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Ca^{2+}-Permeable Acid-sensing Ion Channels and Ischemic Brain Injury

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Abstract. Acidosis is a common feature of brain in acute neurological injury, particularly in ischemia where low pH has been assumed to play an important role in the pathological process. However, the cellular and molecular mechanisms underlying acidosis-induced injury remain unclear. Recent studies have demonstrated that activation of Ca^{2+}-permeable acid-sensing ion channels (ASIC1a) is largely responsible for acidosis-mediated, glutamate receptor-independent, neuronal injury. In cultured mouse cortical neurons, lowering extracellular pH to the level commonly seen in ischemic brain activates amiloride-sensitive ASIC currents. In the majority of these neurons, ASICs are permeable to Ca^{2+}, and an activation of these channels induces increases in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$). Activation of ASICs with resultant $[Ca^{2+}]_i$ loading induces time-dependent neuronal injury occurring in the presence of the blockers for voltage-gated Ca^{2+} channels and the glutamate receptors. This acid-induced injury is, however, inhibited by the blockers of ASICs, and by reducing $[Ca^{2+}]_o$. In focal ischemia, intracerebroventricular administration of ASIC1a blockers, or knockout of the ASIC1a gene protects brain from injury and does so more potently than glutamate antagonism. Furthermore, pharmacological blockade of ASICs has up to a 5 h therapeutic time window, far beyond that of glutamate antagonists. Thus, targeting the Ca^{2+}-permeable acid-sensing ion channels may prove to be a novel neuroprotective strategy for stroke patients.

Key words: Acidosis — Ischemia — Ca^{2+} toxicity — Neurons — Patch clamp

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Introduction

Stroke or brain ischemia is a leading cause of morbidity and mortality and a common reason for long-term disability. Although in recent years enormous progress has been made towards defining the cellular and molecular responses of the brain to ischemia, there is still no effective neuroprotective treatment for stroke patients. Brain ischemia, caused by an interruption of blood flow to the brain, results in a progressive loss of specific subpopulations of neurons (Hsu et al., 1994; Sweeney et al., 1995; Du et al., 1996; Rosenblum, 1997). For several decades, the mechanisms underlying damage of neurons destined to die have been a central focus of stroke research. Over the past 20 years, activation of glutamate receptors resulting in intracellular Ca^{2+} overload and excitotoxicity has drawn the most attention (Simon et al., 1984; Choi and Rothman, 1990; Siesjo, 1992; Tymianski et al., 1993; Lee, Zipfel & Choi, 1999). Large numbers of studies have demonstrated that antagonists of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors are effective in preventing or reducing ischemic neuronal injury (Simon et al., 1984; Wieloch, 1985; Choi, Koh & Peters, 1988; Albers, Goldberg & Choi, 1989; Tymianski et al., 1993). However, recent clinical trials using these antagonists to prevent ischemic brain injury in humans have been largely disappointing (Lee et al., 1999; Wahlgren & Ahmed, 2004). Although multiple factors including difficulty in early initiation of treatment and intolerance of severe side effects may have contributed to the failures, emerging new studies have suggested that activation of several glutamate receptor-independent Ca^{2+}-toxicity pathways also contribute to ischemic brain injury. This review focuses on the role of Ca^{2+}-permeable acid-sensing ion channels in acidosis-mediated glutamate receptor-independent neuronal injury.

Glutamate Receptors and Excitotoxicity

Glutamate is the principal excitatory neurotransmitter in the central nervous system (CNS) (Curtis & Watkins, 1960; Krnjevic, 1970; Nakanishi, 1992). Activation of glutamate receptors is essential for normal neurological functions including cognition, movement and sensation, and learning and memory (Nakanishi, 1992; Gasic & Hollmann, 1992). Due to the high permeability to Ca^{2+} ions, activation of NMDA receptors and subsequent intracellular Ca^{2+} accumulation is essential for glutamate excitotoxicity associated with various neurological disorders including brain ischemia (Choi, 1988a; Rothman & Olney, 1995; Mori & Mishina, 1995; Sucher et al., 1996; Tymianski, 1996).

