Coupling between NMDA Receptor and Acid-Sensing Ion Channel Contributes to Ischemic Neuronal Death

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Summary

Acid-sensing ion channels (ASICs) composed of ASIC1a subunit exhibit a high Ca^{2+} permeability and play important roles in synaptic plasticity and acid-induced cell death. Here, we show that ischemia enhances ASIC currents through the phosphorylation at Ser478 and Ser479 of ASIC1a, leading to exacerbated ischemic cell death. The phosphorylation is catalyzed by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) activity, as a result of activation of NR2B-containing *N*-methyl-D-aspartate subtype of glutamate receptors (NMDARs) during ischemia. Furthermore, NR2B-specific antagonist, CaMKII inhibitor, or overexpression of mutated form of ASIC1a with Ser478 or Ser479 replaced by alanine (ASIC1a-S478A, ASIC1a-S479A) in cultured hippocampal neurons prevented ischemia-induced enhancement of ASIC currents, cytoplasmic Ca^{2+} elevation, as well as neuronal death. Thus, NMDAR-CaMKII cascade is functionally coupled to ASICs and contributes to acidotoxicity during ischemia. Specific blockade of NMDAR/CaMKII-ASIC coupling may reduce neuronal death after ischemia and other pathological conditions involving excessive glutamate release and acidosis.

Introduction

Certain types of neurons, especially hippocampal CA1 pyramidal neurons, are highly vulnerable to ischemic insults. Among various hypotheses on the mechanism of ischemic neuronal injury, excitotoxicity mediated by *N*-methyl-D-aspartate subtype of glutamate receptors (NMDARs) has attracted much attention (Choi and Rothman, 1990). However, recent data demonstrated that in addition to NMDARs, activation of acid-sensing ion channels (ASICs) is also involved in the ischemic cell injury (Xiong et al., 2004). The precise mechanism by which ASIC activation causes neuronal injury and whether there is a relationship between NMDAR activation and ASIC-dependent acidotoxicity remain unclear.

The ASICs belong to the degenerin/epithelial Na^+ channel superfamily. These channels are cation selective and diuretic amiloride sensitive (Alvarez de la Rosa et al., 2000; Waldmann et al., 1997). To date, six subunits of ASICs have been identified: 1a, 1b, 2a, 2b, 3, and 4 (Krishtal, 2003), among which 1b and 3 are mainly expressed in periphery sensory neurons. Moreover, the homomeric channel composed of ASIC1a is highly permeant to Ca^{2+}, whereas other homomeric or heteromeric ASICs are largely impermeant to Ca^{2+} (Waldmann et al., 1997; Wu et al., 2004; Yermolaieva et al., 2004). This suggests the potential involvement of ASIC1a in Ca^{2+}-dependent cellular processes. Indeed, deletion of mouse ASIC1 gene markedly impaired synaptic plasticity and caused learning and memory deficits (Bianchi and Driscoll, 2002; Wemmie et al., 2002). Under pathological conditions, activation of homomeric ASIC1a channels may also play a key role in acidosis-induced neuronal death. Xiong et al. (2004) recently reported that acidosis activates ASIC1a, inducing Ca^{2+}-dependent but glutamate receptor-independent neuronal injury and death.

It is well known that NMDAR activation and subsequent Ca^{2+} overload are critical for ischemia-induced cell death. The NMDAR is a heteromeric complex containing an essential subunit NR1 and one or more regulatory subunits, NR2A, 2B, 2C, 2D, or NR3. Agonist binding of NMDARs increases the Ca^{2+} influx and activates multiple intracellular signaling cascades, including autophosphorylation and membrane translocation of Ca^{2+}/calmodulin kinase II (CaMKII) (Aronowski et al., 1992; Suzuki et al., 1994), which in turn enhances NMDAR-mediated Ca^{2+} influx and ischemic neuronal death (Bayer et al., 2001; Leonard et al., 1999). Membrane proteins phosphorylated by CaMKII may also include ASIC1a, which contributes to acidosis-induced Ca^{2+} influx and cell death.

Because excess glutamate release and acidosis often occur concurrently during brain ischemia, we asked whether ischemia may coactivate NMDARs and ASICs and whether NMDAR-ASIC coupling participates in ischemic neuronal death. We examined specifically the possibility that NMDAR activation leads to exacerbated cell death by enhancing ASIC1a functions in the rat hippocampus. This possibility was suggested by the following observations: (1) excess glutamate release and tissue acidosis are both associated with ischemic injury (Lipton, 1999), (2) subunits of both NMDARs (Simon et al., 1984) and ASICs (Waldmann et al., 1997) are abundant in the hippocampus, (3) ischemia triggers Ca^{2+} signaling through activation of either NMDARs (Sattler and Tymianski, 2001) or ASIC1a (Xiong et al., 2004),

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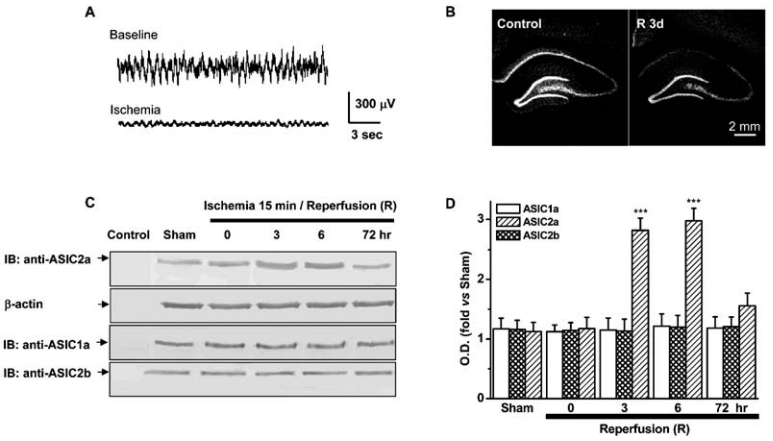

Figure 1. Western Blot Analysis of ASIC1a, 2a, and 2b Levels in Lysates from Rat Hippocampi after Transient Global Ischemia

Proteins were extracted from hippocampi of rats treated with either sham operation (Sham) or 15 min ischemia followed by 0, 3, 6, or 72 hr reperfusion (see Experimental Procedures). Affinity-purified rabbit polyclonal antibodies against various ASIC subunits were used to detect the protein at 70 kDa. (A) Real-time EEG recording before (baseline) and during ischemic insult. (B) Sections (30 μm) of hippocampus immunostained with NeuN, indicating the selective loss of NeuN-positive cells in hippocampal CA1 region after ischemia and reperfusion for 3 days (R 3d). (C) The expression of ASIC2a was enhanced after 3 hr of reperfusion (R) and returned to normal levels at 72 hr, whereas 1a and 2b did not change significantly. Control, negative control without anti-ASIC antibody treatment. β-Actin was used as the loading control. (D) Results from three experiments similar to that shown in (C). Data (mean ± SE) were normalized to the basal expression level of individual ASIC subunits. Triple asterisk indicates $p < 0.001$, compared with "Sham" (one-way ANOVA).

(4) activation of either NMDARs or ASIC1a (Xiong et al., 2004) directly mediates neuronal death, and (5) the magnitude of ASIC currents is enhanced by ischemia-related factors (Allen and Attwell, 2002; Immke and McCleskey, 2001) and by experimental ischemia in neuronal cultures (Xiong et al., 2004).

Results

Global Ischemia Induces CaMKII-Dependent ASIC1a Phosphorylation

To explore the role of ASICs in ischemia signaling, we first examined the expression and phosphorylation of ASICs after transient global ischemia, which was induced by the four-vessel occlusion method (Pulsinelli and Brierley, 1979). The effectiveness of this method in inducing global ischemia was verified by changes in EEG and neuronal loss in the CA1 region of the hippocampus (Figures 1A and 1B).

The effect of transient ischemia on the expression of three isoforms of ASIC subunits, 1a, 2a, and 2b, which are known to be predominantly expressed in hippocampal neurons (Waldmann, 2001), was assayed by immunoblotting. We found the expression of ASIC2a was significantly increased after ischemia/reperfusion, consistent with a previous report (Johnson et al., 2001), whereas the level of 1a and 2b remained unchanged (Figures 1C and 1D).

