acidosis, thus potentiating toxic Ca^{2+} loading in ischemic neurons. Furthermore, ischemia dramatically reduces desensitization of ASIC currents, signifying a possibility of long-lasting activity of ASICs during prolonged ischemic acidosis in vivo.

It has been shown in intact animals that brief global reductions of brain pH to 6.5 alone do not produce brain injury (Litt et al., 1985), nor does hypoxia alone (Miyamoto and Auer, 2000; Pearigen et al., 1996). However, our in vitro data suggest that the combination of ischemia (hypoxia) with acidosis (ischemic acidosis), as occurs in vivo, may cause marked brain injury through ischemia enhancing the toxic effect of ASIC1a channels. This argument is strongly supported by the finding that both ASIC1a blockade and ASIC1a gene knockout produce substantial (~60%) reduction in infarct volume.

Acidosis, apart from affecting ischemic brain injury via ASICs, affects the function of other channels as well. Particularly pertinent in ischemia is the acid blockade of the NMDA channels (Tang et al., 1990; Traynelis and Cull-Candy, 1990), which is protective against in vitro ischemic neuronal injury (Kaku et al., 1993; Giffard et al., 1990). This NMDA blockade in the ischemic brain by acidosis might in part explain the failure of NMDA antagonists in human stroke trials. Treatment of stroke with ASIC1a blockade could be particularly effective, as ischemic acidosis is serving as an additional therapy by blocking NMDA function.

As our in vitro studies showing a protective effect of ASIC1a blockade were performed in the presence of antagonists of NMDA, AMPA, and voltage-gated Ca^{2+} channels, the findings reported here may offer a new and robust neuroprotective strategy for stroke, either alone or in combination with other therapies (MacGregor et al., 2003). Further, we demonstrate in vivo that pharmacologic ASIC1a blockade or ASIC1a gene deletion offer more potent neuroprotection against stroke than NMDA antagonism.

Together, our studies suggest that activation of Ca^{2+}-permeable ASIC1a is a novel, glutamate-independent biological mechanism underlying ischemic brain injury. As the regulation of other potentially protective ASIC subunits also occurs in the ischemic brain (Johnson et al., 2001), these findings may help the design of novel therapeutic neuroprotective strategies for brain ischemia.

Experimental Procedures

Neuronal Culture

Following anesthesia with halothane, cerebral cortices were dissected from E16 Swiss mice or P1 $ASIC1^{+/+}$ and $ASIC1^{-/-}$ mice and incubated with 0.05% trypsin-EDTA for 10 min at 37°C. Tissues were then triturated with fire-polished glass pipettes and plated on poly-L-ornithine-coated 24-well plates or 25 × 25 mm glass coverslips at a density of 2.5×10^5 cells per well or 10^6 cells per coverslip. Neurons were cultured with MEM supplemented with 10% horse

serum (for E16 cultures) or Neurobasal medium supplemented with B27 (for P1 cultures) and used for electrophysiology and toxicity studies after 12 days. Glial growth was suppressed by addition of 5-fluoro-2-deoxyuridine and uridine, yielding cultured cells with ~90% neurons as determined by NeuN and GFAP staining (data not shown).

Electrophysiology

ASIC currents were recorded with whole-cell patch-clamp and fast-perfusion techniques. The normal extracellular solution (ECF) contained (in mM) 140 NaCl, 5.4 KCl, 25 HEPES, 20 glucose, 1.3 $CaCl_2$, 1.0 $MgCl_2$, 0.0005 TTX (pH 7.4), 320–335 mOsm. For low pH solutions, various amounts of HCl were added. For solutions with pH $<$ 6.0, MES instead of HEPES was used for more reliable pH buffering. Patch electrodes contained (in mM) 140 CsF, 2.0 $MgCl_2$, 1.0 $CaCl_2$, 10 HEPES, 11 EGTA, 4 MgATP (pH 7.3), 300 mOsm. The Na^+-free solution consisted of 10 mM $CaCl_2$, 25 mM HEPES with equiosmotic NMDG or sucrose substituting for NaCl (Chu et al., 2002). A multibarrel perfusion system (SF-77B, Warner Instrument Co.) was employed for rapid exchange of solutions.

Cell Injury Assay—LDH Measurement

Cells were washed three times with ECF and randomly divided into treatment groups. MK801 (10 μM), CNQX (20 μM), and nimodipine (5 μM) were added in all groups to eliminate potential secondary activation of glutamate receptors and voltage-gated Ca^{2+} channels. Following acid incubation, neurons were washed and incubated in Neurobasal medium at 37°C. LDH release was measured in culture medium using the LDH assay kit (Roche Molecular Biochemicals). Medium (100 μl) was transferred from culture wells to 96-well plates and mixed with 100 μl reaction solution provided by the kit. Optical density was measured at 492 nm 30 min later, utilizing a microplate reader (Spectra Max Plus, Molecular Devices). Background absorbance at 620 was subtracted. The maximal releasable LDH was obtained in each well by 15 min incubation with 1% Triton X-100 at the end of each experiment.