The term "excitotoxicity" was offered as a concept whereby neurons are over-stimulated or excited to the point of death through an overabundance of excitatory neurotransmission (Olney, Ho & Rhee, 1971; Olney et al., 1987; Choi, 1992). During ischemia, neurons and glial cells deprived of oxygen and glucose rapidly lose ATP and become depolarized, leading to excessive synaptic release of glutamate and reduced glutamate uptake (Benveniste et al., 1984; Nicholls & Attwell, 1990; Siesjo, 1992). Accumulation of glutamate in the extracellular space over-activates the postsynaptic NMDA receptors, resulting in intracellular Ca^{2+} overload and subsequent neuronal cell death (Choi, 1988b; Choi, 1992; Sattler & Tymianski, 2001). It is well recognized that glutamate excitoxicity is largely mediated by excessive Ca^{2+} influx directly through the NMDA channels (Murphy & Horrocks, 1993; Sucher, Lipton & Dreyer, 1997). Excessive intracellular Ca^{2+} accumulation leads to inappropriate activation of several enzyme systems including nitric oxide synthase (NOS), proteases, phospholipase A2 (PLA2), and the endonucleases. Over-activation of these enzymes induces breakdown of proteins, lipids and nucleic acids, either directly, or indirectly through downstream signaling molecules (Coyle & Puttfarcken, 1993; Simonian & Coyle, 1996; Lee et al., 1999; Sattler et al., 1999).

Despite the fact that most studies, using both in vitro neuronal cell culture or in vivo animal models, demonstrated clear neuroprotection by antagonists of glutamate receptors, recent clinical trials have failed to show a satisfactory effect against ischemic brain injury (Lee et al., 1999; Ikonomidou & Turski, 2002; Gladstone et al., 2002; Hoyte et al., 2004). The failure of these agents in human clinical trials has been attributed to a number of factors, for example, intolerance of glutamate receptor antagonists and the inability to initiate treatment at an early stage (Lee et al., 1999; Ikonomidou & Turski, 2002; Hoyte et al., 2004). It is worth mentioning that even in well-controlled animal studies, the protective effect of glutamate antagonists had only a narrow time window of less than an hour (Rod & Auer, 1989; Chen et al., 1991; Gladstone et al., 2002; Biegon et al., 2004). This finding may have predicted the failure of clinical trials. Interestingly, new studies in the past 2–3 years have suggested that blocking the function of glutamate receptors in the late stage of ischemia or in chronic neurodegenerative disorders may actually be detrimental to neuronal survival (Ikonomidou, Steforska & Turski, 2000; Biegon et al., 2004). Using a mouse model of traumatic brain injury and quantitative autoradiography of the activity-dependent NMDA receptor antagonist MK801, Biegon and colleagues (2004) showed that hyperactivation of NMDA receptors after traumatic brain injury was short-lived (< 1 h) and was followed by a profound and long-lasting loss of function. Furthermore, stimulation of NMDA receptors 24–48 h postinjury produced a significant attenuation of neurological deficits and restored cognitive performance 14 days after injury. These findings may suggest that glutamate toxicity is only involved in the acute neurodestructive phase that occurs immediately after traumatic or ischemic injury (excitotoxicity), but after this period, it assumes its normal physiological functions, which include promotion of neuronal survival. Therefore, the failure of NMDA receptor antagonists in stroke and traumatic brain injury trials may involve the inhibition of neuronal survival by NMDA receptor blockade (Ikonomidou & Turski, 2002; Hoyte et al., 2004; Biegon et al., 2004).

Although this finding is intriguing, it is likely only part of the story. Emerging new studies in the past year have also provided other glutamate receptor-independent Ca^{2+}-toxicity pathways activated in ischemia which contribute to ischemic neuronal injury (Aarts et al., 2003; Huang & McNamara, 2004; Xiong et al., 2004; Benveniste & Dingledine, 2005). This review will focus on the role of one of these, the acid-sensing ion channels, in ischemic brain injury.