Transient global ischemia causes serine phosphorylation of a number of membrane proteins such as NMDA receptor subunits and CaMKII in vulnerable CA1 pyramidal neurons (Figure S1). Therefore, we examined the change in phosphorylation of ASICs in the rat hippocampus after ischemic insults. Immunoprecipitation (IP) of hippocampal extracts with anti-phosphoserine antibody and subsequent immunoblotting (IB) with anti-ASIC1a antibody showed that the phosphorylation of ASIC1a was significantly elevated and reached an apparent plateau after 5 min of ischemic insult, whereas the phosphorylation of 2a and 2b isoforms showed no significant change (Figure 2A). After 1 hr reperfusion, the ASIC1a phosphorylation returned to the basal level

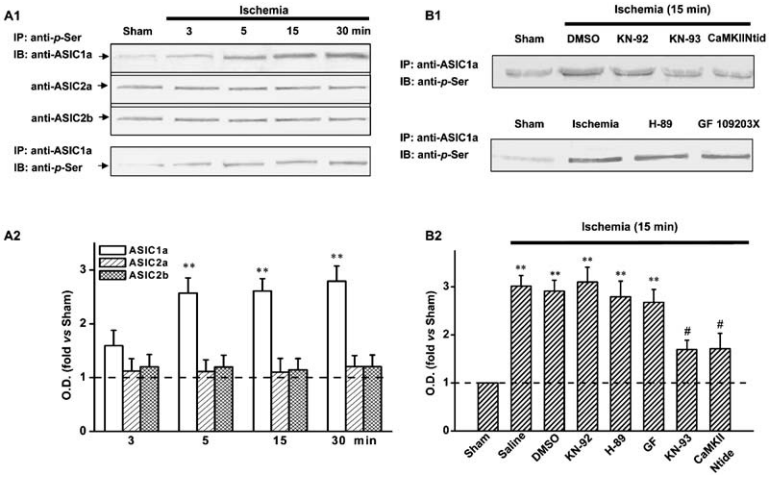

Figure 2. Global Ischemia Rapidly Elevated Serine Phosphorylation of ASIC1a Subunit

Proteins were extracted from hippocampi of rats treated either with sham operation or with 3, 5, 15, or 30 min of ischemia. (A1) Upper blots show the immunoreactive bands with antibodies against ASIC1a, 2a, and 2b after immunoprecipitation (IP) of extracted proteins with an anti-phosphoserine antibody. Lowest blot shows immunobands for phosphoserine after IP of extracted proteins with the ASIC1a antibody. (A2) Measurements of the level of serine phosphorylation of ASIC1a, 2a, and 2b, normalized to the "Sham" level (dashed line) of the respective subunit. (B1) The effect of pharmacological blockers of CaMKII (KN-93 or CaMKIINtide), PKA (H-89), and PKC (GF 109203X) on the ischemia-induced enhancement of ASIC1a phosphorylation. KN-92, an inactive analog of KN-93. DMSO, vehicle for KN-93, KN-92, and CaMKIINtide. Saline, vehicle for H-89 and GF 109203X. (B2) Summary of results from experiments similar to that described in (B1), normalized to the "Sham" level (dashed line). Data are mean ± SE (n = 3). Double asterisk indicates $p < 0.01$, compared with "Sham"; pound sign indicates $p < 0.05$, compared with DMSO (one-way ANOVA).

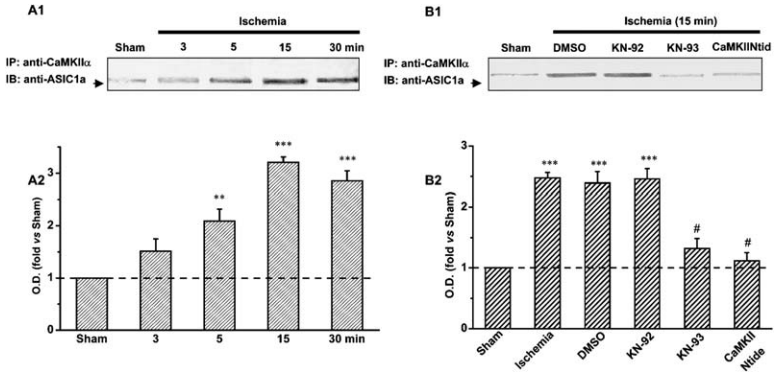

Figure 3. Global Ischemia Increased the Association of CaMKIIα with ASIC1a

Immunoprecipitation (IP) was performed with anti-CaMKIIα antibody and Western blot with anti-ASIC1a antibody. (A1) Enhanced coimmunoprecipitation (CoIP) of ASIC1a and CaMKIIα found in extracts from ischemic hippocampi. (A2) Semiquantitative densitometric measurements of the extent of CoIP were determined for samples from rats treated with different duration of ischemia. (B1) Blocking CaMKII activity with KN-93 or CaMKIINtide decreased the CoIP of CaMKIIα with ASIC1a. (B2) Summary of results from experiments similar to that described in (B1). All data are shown as mean ± SE (n = 3), normalized to the value of "Sham" (dashed line). Double asterisk indicates $p < 0.01$, and triple asterisk indicates $p < 0.001$, compared with "Sham"; pound sign indicates $p < 0.05$, compared with DMSO (one-way ANOVA).

observed prior to the ischemic insult (data not shown). This increased phosphorylation of ASIC1a by the ischemic insult was further confirmed by reverse IP with ASIC1a antibody and subsequent IB with anti-phosphoserine antibody (Figure 2A1, bottom band). Intracerebroventricular injection of KN-93 (5 μl at 1 mg/ml), a specific CaMKII inhibitor, completely abolished the increase in ASIC1a phosphorylation, whereas injection of H-89 (5 μl at 10 mM) and GF 109203X (5 μl at 4 mM), specific inhibitors of cAMP-dependent protein kinase (PKA) and protein kinase C (PKC), respectively, had no effect on the ASIC1a phosphorylation. Moreover, the ASIC1a phosphorylation was also unaffected by KN-92 (5 μl at 1 mg/ml) (Figure 2B), an inactive analog of KN-93. The specific action of CaMKII in ASIC1a phosphorylation was further confirmed by intracerebroventricular injection of a CaMKII inhibitor peptide, CaMKIINtide (5 μl at 10 mM), which significantly reduced the level of ASICla phosphorylation (Figure 2B). Together, these results suggest that ischemic insult selectively increased serine phosphorylation of ASIC1a in rat hippocampus via a CaMKII-dependent process.

Increased CaMKIIα/ASIC1a Interaction during Transient Ischemia

The ASIC1a channels are expressed on the surface of hippocampal neurons (Alvarez de la Rosa et al., 2003; Wemmie et al., 2002), and global ischemia induces autophosphorylation and translocation of CaMKII to the plasma membrane (Aronowski et al., 1992; Suzuki et al., 1994). We thus hypothesized that interaction between membrane bound CaMKII with ASIC1a after ischemic insult may be responsible for the increased phosphorylation of ASIC1a. Consistent with this hypothesis, IP with anti-CaMKIIα obtained from hippocampal extracts and subsequent IB with anti-ASIC1a antibody showed a significant increase in the amount of ASIC1a in the CaMKIIα precipitate after 5 min of ischemic insult (Figure 3A), and this increase was diminished by intracerebroventricular injection of KN-93 (5 μ1 at 1 mg/ml) or CaMKIINtide (5 μ1 at 10 mM), but not by KN-92 (5 μ1 at 1 mg/ml) (Figure 3B). However, when the same protocol was applied to 2a or 2b isoforms, no coprecipitation of these isoforms with CaMKIIα was detected (data not shown). Thus, ischemia-activated CaMKIIα appears to interact with ASICla and is responsible for the selective phosphorylation of ASIC1a.

Ischemia-Induced ASIC1a Phosphorylation Requires NR2B-NMDAR Activation

The autophosphorylation and translocation of CaMKII are triggered by elevation of intracellular Ca^{2+}, and the Ca^{2+} influx during the early stage of global ischemia is mostly mediated by NMDARs (Silver and Erecinska, 1990). Thus, we further investigated whether NMDAR activation is required for the increased phosphorylation of ASIC1a during ischemia. As shown in Figure 4A, we found that the enhancement of ASIC1a phosphorylation was totally abolished by intraperitoneal injection of MK-801 (1 mg/kg) or intracerebroventricular injection of APV (5 μl at 50 mM), two selective NMDAR antagonists, 20 min before global ischemia, whereas intracerebroventricular injection of CNQX (5 μl at 10 mM), a selective AMPA receptor antagonist, had no significant effect (Figure 4A), consistent with the predominant role of NMDARs in the early stage of ischemic signaling.

Among various NMDAR subunits, NR2B is known to bind CaMKII. This binding facilitates CaMKII autophosphorylation and its targeting to postsynaptic density as well as phosphorylation and activation of NR2B by CaMKII. To examine whether NR2B-containing NMDARs (NR2B-NMDARs) are involved in the CaMKII-mediated ASIC1a phosphorylation demonstrated above, we studied the effects of two selective NR2B-NMDAR inhibitors, ifenprodil and Ro 25-6981. As shown in Figure 4B, ifenprodil (5 μl at 3 or 5 mM) or Ro 25-6981 (5 μl at 0.1 mM) effectively blocked the increased phosphorylation of ASIC1a induced by the ischemic insult (Figure 4B). Thus, NR2B-NMDAR activation is required for the increased ASIC1a phosphorylation induced by global ischemia.