Ca^{2+} Imaging

Cortical neurons grown on 25 × 25 mm glass coverslips were washed three times with ECF and incubated with 5 μM fura-2-acetoxymethyl ester for 40 min at 22°C, washed three times, and incubated in normal ECF for 30 min. Coverslips were transferred to a perfusion chamber on an inverted microscope (Nikon TE300). Cells were illuminated using a xenon lamp and observed with a 40× UV fluor oil-immersion objective lens, and video images were obtained using a cooled CCD camera (Sensys KAF 1401, Photometrics). Digitized images were acquired and analyzed in a PC controlled by Axon Imaging Workbench software (Axon Instruments). The shutter and filter wheel (Lambda 10-2) were controlled by the software to allow timed illumination of cells at 340 or 380 nm excitation wavelengths. Fura-2 fluorescence was detected at emission wavelength of 510 nm. Ratio images (340/380) were analyzed by averaging pixel ratio values in circumscribed regions of cells in the field of view. The values were exported to SigmaPlot for further analysis.

Fluorescein-Diacetate Staining and Propidium Iodide Uptake

Cells were incubated in ECF containing fluorescein-diacetate (FDA) (5 μM) and propidium iodide (PI) (2 μM) for 30 min followed by wash with dye-free ECF. Alive (FDA-positive) and dead (PI-positive) cells were viewed and counted on a microscope (Zeiss) equipped with epifluorescence at 580/630 nm excitation/emission for PI and 500/550 nm for FDA. Images were collected using an Optronics DEI-730 camera equipped with a BQ 8000 sVGA frame grabber and analyzed using computer software (Bioquant, TN).

Transfection of COS-7 Cells

COS-7 cells were cultured in MEM with 10% HS and 1% PenStrep (GIBCO). At ~50% confluence, cells were cotransfected with cDNAs for ASICs and GFP in pc^{DNA3} vector using FuGENE6 transfection reagents (Roche Molecular Biochemicals). DNA for ASICs (0.75 μg) and 0.25 μg of DNA for GFP were used for each 35 mm dish. GFP-positive cells were selected for patch-clamp recording 48 hr after transfection. For stable transfection of ASIC1a, 500 μg/ml G418 was added to culture medium 1 week following the transfection. The surviving G418-resistant cells were further plated and passed for $>$5 passages in the presence of G418. Cells were then checked with patch-clamp and immunofluorescent staining for the expression of ASIC1a.

Oxygen-Glucose Deprivation

Neurons were washed three times and incubated with glucose-free ECF at pH 7.4 or 6.0 in an anaerobic chamber (Model 1025, Forma Scientific) with an atmosphere of 85% N_2, 10% H_2, and 5% CO_2 at 35°C. Oxygen-glucose deprivation (OGD) was terminated after 1 hr by replacing the glucose-free ECF with Neurobasal medium and incubating the cultures in a normal cell culture incubator. With HEPES-buffered ECF used, 1 hr OGD slightly reduced pH from 7.38 to 7.28 (n = 3) and from 6.0 to 5.96 (n = 4).

Focal Ischemia

Transient focal ischemia was induced by suture occlusion of the middle cerebral artery (MCAO) in male rats (SD, 250–300 g) and mice (with congenic C57Bl6 background, ~25 g) anesthetized using 1.5% isoflurane, 70% N_2O, and 28.5% O_2 with intubation and ventilation. Rectal and temporalis muscle temperature was maintained at 37°C ± 0.5°C with a thermostatically controlled heating pad and lamp. Cerebral blood flow was monitored by transcranical LASER doppler. Animals with blood flow not reduced below 20% were excluded.

Animals were killed with chloral hydrate 24 hr after ischemia. Brains were rapidly removed, sectioned coronally at 1 mm (mice) or 2 mm (rats) intervals, and stained by immersion in vital dye (2%) 2,3,5-triphenyltetrazolium hydrochloride (TTC). Infarction area was calculated by subtracting the normal area stained with TTC in the ischemic hemisphere from the area of the nonischemic hemisphere. Infarct volume was calculated by summing infarction areas of all sections and multiplying by slice thickness. Rat intraventricular injection was performed by stereotaxic technique using a microsyringe pump with cannula inserted stereotactically at 0.8 mm posterior to bregma, 1.5 mm lateral to midline, and 3.8 mm ventral to the dura. All manipulations and analyses were performed by individuals blinded to treatment groups.

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