Brain Acidosis

A stable pH is critical for normal neuronal function. Like the cells in other tissues, brain cells maintain their pH level through H^+-transporting mechanisms including Na^+/H^+ and Cl^-/HCO_3^- systems (for review *see* Chesler, 1990). In normal brain tissue, for example, extracellular pH (pH_o) is at $\sim$7.3 while intracellular pH (pH_i) is at $\sim$7.0 (Chesler, 1990; Nedergaard et al., 1991a; Back et al., 2000). It has been recognized for several decades that brain ischemia is accompanied by marked reduction of tissue pH, a condition termed ischemic acidosis (Thorn & Heitmann, 1954; Crowell & Kaufmann, 1961; Ljunggren, Norberg & Siesjo, 1974; Rehncrona, 1985; Siesjo, 1988; Nedergaard et al., 1991a). The shortage of oxygen, due to the lack of blood supply,

results in increased anaerobic glycolysis, which leads to lactic acid accumulation (*for review see*: Tombaugh & Sapolsky, 1993; Siesjo, Katsura & Kristian, 1996). Accumulation of lactic acid, along with H^+ release from ATP hydrolysis, results in a dramatic decrease in tissue pH. Brain pH typically falls to 6.5 during ischemia under normoglycemic conditions, and to 6.0 or below during severe ischemia or under hyperglycemic conditions (Ljunggren et al., 1974; Siemkowicz & Hansen, 1981; Rehncrona, 1985; Kraig, Pulsinelli & Plum, 1985; Nedergaard et al., 1991a). It may be worth mentioning that the degree and the direction of the changes for pH_o and pH_i in ischemic brain tissues are not always the same. For example, studies by Nedergaard and colleagues have demonstrated that focal ischemia in rats preferentially causes decreases of pH_o rather than pH_i in the penumbra regions of the brain. In their studies, pH_o in cortical region surrounding the ischemic core decreased to below 6.0 in hyperglycemic conditions, while pH_i remained relatively stable at ~6.8 (Nedergaard et al., 1991a). In other studies, an alkaline pH_i to as high as ~7.3 has also been reported in the cortical penumbra regions following focal ischemia (Back et al., 2000). Thus, it is conceivable that in regions of the brain where H^+ transporting mechanisms are not severely compromised by ischemia, cells may continue to extrude H^+ to maintain a relatively stable pH_i. In these regions of the brain, pH_o may drop significantly with relatively normal pH_i (Nedergaard et al., 1991a; Back et al., 2000). In other regions where the tight regulation of tissue pH via Na^+/H^+ and Cl^-/HCO_3^- exchangers and the availability of intracellular anionic buffers are disturbed, greater diversity of the extracellular and intracellular pH may occur (Back et al., 2000).

Acidosis Induces Neuronal Injury

Acidosis has long been considered among the principal mechanisms responsible for ischemic brain injury (Siesjo, 1988; Tombaugh & Sapolsky, 1993; Siesjo et al., 1996). A large number of studies, conducted both in vitro and in vivo, have demonstrated that acidosis aggravates ischemic neuronal injury (Kraig et al., 1987; Goldman et al., 1989; Kristian et al., 1994; Siesjo et al., 1996), and a direct correlation of brain acidosis with infarct size has been described (Siesjo, 1988; Back et al., 2000).

Although it was assumed that acidosis generated in ischemia is deleterious for neurons, the exact mechanism(s) was largely unknown. Over the last two decades, a number of mechanisms have been proposed. For example, it has been suggested that low tissue pH may cause a non-selective denaturation of proteins and nucleic acids (Kalimo et al., 1981). Alternately, triggering of cell swelling via stimulation of the Na^+/H^+ and Cl^-/HCO_3^- exchangers, leading to cellular edema and osmolysis has been proposed (Kimelberg et al., 1990). Others have proposed that acidosis hinders postischemic metabolic recovery by inhibiting mitochondrial energy metabolism (Hillered, Smith & Siesjo, 1985) and by impairing postischemic blood flow via vascular edema. Stimulation of pathologic free radical formation has also been shown in some systems (Rehncrona, Hauge & Siesjo, 1989; Tombaugh & Sapolsky, 1993). At the neurotransmitter level, profound acidosis inhibits astrocyte glutamate uptake, which may contribute to excitatory neuronal injury (Swanson, Farrell & Simon, 1995). Marked acidosis, with tissue pH < 5.5, may also influence neuronal vulnerability indirectly by damaging glial cells (Goldman et al., 1989; Giffard, Monyer & Choi, 1990; Tombaugh & Sapolsky, 1993). It has also been suggested that acidosis may potentiate AMPA/kinate receptor mediated toxicity (McDonald et al., 1998).