NMDAR Activation Enhances ASIC1a Currents via NR2B

To investigate whether NR2B-NMDAR activity is functionally coupled to the activation of ASICs, we examined the effect of NMDAR activation on acid-induced membrane currents in acutely dissociated hippocampal CA1

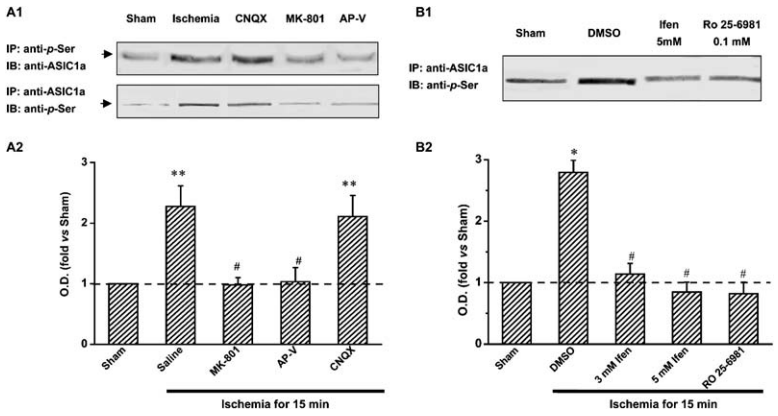

Figure 4. NMDAR Activation Is Required for Ischemia-Induced Enhancement of ASIC1a Phosphorylation

(A1) Effects of blockers of NMDARs (MK-801 or APV) and AMPA receptors (CNQX) on the enhancement of ASIC1a phosphorylation induced by 15 min ischemia.

(A2) Densitometric measurements of results obtained from experiments described in (A1). Data were normalized to the "Sham" control.

(B1) Effect of NR2B-specific blocker of NMDARs (ifenprodil and Ro 25-6981) on the ischemia-induced enhancement of ASIC1a phosphrylation.

(B2) Summary of results from experiments similar to that described in (B1). All data (mean ± SE, n = 3) were normalized to the value of "Sham" (dashed line). DMSO represents vehicle control for ifenprodil or Ro 25-6981, and saline for CNOX and APV. Asterisk indicates $p < 0.05$, compared with "Sham"; pound sign indicates $p < 0.05$, compared with DMSO (one-way ANOVA).

neurons (Gao et al., 2004). As shown in Figure 5A, we found that in 15/21 neurons examined, brief activation of NMDARs with aspartate (0.1 mM) led to an increase (48.3% ± 1.9%) (Figures 5A and 5C) in the amplitude of ASIC currents induced by subsequent application of an acidic external solution. This increase was inhibited by bath application of APV (100 μM) or ifenprodil (3 μM), which by itself did not affect ASIC currents (data not shown). In the rest of cells (6/21), no effect of aspartate on ASIC currents was observed. The heterogenous effects of aspartate may be due to the differential expression of NR2B or CaMKII in different types of hippocampal CA1 neurons (Law et al., 2003).

The specific modulatory effect of NR2B on ASIC currents was further examined by using HEK293T cells cotransfected with cDNAs of ASIC1a and NR1, together with that of either NR2B-GFP or NR2A-GFP. Although endogenous ASIC1a was found in HEK293 cells (Gunthorpe et al., 2001), exogenous ASIC1a was transfected to ensure a high-level ASIC1a expression. Our immunochemical staining data indicated the presence of abundant endogenous CaMKIIα in HEK293T cells (data not shown); thus, no cotransfection of CaMKIIα was performed. As shown in Figures 5B and 5C, brief application of aspartate (0.1 mM) led to a subsequent enhancement of ASIC1a-mediated currents in cells expressing NR2B-NMDARs but not in those expressing NR2A-NMDARs. Consistent with the results on acutely dissociated hippocampal neurons, the aspartate-induced effects on ASIC1a currents were abolished by NMDAR or NR2B antagonist, APV or ifenprodil, respectively (Figure 5C). Thus, activation of NMDARs exerts a modulatory action on ASIC currents that depends on NR2B, presumably through the NR2B-NMDAR-mediated Ca^{2+} influx and the subsequent activation of CaMKII, which phosphorylates ASIC1a (Figure 2).

Experimental Ischemia Enhances ASIC Currents

To further gain insights into the relationship of NMDAR activation and ASIC regulation during brain ischemia, we examined whether ASIC currents in hippocampal CA1 neurons are affected by experimental ischemia induced by oxygen and glucose deprivation (OGD), which mediates neuronal death through NMDAR activation

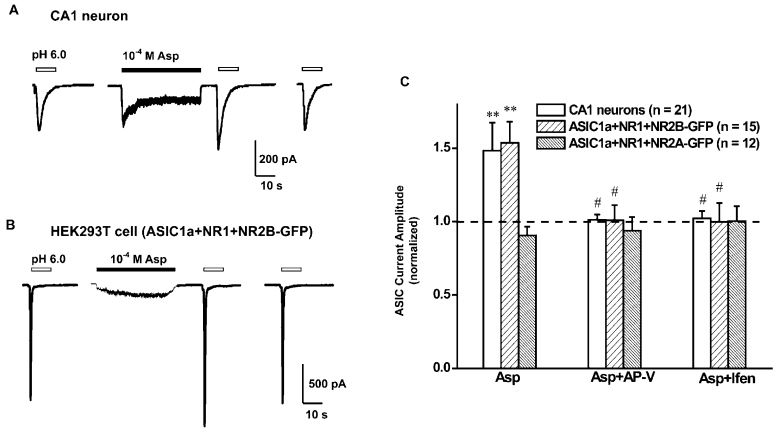

Figure 5. Activation of NR2B-NMDARs Enhanced ASIC Currents

(A) Activation of NMDARs by aspartate enhanced ASIC currents induced by an acidic solution of pH 6.0 in acutely dissociated CA1 hippocampal neurons. Traces shown are samples of currents before and after aspartate application.

(B) The effect of aspartate on ASIC1a currents recorded in HEK293T cells cotransfected with ASIC1a and various subunits of NMDARs. Sampled traces show the effect of activation of NR2B-NMDARs. Note the decay of ASIC1a currents in HEK293T cells was faster than that of the native ASIC currents in hippocampal neurons that shown in (A).

(C) Summary of results on the effect of NMDAR activation on ASIC currents in CA1 neurons and HEK293T cells. The amplitude of ASIC currents was normalized to the mean value before aspartate application (dashed line). Note that activation of NR2A-containing NMDAR had no effect on ASIC1a currents in HEK293T cells. Data are mean ± SE. Double asterisk indicates $p < 0.01$, compared with the control value without aspartate pretreatment; pound sign indicates $p < 0.05$, compared with aspartate group (Student's paired t test).

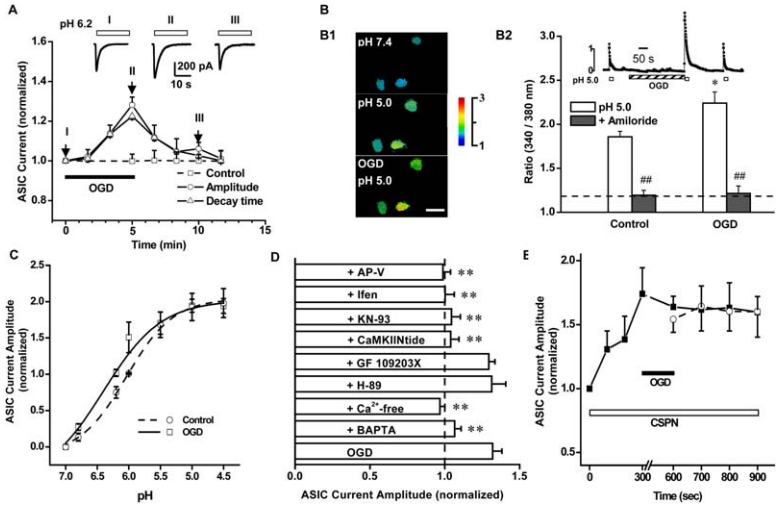

Figure 6. NR2B Antagonist or CaMKII Inhibitor Prevented Oxygen and Glucose Deprivation-Induced Enhancement of ASIC Currents in Hippocampal Neurons

(A) Both the amplitude and the decay time of the ASIC current ($I_{6.2}$) were enhanced by oxygen and glucose deprivation (OGD) (n = 8–13). Dashed line indicates control $I_{6.2}$. Inset, samples of ASIC currents traces recorded from one neuron at different times before and after OGD (marked by arrows).