Mild acidosis, in contrast, has been shown to be beneficial in protecting neurons from excitotoxic injury (Giffard et al., 1990b; Kaku et al., 1993; Sapolsky, Trafton & Tombaugh, 1996). An explanation for this effect is that a decrease in pH_o inhibits the NMDA channel activity (Tang, Dichter & Morad, 1990; Traynelis & Cull-Candy, 1990). This finding may imply that, during brain ischemia where acidosis takes place, Ca^{2+} entry through NMDA receptor-gated channels does not play an essential role in neuronal injury since the activity of NMDA channels is largely inhibited by the ischemic acidosis. Therefore, additional Ca^{2+} entry pathways other than NMDA channels must have played a role in ischemic brain injury.

Acid-sensing Ion Channels

The recent finding that acidosis activates a distinct family of membrane channels, the acid-sensing ion channels (ASICs), in both peripheral sensory neurons and in neurons of the central nervous system (Krishtal & Pidoplichko, 1980; Grantyn et al., 1989; Kovalchuk, Krishtal & Nowycky, 1990; Ueno, Nakaye & Akaike, 1992; Price, Snyder, & Welsh, et al., 1996; Waldmann et al., 1997a; Waldmann & Lazdunski, 1998; Varming, 1999; Wemmie et al., 2002; Baron, Waldmann & Lazdunski, 2002; De La Rosa et al., 2003) has dramatically changed the landscape of brain ischemia and provided a new mechanism for glutamate-independent neuronal injury (Yermolaieva et al., 2004; Huang & McNamara, 2004; Xiong et al., 2004; Benveniste & Dingledine, 2005).

ASICs, activated by a drop of the extracellular pH (pH_o) or by an increase of proton concentration, belong to the amiloride-sensitive epithelial Na^+-channel/degenerin superfamily. This family of ion channels contains two tran membrane-spanning

regions flanked by a large extracellular loops and short intracellular N- and C-termini (Corey & Garcia-Anoveros, 1996; Waldmann et al., 1997a; Waldmann & Lazdunski, 1998; Benos & Stanton, 1999; Alvarez et al., 2000; Bianchi & Driscoll, 2002; Krishtal, 2003; Saugstad et al., 2004). Four genes (ASIC1 - ASIC4) encoding six different ASIC subunits have been cloned to date. ASIC1a (also named ASIC or BNaC2) subunits are enriched in primary sensory neurons of dorsal root and trigeminal ganglia, and are also expressed in most brain regions (Garcia-Anoveros et al., 1997; Waldmann et al., 1997a; De La Rosa et al., 2003). These channels can be activated by moderate decreases of pH_o; the pH for half maximal activation ($pH_{0.5}$) is ~6.2 (Waldmann et al., 1997a). In addition to being selective for Na^+, homomeric ASIC1a channels are also permeable to Ca^{2+} ions (Waldmann et al., 1997a; Chu et al., 2002a; Yermolaieva et al., 2004). ASIC1β (or ASIC1b), a splice variant of ASIC1a, is expressed only in sensory neurons (Chen et al., 1998; Bassler et al., 2001). When expressed in heterologous systems, ASIC1β forms homomeric channels with a $pH_{0.5}$ of ~5.9 (Chen et al., 1998). Different from ASIC1a, which is Ca^{2+}-permeable, ASIC1β (ASIC1b) has little Ca^{2+} permeability (Chen et al., 1998; Bassler et al., 2001). Similar to the ASIC1 gene, the ASIC2 gene is alternatively spliced to code for two variants: ASIC2a and 2b. ASIC2a subunits (also named MDEG, or BNaC1) have widespread distribution in both peripheral sensory and central neurons (Waldmann et al., 1996; Price et al., 1996; Garcia-Anoveros et al., 1997). Homomeric ASIC2a channels have a low sensitivity to H^+ with a $pH_{0.5}$ of 4.4 (Waldmann et al., 1996; Price et al., 1996; Lingueglia et al., 1997). ASIC2b subunits (or MDEG2) are expressed in both peripheral sensory and central neurons (Lingueglia et al., 1997). They do not form functional proton-gated channels by themselves, but may associate with other ASIC subunits (e.g., ASIC3) to form heteromultimeric channels (Lingueglia et al., 1997). ASIC3 (also named DRASIC) is expressed predominantly in neurons of dorsal root ganglia (Waldmann et al., 1997b; Sutherland et al., 2001). Homomeric ASIC3 respond to pH drops biphasically with a fast desensitizing current followed by a sustained component (Waldmann et al., 1997b; De Weille et al., 1998; Sutherland et al., 2001). Recently cloned, ASIC4 subunits show high levels of expression in the pituitary gland (Grunder, Geissler & Bassler, 2000; Akopian et al., 2000). However, they do not seem to form functional acid-sensing channels on their own (Grunder et al., 2000).

Like other ligand-gated ion channels, ASICs are believed to assemble from homomultimeric or heteromultimeric subunits (Waldmann et al., 1997a; Krishtal, 2003). The exact subunit combination of ASICs in native neurons, however, is not clear. In the past five years, the electrophysiological properties and pharmacological profiles of recombinant homomeric and heteromeric ASICs in heterologous expression systems have been extensively investigated (Bassilana et al., 1997; Waldmann et al., 1997a; Babinski et al., 2000; Baron et al., 2001; Chu et al., 2004). These studies have provided information critical for elucidating the subunit composition of ASICs in native neurons, since different homomeric and heteromeric ASICs have distinct pH sensitivity, ion selectivity, and channel kinetics. The recent findings that *tarantula* toxin PcTX1 specifically blocks homomeric ASIC1a channels (Escoubas et al., 2000), while sea anemone peptide APETx2 specifically blocks the ASIC3 channels (Diochot et al., 2004), have provided additional means by which one could investigate the subunit composition of native ASICs. More significant findings related to the subunit composition and functions of ASICs in the brain have been made by gene knockout approaches, combined with ectrophysiological recordings and behavioral testing (Wemmie et al., 2002; Askwith et al., 2004).

The detailed role that ASICs can play is still under active investigation. In peripheral sensory neurons ASICs have been implicated in mechanosensation (Price et al., 2000, 2001; Page et al., 2005) and perception of pain during tissue acidosis (Krishtal & Pidoplichko, 1981; Bevan & Yeats, 1991; Chen et al., 2002; Ugawa et al., 2002; Sluka et al., 2003; Wu et al., 2004), particularly in the ischemic myocardium where ASICs likely transduce anginal pain (Benson, Eckert & McCleskey, 1999). Recent studies also suggested that activation of ASICs is involved in taste transduction (Lin, Ogura & Kinnamon, 2002; Ugawa et al., 2003; Ugawa, 2003), and maintenance of retinal integrity (Ettaiche et al., 2004). The presence of ASICs in the brain, which lacks nociceptors, suggests that these channels have functions beyond nociception (Johnson et al., 2001; Allen and Attwell, 2002). Indeed, recent studies have indicated that ASIC1a is involved in synaptic plasticity, learning/memory, and fear conditioning (Wemmie et al., 2002; Wemmie et al., 2003). Using a combination of patch-clamp recording, Ca^{2+}-imaging, in vitro cell-toxicity assays, and in vivo ischemia models combined with ASIC gene knock-out mice, we have recently demonstrated that activation of Ca^{2+}-permeable ASIC1a is also responsible for glutamate-independent, acidosis mediated, ischemic brain injury (Xiong et al., 2004).

Activation of ASICs in Mouse Cortical Neurons

The presence of acid-sensing ion channels in cultured mouse cortical neurons, a preparation commonly used for cell toxicity studies (Sattler et al., 1999; Aarts et al., 2003), was described and detailed electrophysiological properties of these channels were studied (Varming,

1999; Xiong et al., 2004; Chu et al., 2004). With neurons voltage-clamped at −60 mV, lowering pH_o from 7.4 to below 7.0 evoked a transient inward current in all neurons tested (Chu et al., 2004; Xiong et al., 2004). The amplitude of the peak current increases in a sigmoidal fashion as pH_o decreases, and a detailed dose-response analysis yielded an average $pH_{0.5}$ of ~6.1 (Chu et al., 2004; Xiong et al., 2004). This pH sensitivity of ASICs is close to the homomeric ASIC1a and/or heteromeric ASIC1a/2a channels expressed in heterologous systems (Waldmann et al., 1997a; Baron et al., 2001). Further studies demonstrate that the electrophysiological property and the pharmacological profile of ASICs in mouse cortical neurons may undergo significant changes at different developmental or maturation stages (Li & Xiong, 2005).