(B1) Representative images showing enhancement of the acid-induced $[Ca^{2+}]_i$ elevation by OGD in cultured hippocampal neurons.

(B2) Summary of results on the effect of OGD on the ASIC-mediated $[Ca^{2+}]_i$ elevation. Dashed line indicates the basal level of fluorescence ratio (340/380). Inset, representative ratiometric measurements of $[Ca^{2+}]_i$ elevation induced by a solution of pH 5.0 before and after OGD. Data are mean ± SE (n = 13). Asterisk indicates $p < 0.05$, compared with the ratio induced by pH 5.0 before OGD; double pound sign indicates $p < 0.01$, compared with the ratio in the absence of amiloride, the inhibitor of ASICs (Student's paired t test).

(C) OGD enhanced the ASIC currents by inducing a leftward shift of pH-dose response curve for the ASICs. The EC_{50} was 6.05 ± 0.25 and 6.40 ± 0.26 before and after OGD, and the difference is statistically significant ($p < 0.05$, n = 6–8, Student's paired t test). The Hill coefficient was not significantly affected by OGD (1.44 and 1.49 before and after OGD).

(D) Summary of results on the effect of various pharmacological treatments on $I_{6.2}$ at 5 min after the onset of OGD. Data (mean ± SE) were normalized to the mean amplitude of $I_{6.2}$ before OGD (dashed line) for each neuron before averaging. Double asterisk indicates $p < 0.01$ (n = 6–8, Student's paired t test).

(E) The effect of the calcineurin inhibitor cyclosporin A (CSPN, 1 μM) on $I_{6.2}$. Note that CSPN alone increased the amplitude of $I_{6.2}$ (squares) and effectively prevented the enhancement of $I_{6.2}$ by OGD (circles). Data were normalized as in (D).

(Goldberg and Choi, 1993). The ASIC current was evoked by applying an acidic external solution (Figure 6A, inset). The peak amplitude of acidosis-induced current at pH 6.2 ($I_{6.2}$) did not change during repeated applications of the same acidic solution (Figure 6A, square). However, after 5 min of OGD, the amplitude and the decay time of $I_{6.2}$ were increased, both of which returned gradually to the original level within 5 min after the termination of OGD (Figure 6A). Consistent with the enhancement of ASIC current, ratiometric Ca^{2+} imaging showed that hippocampal neurons responded to a pH drop with an increase in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$), an effect that was largely inhibited by 10^{-4} M amiloride (Figure 6B). After 5-min of OGD, the magnitude of $[Ca^{2+}]_i$ elevation evoked by acidic external solution was significantly enhanced (Figure 6B) (n = 13). Thus, OGD-induced enhancement of ASIC currents was accompanied by an increased acidosis-induced $[Ca^{2+}]_i$ elevation.

To quantitatively characterize the changes in the ASIC function after ischemic insult, we examined pH dose response before and after OGD. As shown in Figure 6C, OGD induced a significant increase in the amplitude of ASIC currents in the range of pH 7.0–5.0, but not below pH 5.0, as reflected by a leftward shift of the pH dose-response curve. This finding indicates that OGD significantly increases ASIC sensitivity to the pH reduction, perhaps because of an increase in the affinity of ASICs to proton.

CaMKII Mediates Functional Coupling between NR2B-NMDAR and ASIC1a

The OGD effect on the $I_{6.2}$ (Figure 6A, circle) exhibited a delay of onset of about 5 min, suggesting involvement of intracellular signal transduction. As shown in Figure 6D, when BAPTA (10 mM) was loaded into the neuron via the patch recording pipette or when Ca^{2+}-free external solution was used, the enhancement of the $I_{6.2}$ by OGD was abolished. Thus, $[Ca^{2+}]_i$ elevation, presumably because of Ca^{2+} influx, is required for the $I_{6.2}$ enhancement by OGD. To further explore downstream effectors activated by $[Ca^{2+}]_i$ elevation, we loaded the neurons with the inhibitor for PKA, PKC, or CaMKII, kinases that are known to be involved in mediating the cellular effects of ischemia (Lipton, 1999). The enhancement of $I_{6.2}$ by OGD was unaffected by either H-89 (10 μM, n = 6) or GF 109203X (4 μM, n = 6), selective inhibitor for PKA or PKC, respectively. In contrast, inhibition of CaMKII by KN-93 (5 μM) or CaMKIINtide (10 μM) abolished the enhancement of $I_{6.2}$ induced by OGD (Figure 6D). These results suggest that CaMKII mediates OGD-induced enhancement of $I_{6.2}$ in hippocampal neurons, consistent with our observation that transient global ischemia induced CaMKII-dependent phosphorylation of ASIC1a (Figures 2B and 3). That phosphorylation of ASIC1a is indeed important was further supported by the finding that intracellular loading of cyclosporin A (CSPN, 1 μM), a specific inhibitor of the Ca^{2+}-dependent phosphatase calcineurin. Loading of CSPN by itself resulted in an increase in the $I_{6.2}$ amplitude by 162.1% ± 2.8% (Figure 6E, solid square) (n = 8, $p < 0.01$) and occluded the OGD-induced enhancement of $I_{6.2}$ observed in control neurons (Figure 6E, circle). This suggests that ASIC activity depends on its state of phosphorylation and is consistent with the notion that increased serine phosphorylation of ASICs by acute CaMKII activation may exert rapid modulation of ASIC currents.

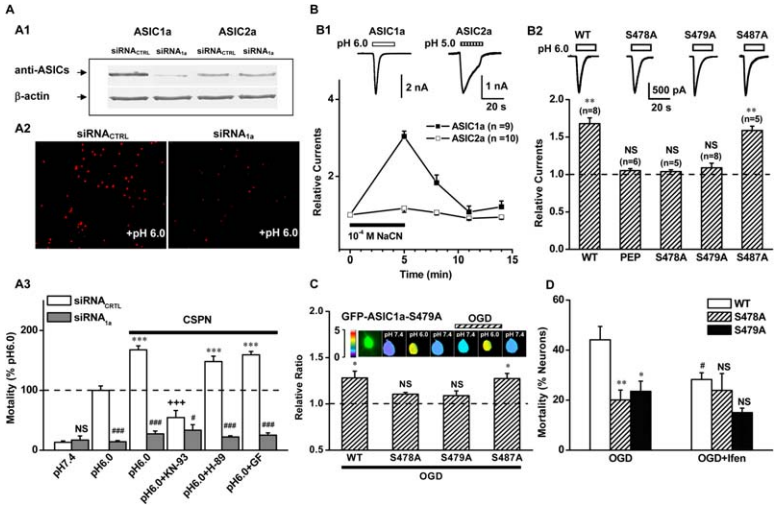

Figure 7. Neuroprotection by ASIC1a Gene Deficiency and ASIC1a Mutants in Cultured Hippocampal Neurons or COS7 Cells

(A1) Representative Western blots showing specific downregulation of ASIC1a by corresponding siRNAs. The $siRNA_{CTRL}$ and $siRNA_{1a}$ represent nonsilencing siRNA control and siRNA of ASIC1a, respectively.

(A2) Representative image illustrating the protective effect of $siRNA_{1a}$ on acidosis (pH 6.0)-induced neuron death. Prominent propidium-iodide (PI) staining of nuclei marked the population of dying neurons.

(A3) CSPN (1 μM) significantly enhanced the acidosis-induced cell death, which was blocked by $siRNA_{1a}$ and KN-93, respectively (n = 20–25 wells). Neither H-89, the inhibitor of PKA, nor GF 109203X (GF), the inhibitor of PKC, affected the cell death. Triple asterisk indicates $p < 0.01$, compared with $siRNA_{CTRL}$ at pH 6.0. Pound sign indicates $p < 0.05$, and triple pound sign indicates $p < 0.001$, compared $siRNA_{1a}$ with $siRNA_{CTRL}$. Triple plus sign indicates $p < 0.001$, compared with $siRNA_{CTRL}$ treated with pH 6.0 plus CSPN (ANOVA with Bonferoni correction). In this and subsequent figures, NS means no significant difference.

(B1) Subunit-specificity of ASIC regulation by ischemia (5 min treatment with 10^{-4} M NaCN, see text for details). Inset, representative ASIC currents induced by acidic solutions in COS7 cell transfected with GFP-ASIC1a or GFP-ASIC2a plasmids.

(B2) The enhancing effect of NaCN (10^{-4} M, 5 min) on ASIC1a currents was markedly reduced by ASIC1a-PEP, ASIC1a-S478A, and ASIC1a-S479A, but not by ASIC1a-S487A mutations in cultured hippocampal neurons. Dashed line indicates control ASIC1a currents induced by pH 6.0 ($I_{6.0}$). Inset, representative traces of ASIC1a currents recorded from neurons transfected with wild-type (wt) or mutated ASIC1a, as indicated above each bar. Double asterisk indicates $p < 0.01$ and "NS", compared with $I_{6.0}$ before NaCN treatment (Student's paired t test).