Pharmacology of ASICs in Mouse Cortical Neurons

The effect of amiloride, a known blocker of ASICs (Waldmann et al., 1997a; Chen et al., 1998; Benson et al., 1999) has been tested on the acid-activated currents in mouse cortical neurons. As expected, the ASIC currents in mouse cortical neurons were dose-dependently and reversibly inhibited by amiloride at low micromolar concentrations (Varming, 1999; Xiong et al., 2004). These concentrations of amiloride are, however, not different from that required to block other ion channels or ion transporter systems including Na^+-H^+ and Na^+-Ca^{2+} exchangers (Kleyman and Cragoe, Jr., 1988). Therefore, using amiloride alone would not be very useful in defining the functions of ASICs in either physiological or pathological conditions. Fortunately, at least for homomeric ASIC1a channels, a specific and potent blocker, *Psalmotoxin-1* or PcTX1, has been found in the venom of tarantula *Psalmopoeus cambridgei* by Escoubas and colleagues (Escoubas et al., 2000). This toxin specifically blocks the acid-activated currents mediated by homomeric ASIC1a channels expressed in heterologous systems and does so at nanomolar concentrations (Escoubas et al., 2000). PcTX1 also blocks the ASIC currents in a subpopulation of DRG neurons and in a majority of mouse cerebellar granule neurons, indicating the presence of homomeric ASIC1a channels in native neurons (Escoubas et al., 2000). Similar to cerebellar granule neurons, ASIC currents in the majority of mouse cortical neurons were sensitive to PcTX1 blockade with >50% reduction in the peak amplitude, indicating the expression of homomeric ASIC1a in these neurons (Xiong et al., 2004).

Activation of ASICs Induces Inward Ca^{2+} Current and Increased Intracellular Ca^{2+} Concentrations

Two studies were carried out to determine whether ASICs in cultured cortical neurons are permeable to Ca^{2+} ion-substitution experiment and fura-2 fluorescent Ca^{2+}-imaging. With a Na^+-and K^+-free extracellular solution containing only 10 mm Ca^{2+} as the charge carrier, decreasing pH_o was able to activate amiloride- and PcTX1-sensitive inward currents in the majority of cortical neurons, providing the first evidence that ASICs in these neurons are permeable to Ca^{2+}. Fura-2 fluorescent Ca^{2+}-imaging was then used to demonstrate an increase in intracellular Ca^{2+} signal in response to a decrease in pH_o. This acid-induced increase of $[Ca^{2+}]_i$ was not blocked by a combination of antagonists of voltage-gated Ca^{2+} channels and glutamate receptors, but was eliminated either by amiloride or PcTX1, suggesting an entry of Ca^{2+} directly through the ASIC1a channels. To provide additional evidence that activation of the ASIC1a channels is indeed responsible for acid-induced increase of $[Ca^{2+}]_i$ in mouse cortical neurons, neurons cultured from the ASIC1 knock-out mice (Wemmie et al., 2002) were also used. In these neurons, decreasing pH_o did not induce any change in $[Ca^{2+}]_i$ (Xiong et al., 2004).

Acidosis Induces Injury of Cultured Cortical Neurons Independently of Glutamate Receptors and Voltage-gated Ca^{2+} Channels

Acidosis-induced neuronal injury was first studied in cultured mouse cortical neurons grown on 24-well culture plates. Cells were randomly incubated in normal (pH 7.4) or acidic (pH 6.0) solution with or without ASIC blockers. Cell injury in different treatment groups was analyzed by measurement of lactate dehydrogenase (LDH) release. Blockers of glutamate receptors and voltage-gated Ca^{2+} channels were included in the treatment solutions to eliminate potential secondary activation of these receptors/channels. Similar to previous reports (Nedergaard et al., 1991b; Yermolaieva et al., 2004), brief acid incubation induced significant neuronal injury, as indicated by increased LDH release and fluorescent staining of alive/dead cells. Importantly, this acid-induced neuronal injury was inhibited by either amiloride or PcTX1, suggesting the involvement of ASIC1a activation in acid-induced neuronal injury. Unlike neurons from wild-type mice, cortical neurons cultured from the ASIC1a knock-out mice did not show increased LDH release following the acid incubation, further supporting a critical role of ASIC1a in acidosis-mediated neuronal injury. Moreover, attenuation of acidosis-mediated neuronal injury by reducing the concentration of extracellular Ca^{2+} also supports the involvement of Ca^{2+}-permeable ASIC1a in acid-mediated neuronal injury.

In addition to native neurons, involvement of ASIC1a in acidosis-induced cell injury was studied in COS-7 cells, a cell line lacking endogenous ASICs

(Chen et al., 1998; Escoubas et al., 2000; Immke & McCleskey, 2001). If ASIC1a is indeed involved in acidosis-induced injury, one can expect that the lack of functional ASICs in COS-7 cells should make these cells less sensitive to acid injury, while expression of exogenous ASIC1a in these cells should increase their acid-sensitivity. Indeed, incubation of COS-7 cells with pH 6.0 solution for 1 h did not induce a time-dependent increase of LDH release when compared to cells treated with pH 7.4 solution. However, after being stably transfected with ASIC1a subunits, COS-7 cells became sensitive to acid treatment with significant injury (Xiong et al., 2004).

Hypoxia/Ischemia Facilitates the Activation of ASICs

Although the degree of acidosis generated in brain ischemia is known to activate the ASICs in non-ischemic condition, it is not clear whether the same acidosis can activate these channels in ischemic conditions since hypoxia/ischemia is known to cause either down- or up-regulation of various ion channels. It has been shown, for example, in turtle brain that voltage-gated K^+ channel and NMDA channel activity decreases dramatically following anoxia, a phenomena called "channel arrest" (Buck & Bickler, 1998; Lutz & Milton, 2004). A similar reduction of K^+ channel gene expression and channel activity was also reported in rat brain following sub-lethal ischemia, a process partially responsible for ischemic tolerance (Stenzel-Poore et al., 2003). Before a potential pathological role for ASICs in brain ischemia can be established, it is important to know whether these channels can be activated under ischemic condition. Thus, ASICs currents were also studied in neurons following oxygen-glucose deprivation (OGD), a common in vitro ischemia model (Grabb & Choi, 1999; Sattler et al., 2000). Unlike NMDA or voltage-gated K^+ channels in anoxic turtle brain, the activity of ASICs in mouse cortical neurons was significantly enhanced following OGD (Xiong et al., 2004). In addition to an increase in the amplitude of the ASIC current, OGD also reduced its desensitization, as indicated by slowing down of the current decay. The exact mechanism underlying the potentiation of the ASIC current is not clear. One possible explanation is that OGD treatment induced an increase in the affinity of ASICs to the proton, as demonstrated by a shift of the pH dose response relationship towards less acidic pH (Chu et al., 2002b). A recent study by Gao et al. has suggested that an increased phosphorylation of the ASIC1a subunits by CaMKII following NMDAR activation is involved in ischemia-induced enhancement of the ASIC responses (Gao et al., 2005). These findings, together with previous reports that the activation of ASICs is promoted by stretching of the membrane, the release of arachidonic acid (Allen & Attwell, 2002), the production of lactate (Immke & McCleskey, 2001) or a drop in extracellular calcium concentrations (Immke & McCleskey, 2003)—conditions that occur in neurons subjected to ischemia—strongly suggest that activation of ASIC1a should play an important role in the pathophysiology of brain ischemia.

Activation of ASICs Is Involved in Ischemic Brain Injury in Vivo

To gain more evidence that activation of ASICs is indeed involved in ischemic brain injury, middle cerebral artery occlusion in rats and mice, a common in vivo model of brain ischemia (Longa et al., 1989), was then used to test the effect of ASIC blockade and ASIC gene manipulation. Both pharmacological intervention (i.e., by intracerebral ventricular injection of ASIC1a blockers amiloride and PcTX1) and ASIC1 gene knockout, dramatically reduced the infarct volume by up to 60%. Furthermore, both ASIC1a blockade and ASIC1 gene knockout provided additional protection even in the presence of glutamate receptor antagonist memantine. These studies provided convincing evidence that activation of Ca^{2+}-permeable ASIC1a channels is indeed involved in glutamate receptor-independent ischemic brain injury in vivo.