(C) The ASIC1a-S478A and ASIC1a-S479A mutants prevented OGD-induced increase of ASIC1a-mediated $[Ca^{2+}]_i$ elevation in cultured hippocampal neurons. Dashed line indicates control ratiometry induced with pH 6.0 solution without OGD treatment. Inset, representative images demonstrating lack of OGD-induced enhancement of ASIC1a-mediated $[Ca^{2+}]_i$ elevation in ASIC1a-S479A transfected neurons. Asterisk indicates $p < 0.05$ and "NS", compared with control (n = 6, Student's paired t test).

(D) The ASIC1a-S478A and ASIC1a-S479A mutants protected cultured hippocampal neurons from OGD-induced cell injury (n = 20–25 wells). Asterisk indicates $p < 0.05$, and double asterisk indicates $p < 0.01$, compared ASIC1a-S478A or ASIC1a-S479A with wt. Pound sign indicates $p < 0.05$ and "NS", compared with corresponding OGD groups without ifenprodil (Ifen, 3 μM) (ANOVA with Bonferoni correction).

The above studies have shown that NR2B-NMDARs are functionally coupled to ASIC1a channels in both hippocampal neurons and transfected HEK293T cells under nonischemic conditions. That such coupling is also important for OGD-induced enhancement of $I_{6.2}$ was further confirmed by the finding that application of either APV (100 μM) or ifenprodil (3 μM) prevented the enhancement of $I_{6.2}$ induced by OGD (Figure 6D). Together with our biochemical observations on the interaction between CaMKII and ASIC1a (Figure 3) and on the blockade of ASIC phosphorylation by NR2B-NMDAR antagonists (Figure 4), these results strongly support a functional link between NR2B-NMDARs and ASIC1a channels through CaMKII activation during ischemic signaling.

CaMKII-Dependent Phosphorylation of ASIC1a Contributes to Acidotoxicity on Cultured Hippocampal Neurons

To explore whether CaMKII phosphorylation of ASIC1a contributes to acidotoxicity, we used propidium iodide (PI) staining of the nuclei of dying neurons to assess acidosis-induced neuronal death in cultured hippocampal neurons transfected with siRNA of ASIC1a ($siRNA_{1a}$). Western blotting analysis revealed that $siRNA_{1a}$ effectively reduced ASIC1a but not ASIC2a expression (Figure 7A1). In addition, $siRNA_{1a}$ did not affect NMDAR currents in cultured hippocampal neurons (Figure S2). It has been shown previously that knockout of the ASIC1a gene protects the brain from focal ischemic injury (Xiong et al., 2004). In the present experiment, we also observed neuronal protection effect of the downregulation of ASIC1a (Figures 7A2 and 7A3). If ASIC1a is the most relevant target for CaMKII, downregulation of ASIC1a expression through siRNA should occlude the effect of pharmacological inhibition of CaMKII. This was indeed the case. As shown in Figure 7A3, protein phosphatase inhibitor CSPN (1 μM, pretreatment for 30 min), which enhanced the ASIC currents (Figure 6E) possibly through raising basal phosphorylation, significantly exacerbated the acidosis-induced cell death. Under such a condition, both KN-93 (5 μM), the selective inhibitor of CaMKII, and $siRNA_{1a}$ prevented acidosis-induced cell death. However, neither H-89, the inhibitor of PKA, nor GF 109203X, the inhibitor of PKC, had significant effect on the cell death (Figure 7A3). Collectively, these data strongly support the notion that CaMKII-dependent phosphorylation of ASIC1a plays an essential role in ischemia-induced cell death.

Mutation at S478 or S479 Inhibits ASIC1a Activity and Prevents OGD-Induced Neuronal Death

In COS7 cells transfected with GFP-ASIC1a or GFP-ASIC2a, we found that NaCN (10^{-4} M, 5 min) treatment, which mimics ischemic condition by causing $[Ca^{2+}]_i$ elevation through metabolic inhibition (Duchen et al., 1990), significantly enhanced ASIC1a- but not ASIC2a-mediated currents (Figure 7B1), consistent with our

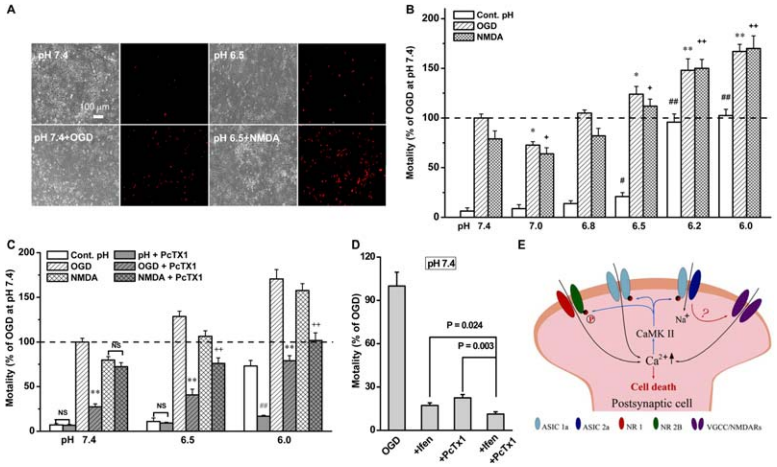

Figure 8. OGD Exacerbated Acidosis-Induced Neuronal Death.

(A) Microscopic images of cultured hippocampal neurons illustrating the effect of OGD and NMDA on neuronal death. Prominent propidium iodide (PI) staining of nuclei marked the population of dying neurons. Note that OGD induced cell death at pH 7.4 and NMDA exacerbated the acid-induced cell death.

(B) Summary of results on the effect of OGD and NMDA on acidotoxic cell death (n = 20–25 wells). All data were normalized to the extent of OGD-induced neuronal death at normal pH (7.4). Note that moderate acidosis (pH 7.0) was neuroprotective. Pound sign indicates $p < 0.05$, and double pound sign indicates $p < 0.01$, compared with corresponding group at pH 7.4; asterisk indicates $p < 0.05$, and double asterisk indicates $p < 0.01$, compared with OGD (pH 7.4) group; and plus sign indicates $p < 0.05$, and double plus sign indicates $p < 0.01$, compared with NMDA (pH 7.4) group, ANOVA with Bonferoni correction).

(C) Blocking ASIC1a channels with PcTX1 significantly inhibited the enhanced acidotoxic cell death induced by OGD or NMDA (n = 20–25 wells). Double pound sign indicates $p < 0.01$, compared with the corresponding group in the absence of PcTX1 (at pH 6.0); double asterisk indicates $p < 0.01$, compared with OGD group in the absence of PcTX1 at corresponding pH; and double plus sign indicates $p < 0.01$, compared with NMDA group in the absence of PcTX1 at corresponding pH (ANOVA with Bonferoni correction).

(D) OGD-induced cell death was prevented by inhibitors of NR2B-NMDAR and ASIC1a, ifenprodil (Ifen) and PcTX1, respectively (n = 20–25 wells).

(E) A proposed model for the interaction of NMDAR and ASIC pathways in ischemic signaling. Stimulation of NR2B-NMDARs by excessive glutamate leads to rapid influx of Ca^{2+} that induces CaMKII activation, which in turn phosphorylates ASIC1a (or its interacting proteins), leading to enhanced ASIC1a currents and the $[Ca^{2+}]_i$ elevation that promotes neuronal death. Membrane depolarization induced by Na^+ influx through activated homomeric ASIC1a and heteromeric ASIC1a-containing ASIC channels also contributes to $[Ca^{2+}]_i$ elevation by opening voltage-gated Ca^{2+} channels (VGCCs) and NMDARs.

above observations that the ischemic insult selectively increased serine phosphorylation of ASIC1a in rat hippocampus via a CaMKII-dependent process (Figures 2 and 6D).