Concluding Remarks

ASICs are highly expressed in neurons of the central nervous systems, where they sense changes of extracellular proton concentrations in both physiological and pathological conditions. A moderate reduction of the pH_o, as expected during normal synaptic transmission, activates ASICs, resulting in membrane depolarization and facilitation of excitatory neurotransmission (Wemmie et al., 2002). Severe acidosis, as accompanies brain ischemia, excessively activates the Ca^{2+}-permable ASIC1a channels resulting in significant Ca^{2+} entry and resultant neuronal injury (Fig. 1). Remarkably, ischemia itself, or ischemia-related factors, markedly enhances the magnitude of the ASIC response and reduces the desensitization of these channels, signifying a possibility of long-lasting and enhanced activation of ASICs during prolonged ischemic acidosis. Indeed, pharmacological blockade of ASIC1a (e.g. by PcTX1) and genetic manipulation of the ASIC1 gene are effective in reducing acidosis-mediated and ischemic neuronal injury both in vitro and in vivo. The protective effect of ASIC1a blockade persists in the presence of antagonists of glutamate receptors and voltage-dependent Ca^{2+} channels. Together, these findings suggest that activation of Ca^{2+}-permeable ASIC1a is a novel, glutamate-

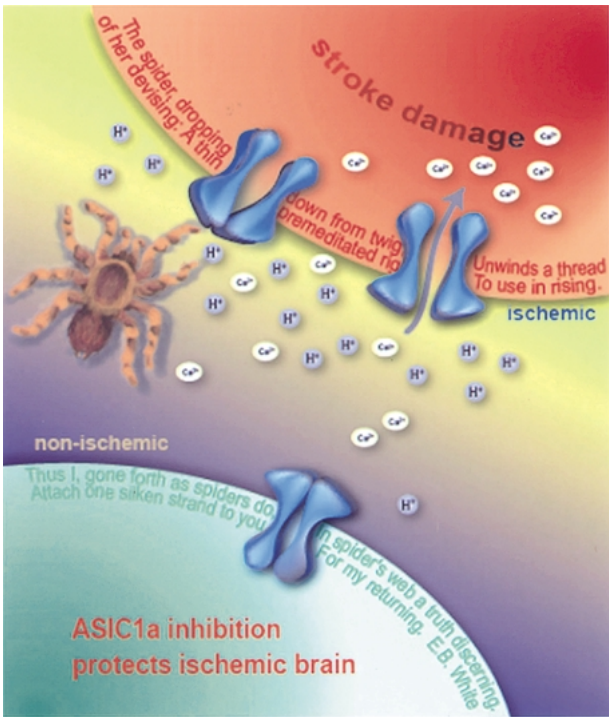

Fig. 1. Schematic diagram demonstrating the role of ASIC1a channels in ischemic neuronal injury. Lower area (*green*) represents neurons in non-ischemic conditions where the concentration of the extracellular protons is low and the ASIC1a channels remain closed. Upper area (*red*) represents neurons in ischemic conditions where the concentration of the extracellular proton increases, resulting in activation of ASIC1a channels and the flux of large amounts of Ca^{2+} into neurons, which leads to neuronal injury. PcTX1, a specific ASIC1a blocker isolated from venom of tarantula *Psalmopoeus cambridgei*, blocks the ASIC1a channels, resulting in neuroprotection. The membrane lipid bilayer is represented by E. B. White's well-known poem.

independent, biological mechanism underlying ischemic brain injury, and that targeting ASIC1a may offer a new and robust neuroprotective strategy for stroke either alone or in combination with other therapies.

Ongoing studies focus on several important aspects of ASIC1a, including the therapeutic time window for ASIC1a blockade and searching for more specific, yet potent ASIC1a blockers. Given the painful lessons learned from the failure of glutamate antagonists in human clinical trials, one would expect that any meaningful neuroprotective intervention should have at least a 3-hour time window in animal studies. Before considering ASICs as a target for future clinical trial, one critical study would be to test the therapeutic time window for the blockade of the ASIC1a channels. Although this study is still preliminary, the finding is encouraging: the neuroprotective effect of the ASIC1a blocker PcTX1 has an effective time window of up to 5 hours following the onset of ischemia (Pignataro et al., 2005).

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