We next performed a series of in vitro assays to evaluate the phosphoryaltion site (or sites) of ASIC1a by CaMKII. Previous studies have reported the phosphorylation of specific residues, including Ser479, on ASIC1a within the C terminus (Leonard et al., 2003). Sequence alignment reveals that Ser478 or Ser487 in C terminus of ASIC1a are unconserved regions of the ASIC1a and 2a subunits. Therefore, we generated a peptide of ASIC1a C terminus (ASIC1a-PEP, residues 467–490 of ASIC1a) (Leonard et al., 2003) containing one conserved (Ser479) and two unconserved serine sites (Ser478 and Ser487) within ASIC1a. To examine the role of the ASIC1a-PEP, we studied NaCN (10^{-4} M)-induced enhancement of ASIC1a currents in cultured hippocampal neurons by whole-cell recordings with pipettes containing ASIC1a-PEP (10 μM). As shown in Figure 7B2, the enhancement of NaCN on ASIC1a currents was abolished by the addition of ASIC1a-PEP in the recording pipette. To evaluate precisely the phosphorylation site (or sites) of the ASIC1a C terminus by CaMKII, we further examined the effect of mutation of each of the three residues on NaCN-induced enhancement of ASIC1a currents. In all cases, serine was mutated to alanine. Of the three ASIC1a mutations, both Ser478A and Ser479A significantly reduced NaCN (10^{-4} M)-induced enhancement of ASIC1a currents (Figure 7B2), whereas replacing Ser487 had no effect. All mutations did not alter the magnitude or kinetics of the ASIC1a currents (Figure 7B2, inset). These results suggest that Ser478 and Ser479 within ASIC1a C terminus are essential for the CaMKII phosphorylation.

Consistent with the reduction of NaCN-induced enhancement of ASIC1a currents, the OGD-induced enhancement of ASIC1a-mediated $[Ca^{2+}]_i$ elevation was also markedly inhibited in cultured hippocampal neurons transfected with ASIC1a-Ser478A or ASIC1a-Ser479A (Figure 7C). More importantly, these two mutations protected cultured hippocampal neurons from OGD-induced cell injury (Figure 7D). Expression of ASIC1a-Ser478A or ASIC1a-Ser479A did not affect NMDAR currents (Figure S2). Thus, the residues of Ser478 and Ser479 within the C terminus of ASIC1a are crucial for ischemia-induced cell damage.

NMDAR-ASIC Coupling Contributes to Ischemic Cell Death

Because the ASIC currents were increased by OGD in an NMDAR-dependent manner (Figure 6D), we expected that NMDAR activation should exacerbate acidosis-induced neuronal death. As shown in Figures 8A and 8B, severe acidic external solution (pH $\leq$ 6.5) produced cell death in hippocampal cultures, as indicated by PI-staining, although mild acidosis had a slight protective effect against OGD (pH 7.0 in Figure 8B), presumably because of the inhibition of NMDARs by H^+ (Giffard et al., 1990). However, treatment with OGD or NMDA in severe acidic solution resulted in a dramatic increase in the number of PI-positive cells (Figures 8A and 8B). It is unlikely that this increased cell death was due to elevated glutamate- or OGD-triggered processes unrelated to ASIC1a under severe acidic conditions because treatment with ASIC1a-selective antagonist Psalmotoxin 1 (PcTX1, 0.5 μl/ml) significantly reduced the increase in both OGD- and NMDA-induced cell death (Figure 8C). Therefore, we conclude that NMDAR-ASIC coupling

contributes to ischemic neuronal death by exacerbating ASIC1a-mediated acidotoxicity.

Interestingly, the OGD effect on acidotoxic cell death was more sensitive than the NMDA effect to the blockade of ASICs by PcTX1 (Figure 8C), suggesting that activation of NMDARs may directly induce cell death through an ASIC-independent pathway. Consistent with this notion, cotreatment of the cultures with PcTX1 (0.5 μl/ml) and ifenprodil (3 μM) reduced the number of dying cells to a greater extent than that found with PcTX1 treatment alone (Figure 8D) ($p < 0.01$). Similarly, PcTX1 and ifenprodil cotreatment also reduced cell death to a greater extent than the ifenprodil treatment alone, consistent with a direct ASIC pathway (Xiong et al., 2004). Taken together, these results suggest that there exist both NMDAR and ASIC pathways leading to ischemic cell death, but the activity of ASIC pathway may be enhanced by the activation of NMDARs.

Discussion

In the present study we have demonstrated that global ischemia selectively enhances the phosphorylation of hippocampal ASIC1a subunit that constitutes homomeric channels of a high Ca^{2+} permeability. This phosphorylation is mediated through the activation of NR2B-NMDARs/CaMKII cascade, which also underlies the enhancement of ASIC currents in hippocampal neurons and transfected cells. Furthermore, under the condition of OGD, the hippocampal ASIC current is also enhanced by NR2B-NMDARs/CaMKII activation, resulting in an elevated intracellular Ca^{2+}. Importantly, activation of NR2B-NMDARs exacerbates acidotoxic neuronal death in hippocampal cultures. These findings support the hypothesis that during brain ischemia, coupling between the NR2B-NMDARs/CaMKII cascade and the ASIC1a channel contributes significantly to acidotoxic neuronal death in the hippocampus.

Subunit-Specific Phosphorylation of ASICs after Brain Ischemia

Despite the popularity of the excitotoxicity hypothesis, clinical trials with NMDAR antagonists as protective agents against ischemic insults failed to show promising results, largely because of nonspecific side effects of the antagonists. Besides NMDARs, other ion channels including ASIC1a homomeric channel, which has a high Ca^{2+} permeability, could produce ischemic neuronal damage (Xiong et al., 2004). The ASIC1a is highly expressed in the brain and is activated by acidification at a level found in ischemic brain tissue (Bereczki and Csiba, 1993). Interestingly, genetic deletion of ASIC1a and pharmacological blockade of ASIC1a channels reduce the neural damage produced by focal ischemia (Xiong et al., 2004). Our present results with $siRNA_{1a}$ (Figure 7A) and the selective antagonist of ASIC1a, PcTX1 (Figure 8D), are consistent with the role of ASIC1a in ischemic neural damage. Furthermore, we expand the above observations by linking the function of ASIC1a channels with NMDAR activation. As depicted in the model shown in Figure 8E, the coupling between NR2B-NMDARs/CaMKII cascade and ASIC1a under ischemic conditions elevates Ca^{2+} influx by enhancing the opening of Ca^{2+}-permeant ASIC1a homomeric channels via CaMKII-dependent phosphorylation. In addition, activation of ASIC1a-containing heteromeric channels may also promote Ca^{2+} influx by generating membrane depolarization, which facilitates opening of NMDARs and/or voltage-gated Ca^{2+} channels (VGCCs) (Figure 8E).

CaMKII-Dependent Phosphorylation of ASIC1a Contributes to Acidotoxicity

Activity of CaMKII declines after ischemia (Churn et al., 1992; Hajimohammadreza et al., 1997). Although the disappearance of CaMKII staining is an established marker of subsequent pyramidal neuron loss, direct evidence linking CaMKII inhibition to neuronal death is limited. In this study, we hypothesized that during global ischemia, coupling between the NMDARs/CaMKII cascade and the ASIC1a channel contributes significantly to acidotoxic neuronal death in the hippocampus. The following observations support this hypothesis. Firstly, inhibition of CaMKII with KN-93 or CaMKIINtide abolished the enhancement of ASIC currents by OGD. Secondly, the phosphorylation of ASIC1a increased after transient global ischemia and intracerebroventricular administration of KN-93 or CaMKIINtide blocked this increase. Thirdly, the increased CoIP of ASIC1a with CaMKIIα after global ischemia was largely inhibited by KN-93 or CaMKIINtide, providing direct evidence for the association and perhaps functional interaction between ASIC1a and CaMKIIα. Previous studies have shown that brain ischemia induces autophosphorylation and translocation of CaMKII to cell membrane (Aronowski et al., 1992). Therefore, the rapid decline in CaMKII activity after ischemia may be a result of a posttranslational modification and/or translocation of the enzyme (Aronowski et al., 1992; Yamamoto et al., 1992). Consistent with this notion, pharmacological inhibition of CaMKII phosphorylation of ASIC1a with KN-93 produced neuroprotection in vitro (Figure 7A3). Furthermore, the mutants of ASIC1a at Ser478A and Ser479A protected cultured hippocampal neurons from OGD-induced cell death (Figure 7D), suggesting that the phosphorylation of ASIC1a by CaMKII at the site of Ser478 and Ser479 plays an essential role in ischemia-induced cell death.

Although CaMKII activation during the period of ischemic preconditioning contributes to neuronal resistance to subsequent ischemic insults (Kawabata et al., 2000; Mabuchi et al., 2001), this effect could be a result of attenuated autophosphorylation and translocation of CaMKII induced by ischemic preconditioning (Shamloo et al., 2000). Therefore, we suggest that the persistent activation and translocation of CaMKII to specific membrane substrates, e.g., NR2B subunit (Omkumar et al., 1996) and ASIC1a (the present observation), after transient global ischemia may promote cell death. Thus, our findings add a new dimension to understanding the complex role of CaMKII in ischemic cell death.

NMDARs Provide Ca^{2+} Entry for ASIC Regulation

The present study indicates that OGD-induced enhancement of ASIC currents depends on $[Ca^{2+}]_i$ elevation and CaMKII activation. It is unlikely that Ca^{2+} entry through ASICs themselves makes major contribution because the amplitude of ASIC currents did not change during repeated application of the same acidic solution

(Figure 6A, dashed line). Given the substantial Ca^{2+} permeability of ASICs (as shown in Figure 6B), we would expect use-dependent facilitation of ASIC currents if the Ca^{2+} influx through this channel contributes significantly to its own phosphorylation. This notion is also supported by the following previous observations. First, only the homomeric ASIC1a channel is Ca^{2+} permeant (Yermolaieva et al., 2004), whereas in the hippocampus, heteromeric channels are predominant (Baron et al., 2002; Gao et al., 2004). Second, during prolonged acid application, ASIC1a currents desensitize rapidly and $[Ca^{2+}]_i$ returns to the basal level (Yermolaieva et al., 2004). Finally, systemic acidosis has no effect on CaMKII activation during ischemia (Katsura et al., 1999).

Studies in several model systems of neuronal injury suggest that NMDARs are largely responsible for the Ca^{2+} influx during the early stage of global ischemia (Silver and Erecinska, 1990). Indeed, our results suggest that the activation of NMDARs is required for the $[Ca^{2+}]_i$ elevation and subsequent CaMKII activation and ASIC1a phosphorylation under ischemic conditions. The involvement of NMDARs is further supported by the fact that blockade of NMDARs reduced ischemia-induced serine phosphorylation of NR2B and CaMKII (Figure S1). Our results, however, do not necessarily exclude other Ca^{2+} rise mechanisms. For example, $[Ca^{2+}]_i$ elevation caused by NaCN through metabolic inhibition (Duchen et al., 1990) enhanced the ASIC1a currents (Figure 7B), suggesting that in addition to Ca^{2+} entry via NMDARs, Ca^{2+} release from internal Ca^{2+} stores may also play a role in ischemia-induced enhancement of ASIC1a function. In addition, the activation of AMPA receptors lacking GluR2 can be responsible for the influx of Ca^{2+} (Geiger et al., 1995; Liu et al., 2004). However, our data show that systemic administration of the AMPA receptor antagonist CNQX before ischemic insults does not inhibit the enhancement of ASIC1a phosphorylation (Figure 4A). Thus, AMPA receptors in ischemic CA1 neurons allow Ca^{2+} entry that may not be involved in the CaMKII/ASIC1a phosphorylation.

NMDAR-ASIC1a Coupling Specifically Involves NR2B

Differences in neuronal NMDAR properties are largely attributed to the NR2 subunits. The C terminus of NR2B contains two sites for regulated CaMKIIα binding (Omkumar et al., 1996) and is necessary for targeting CaMKIIα to the postsynaptic density. Our results point to a specific role of NR2B-NMDAR/CaMKII cascade in ischemic signaling. First, we demonstrated that the activation of NMDARs by aspartate enhanced ASIC currents in CA1 neurons and that the selective NR2B antagonist ifenprodil inhibited the enhancement. Second, in HEK293T cells cotransfected with ASIC1a and different combinations of NMDAR subunits, we demonstrated that the activation of NR2B-NMDARs led to an enhancement of ASIC1a currents, whereas the activation of NR2A-NMDARs had no effect on these currents. Finally, specific inhibition of NR2B-NMDARs largely abolished the ASIC1a phosphorylation during global ischemia.

In CNS neurons, NR2B-NMDARs are localized predominantly at extrasynaptic sites (Tovar and Westbrook, 1999) and are capable of detecting extrasynaptic spillover of glutamate (Scimemi et al., 2004). Because these extrasynaptic NMDARs are known to trigger cell-death pathways (Hardingham et al., 2002), the specific coupling of NR2B-ASIC1a demonstrated in the present study suggests that an excessive glutamate release caused by ischemia may activate extrasynaptic NR2B-NMDARs, leading to exacerbated ASIC1a-mediated acidotoxicity. Indeed, we observed that neuronal ASIC currents were enhanced by OGD or by NMDA application, in parallel with the increased ASIC1a phosphorylation during global ischemia, and both were sensitive to specific NR2B-NMDAR blockade. Activation of NMDARs enhanced both the amplitude and the duration of ASIC currents and broadened the range of acid sensitivity of the ASICs (Figure 6). Thus, NR2B-ASIC coupling amplifies the ASIC function during ischemia. Accordingly, either OGD or NMDA exacerbated acid-induced cell death, which was prevented by selective blockade of NR2B-ASIC coupling (Figure 8).

Interaction between Excitotoxic and Acidotoxic Pathways

Our findings support a model (Figure 8E) that NMDARs allow Ca^{2+} entry that activates CaMKII, which in turn modulates not only NMDARs themselves but also ASICs through phosphorylation. During ischemia, activation of NR2B-NMDARs enhances the ASIC currents, which further increases the influx of Ca^{2+} and Na^+ through homomeric 1a channels and heteromeric-1a-containing ASICs. Membrane depolarization induced by activation of ASICs during acidosis (Baron et al., 2002; Gao et al., 2004) may contribute to aberrant neuronal excitation, which is one of the early events occurring after ischemia, and such depolarization in turn enhances NMDAR function. Therefore, synergistic interaction between NR2B-NMDARs and ASICs, together with the membrane translocation of CaMKII in hippocampal CA1 neurons, may confer the specificity of ischemic signaling to ischemic hippocampal neurons injury.

In summary, our report provides a functional linkage between NMDAR-mediated excitotoxicity and ASIC-mediated acidotoxicity induced by ischemia, which is known to cause excessive glutamate release and acidosis. Both NMDARs and Ca^{2+}-permeant ASICs have now been implicated in ischemic neuronal damage. The interaction between these two pathways shown in the present work adds a new dimension to the complexity of ischemic signaling. Our finding of the specific coupling between NR2B-NMDARs and ASIC1a through CaMKII sheds new light on the development of therapeutic agents against excitotoxic and acidotoxic neuronal damage. Because both NR2B-NMDARs and ASIC1a are also involved in seizure (Biagini et al., 2001) and pain sensation (Wu et al., 2004), similar coupling between these two channels is likely to occur in these diseases as well.

Experimental Procedures

Electrophysiological Recordings

The experimental protocols were approved by the Animal Care and Use Committee of Institute of Neuroscience, Shanghai. The dissociated hippocampal cells were prepared with a mechanical dissociation protocol (Gao et al., 2004). The ASIC currents were recorded by the whole-cell patch-clamp technique. The standard external solution contained (in mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, and

10 glucose, buffered to various pH values with either 10 mM HEPES (pH 6.0–7.4) or 10 mM MES (pH < 6.0), 300–330 mOsm/l. The patch pipette solution for whole-cell patch recording was (in mM): 120 KCl, 30 NaCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, 2 Mg-ATP, and 10 HEPES. The internal solution was adjusted to pH 7.2 with Tris-base. When aspartate-evoked current was recorded, Mg^{2+} was substituted with Ca^{2+} and 1 μM glycine was added to the standard external solution.

All drugs for electrophysiological experiments were purchased from Sigma except GF 109203X (Tocris, Bristol, UK), CaMKIINtide (Calbiochem, California), and ASIC1a-PEP peptide (residues 467–490 of ASIC1a, GL Biochem, Shanghai, China). Statistical comparisons were performed by the appropriate Student's t test. Values are given as mean ± SE.

Ischemia Model

After adult male Sprague-Dawley rats (200–250 g) were anesthetized, transient brain ischemia was induced by the four-vessel occlusion method as described previously (Pulsinelli and Brierley, 1979). Briefly, vertebral arteries were coagulated and transected between the first and second cervical vertebrae. Ischemia was induced for 3, 5, 15, or 30 min by occlusion of the both common carotid arteries with aneurysm clips. Rats that showed a completely flat bitemporal electroencephalogram, maintenance of dilated pupils, and no corneal reflex during occlusion were selected for the experiments. Then, rats were reperfused by releasing the clamp and were killed immediately or 3, 6, or 72 hr thereafter. Rectal temperature was maintained at 37.0°C–37.5°C during ischemia and/or 2 hr of reperfusion. In sham-operated animals killed 6 hr after surgery, all procedures except common carotid artery occlusion were carried out. Intraventricular injection was performed by stereotaxic (Stoelting, Co., IL) technique.

Electroencephalogram Recording

Under anesthesia, the rat electroencephalogram (EEG) activity was recorded by a bioamplifier (Model SMUP-E, Fudan University) with a range of 100 mV and bandpass 0.1–40 Hz by stereotaxic technique with standard stainless steel needle electrode (impedance < 10 KΩ) inserted stereotactically at 1 mm posterior to bregma and 1.5 mm lateral to the midline. Analog data were sampled at 1 kHz.

Immunoprecipitation and Immunoblotting

For immunoprecipitation (IP), tissue homogenates (400 μg of protein) were diluted 4-fold with IP buffer (pH 7.4) containing (in mM): 50 HEPES, 150 NaCl, 50 NaF, 20 NaPPi, 20 β-glycerophosphate, 1 EDTA, 1 EGTA, 1 $ZnCl_2$, 1.5 $MgCl_2$, 1 Na_3VO_3, 0.5 PMSF, 1 *p*-NPP, 10% glycerol, 1% Triton X-100, 0.5% Nonidet P-40, 16 μg/ml benzamidine, and 5 μg/ml each of aprotinin, leupeptin, and pepstatin A. Samples were preincubated for 1 hr with 25 μl protein A/G-Sepharose CL-4B (Amersham Pharmacia, Buckinghamshire, UK) and then centrifuged to remove any nonspecific adhesion to the A/G. The supernatant was incubated with 2–4 μg antiphosphoserine antibody (Santacruz, St. Louis, MO) or anti-CaMKIIα (Sigma, St. Louis, MO) or anti-ASIC1a (Alpha Diagnostic, TX) antibodies for 4 hr or overnight at 4°C. Protein A/G-Sepharose (25 μl) was then added, and the incubation continued for 2 hr. Samples were centrifuged at 10,000 × g, and the pellets were washed three times with immunoprecipitation buffer. Bound proteins were eluted by adding 2 × SDS-PAGE sample buffer (20 ml) and boiled at 100°C for 5 min. Samples were then centrifuged, and supernatants were used for immunoblotting.

For immunoblotting, tissue homogenates or the protein extracted by IP were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose membrane (Amersham Pharmacia). The membrane was probed with anti-ASIC1a, anti-ASIC2a, anti-ASIC2b (1:1000), anti-phosphoserine, or β-actin (1:500) antibodies at 4°C overnight. Detection was carried out by alkaline phosphatase conjugated IgG (Sigma, 1:10,000) and developed with NBT/BCIP color substrate (Promega, Madison, WI). After immunoblotting, the bands on the membrane were scanned and quantified with an image analyzer (LabWorks Software, UVP Upland, CA).

All values shown are means ± SE from three rats. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Duncan's new multiple range method or Newman-Keuls test, as appropriate, with $p < 0.05$ as statistical difference.

Primary Cultures of Hippocampal Neurons

Hippocampal neurons from 18-day-old embryonic Sprague-Dawley rats were isolated by a standard enzyme treatment protocol. Briefly, hippocampi were dissociated in Ca^{2+}-free saline with sucrose (20 mM) and plated (1–5 × 10^5 cell/ml) on poly-D-lysine (Sigma) coated cover glasses. The neurons were grown in DMEM (Gibco) with L-glutamine plus 10% fetal bovine serum (Gibco) and 10% F12. Neuron basic (1.5 ml) with 2% B27 was replaced every 3–4 days. Treatment with 5-fluoro-5′-deoxyuridine (20 μg/ml, Sigma, St. Louis, MO) on the third day after plating was used to block cell division of nonneuronal cells, which helped to stabilize the cell population. The cultures were maintained at 37°C in a 5% CO_2 humidified atmosphere.

Transfection of Plasmids

HEK293T or COS7 cells were transfected by using calcium phosphate. Briefly, 1 day cells (2 ml of medium per culture) were transfected with 1–3 μg of cDNAs encoding ASIC1a and ASIC2a (kindly provided by Dr. J. Xia, University of Science and Technology of Hong Kang, China), NR1, NR2B-GFP, and NR2A-GFP (kindly provided by Dr. J.-H. Luo, Zhejiang University, China). GFP is used for identification of the transfected cells. Electrophysiological recordings were done 1–2 days after transfection.

Oxygen and Glucose Deprivation and Excitotoxicity Assay

The cultures were transferred to an anaerobic chamber containing a 5% CO_2, 10% H_2, and 85% N_2 (<0.2% O_2) atmosphere (Goldberg and Choi, 1993). They were washed three times with deoxygenated glucose-free bicarbonate solution and maintained anoxic for 1.5 hr at 37°C. Oxygen and glucose deprivation (OGD) was terminated by washing the cultures with oxygenated glucose-containing (20 mM) bicarbonate solution. The cultures were maintained for a further 21–24 hr at 37°C in a humidified 5% CO_2 atmosphere.

For studies involving acidosis and glutamate toxicity, delayed toxicity was measured (Koh and Choi, 1987) on 12–14 day old cultures. The culture media was removed and the cells were challenged with Kress-Ringer HEPES buffer of different pH for 2 hr or 100 μM NMDA in Mg^{2+}-free Kress-Ringer HEPES buffer containing 5 μM glycine for 30 min. Then, the acidic solution or the NMDA solution was replaced with normal medium, and the cultures returned to the incubator. On the next day (18–24 hr), cell damage was assessed by propidium-iodide staining.

For evaluating the effect of NMDAR and ASIC1a on the OGD- or NMDA-induced neuronal damage, the respective inhibitors were added for 30 min before and during OGD or NMDA insults, including 10 μM MK-801 (NMDAR antagonist), 3 μM ifenprodil (NR2B-containing NMDAR antagonist), or *Psalmopoeus cambridgei* venom (Yarnell, AZ), which inhibits ASIC1a current because of a toxin contained in the venom, Psalmotoxin 1 (PcTX1) (Escoubas et al., 2000; Poirot et al., 2004). The venom was used at a 1:20,000-fold dilution in all experiments.

Assay of Cell Injury with Propidium-Iodide Staining

Neurodamage was assessed by propidium-iodide (PI) staining. Cultures were rinsed with PBS and incubated with 10 μg/ml PI (Sigma) for 10 min and then rinsed with PBS. They were observed shortly after staining by fluorescent microscopy. The number of apoptotic nuclei was determined by light microscopic examination at 100 × magnification. For each slide, fifteen random fields were enumerated.

Mutagenesis and Expression Vectors

The ASIC1a-S487A mutant was constructed by PCR method (Dieffenbach and Dveksler, 1995). The mutants of ASIC1a-S478A and ASIC1a-S479A were kindly provided by Dr. M.J. Welsh (Howard Hughes Medical Institute, University of Iowa), and the mutated fragments were subcloned into pEGFP-C3. For electrophysiological studies, the ASIC1a mutants were transfected into cultured hippocampal neurons by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). For Ca^{2+}-imaging study and cell-death assay, transfection to the primary hippocampal neurons was immediately carried out with the Rat Neuron Nucleofector Kit (Amaxa, Koeln, Germany) according to Amaxa's optimized protocol for hippocampal neurons.

siRNA Transfection

To determine transfection efficacy, we used a nonsilencing FITC-conjugated siRNA as control: sequence 5′-UUC UCC GAA CGU GUC ACGU-3′ (Shanghai GeneChem, Co. Ltd., China). The siRNAs (Shanghai GeneChem, Co. Ltd.) against ASIC1a correspond to coding regions 416–431 and 1133–1150 relative to the first nucleotide of the start codon of murine ASIC1a. On day six in culture, transfections of siRNA for ASIC1a gene were carried out with TransMessenger transfection reagent (Qiagen, Chatsworth, CA). The siRNA (control or ASIC1a, 1 μg per well) was condensed with Enhanser R and formulated with 4 μl of TransMessenger reagent (Gibco, Grand Island, NY), according to the manufacturer's instructions. The transfection complex was diluted in 900 μl of Neurobasal and was added directly to the cells; it was replaced with Neurobasal culture medium after 3 hr. Transfected cells were used for experiments 2–2.5 days later.

Ca^{2+} Imaging

Hippocampal neurons grown on 8 × 8 mm glass coverslips were washed three times with PBS and incubated with 1 μM fura-2-acetoxymethyl ester (Sigma) for 20 min at 37°C; they were again washed three times and then incubated in standard extracellular solution for 30 min. Coverslips were transferred to a perfusion chamber on an inverted microscope (Nikon TE2000-E). Experiments were performed by using a 40 × UV fluor oil-immersion objective lens, and images were recorded by a cooled CCD camera (HAMAMATSU, Hamamatsu, Japan). The fluorescence excitation source was a 75-W xenon arc lamp. Ratio images were acquired by using alternating excitation wavelengths (340/380 nm) with a monochromators (Till Polychrome IV, Munich, Germany), and fura-2 fluorescence was detected at emission wavelength of 510 nm. Digitized images were acquired and analyzed on a PC with SimplePCI (Compix, Inc., PA). Ratio images (340/380 nm) were analyzed by averaging pixel ratio values in circumscribed regions of cells in the field of view. The values were exported to Origin 7.0 for further analysis.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/48/4/635/DC1/.

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