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Acid sensing ion channels - novel therapeutic targets for ischemic brain injury

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1. ABSTRACT

Ischemic stroke is a leading cause of death and long-term disability in the United States. Unfortunately there is no effective therapeutic intervention other than the use of thrombolytics, which has a limited therapeutic time window of ~3 h and the potential side effect of intracranial hemorrhage. The absence of neuroprotective therapy is particularly apparent following the failure of multiple clinical trials using glutamate antagonists as therapeutic agents. Understanding the detailed biochemical changes associated with brain ischemia and the cellular mechanisms involved in ischemic brain injury are critical for establishing new and effective neuroprotective strategy. Dramatically decreased tissue pH, or acidosis, is a common feature of ischemic brain, and has been suggested to play a role in neuronal injury. However, the detailed cellular and molecular mechanisms of such acid induced injury remain elusive. The recent finding that acidosis activates a distinct family of cation channels, the acid-sensing ion channels (ASICs), in both peripheral and central neurons has dramatically changed the landscape of brain ischemia neurochemistry and provided a novel therapeutic target. In CNS neurons, lowering extracellular pH to the level commonly seen in ischemic brain activates inward ASIC currents resulting in membrane depolarization. In the majority of these neurons, ASICs are also permeable to Ca^{2+}. Therefore, activation of these channels induces an increase of $[Ca^{2+}]_i$. Incubation of neurons with acidic solutions reproduces Ca^{2+}-dependent neuronal injury independent of glutamate receptor activation. The acid-induced currents, membrane depolarization, $[Ca^{2+}]_i$ increase, and neuronal injury can be inhibited by the blockade of ASIC1a. In focal ischemia, ASIC1a blockade, or ASIC1a gene knockout both protect brain from injury. The blockers of ASIC1a also demonstrate a prolonged therapeutic time window, beyond that of the glutamate antagonists. Thus, Ca^{2+}-permeable ASIC1a may represent a novel therapeutic target for ischemic brain injury.

2. INTRODUCTION

Ischemic stroke or brain ischemia, caused by an interruption of blood flow to brain tissue, is a leading cause of death and long-term disabilities in developed countries. Understanding the cellular and molecular mechanisms underlying ischemic brain injury is essential for establishing effective therapeutic interventions. Although multiple biochemical changes take place during brain ischemia, it is generally believed that excessive Ca^{2+} entry and resultant cytosolic Ca^{2+} overload is essential for ischemic brain injury. However, the exact sources of Ca^{2+} loading responsible for neuronal injury remain unclear. For the past two decades, the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors has been recognized as the main entry pathway responsible for toxic Ca^{2+} loading (1, 2, 3, 4, 5). This concept was supported by a large number of studies demonstrating neuroprotection by the antagonists of NMDA receptors (1, 5, 6, 7, 8). It is interesting that neuronal injury is not only dependent on the degree of Ca^{2+} loading but also specific Ca^{2+} entry pathways. For example, in cultured mouse spinal neurons, the same amount of Ca^{2+} loading from NMDA receptors is far more neurotoxic than the Ca^{2+} entry from voltage-gated Ca^{2+} channels (5). This may be explained by the fact that NMDA receptors are specifically coupled to nitric oxide (NO) synthase through a postsynaptic density protein-95 (PSD-95). Therefore, Ca^{2+} entry through NMDA receptors preferentially activates NO synthase resulting in increased NO production and neuronal injury (9). Following successful studies showing neuroprotection by glutamate antagonists in neuronal cell cultures and whole animal models, various clinical trials were conducted. Unfortunately, none of the human trials using the glutamate antagonists showed satisfactory protection. The reasons for the failure of multiple clinical trials have been extensively discussed (3, 10, 11, 12, 13). It is believed that multiple factors, including difficulty in early initiation of treatment and intolerance of severe side effects, may have contributed to the failures. However, recent new studies have suggested that activation of glutamate receptor-independent Ca^{2+} loading pathways, for example, the Ca^{2+}-permeable ASIC1a, also contribute to the ischemic brain injury. Therefore, new stroke therapy should target both glutamate-dependent and independent injury mechanisms.

2.1. NMDA receptors and Ca^{2+}-dependent neuronal injury

Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS) (14, 15, 16). Activation of glutamate receptors is responsible for a variety of physiological functions including neuronal development and differentiation, learning, memory, movement and sensation (14, 17). Glutamate receptors are classified into two major categories: ionotropic receptors, which are ligand-gated ion channels; and metabotropic receptors, which are coupled through G proteins to second messenger systems (18). One subtype of ionotropic glutamate receptor, the N-methyl-D-aspartate (or NMDA) receptor, is highly permeable to Ca^{2+} ions in addition to Na^{+} and K^{+}. The resultant increase in $[Ca^{2+}]_i$ through these receptor-gated channels is critical for both physiological function and "excitotoxicity" (5, 19, 20, 21, 22). One unique feature of the NMDA channels is the voltage-dependent Mg^{2+} blockade (23). At normal resting potential (i.e. -70 mV), NMDA channels are blocked by physiological concentrations of Mg^{2+}. Without membrane depolarization, NMDA channels remain closed even in the presence of the agonist. Membrane depolarization, which can relieve Mg^{2+} blockade, is therefore essential for the activation of NMDA channels (23, 24). During ischemia, neurons and glial cells deprived of oxygen and glucose rapidly lose ATP and become depolarized, leading to release of glutamate from nerve terminals and reduced glutamate uptake by glial cells (4, 25, 26). Accumulation of glutamate over-excites the post-synaptic NMDA receptors, resulting in intracellular Ca^{2+} overload (27, 28, 29, 30, 31). Excessive intracellular Ca^{2+} accumulation can trigger a number of neurotoxic cascades, including inappropriate activation of several enzyme systems such as nitric oxide synthase, calpains, phospholipase A2 (PLA2), and endonucleases. Over-activation of these enzymes results in breakdown of proteins, lipids and nucleic acids (3, 32, 33).

In addition, mitochondria Ca^{2+} loading, along with other factors including oxidative stress, can cause opening of mitochondria permeability transition pore (PTP), a large conductance channel residing in both the inner and outer mitochondrial membrane (34, 35). The role of mitochondria PTP in apoptosis and necrosis has been extensively reviewed (36, 37). It was generally believed that activation of this PTP promotes apoptosis due to the release of cytochrome c and subsequent activation of caspases (38, 39, 40). However, recently studies have also demonstrated that cyclophilin D is a critical component of the mitochondrial permeability transition that mediates neuronal cell death (41, 42, 43).

Although the majority of studies using neuronal cell culture and/or whole animal models demonstrated clear neuroprotection by the antagonists of glutamate receptors, clinical trials in human subjects have failed to show a satisfactory protective effect (3, 10, 11, 12). The difficulty in initiating the treatment at an early stage and the severe side effects associated with the blockade of glutamate receptor functions have been part of the reasons cited for failure (3, 10, 11, 12, 13). It is worth mentioning that, in most animal studies, the neuroprotective effect of glutamate antagonists had only a narrow time window of effectiveness - less than an hour (10, 44, 45, 46). This finding might have predicted the failure of human clinical trials, as most patients arrived in hospitals far beyond this time window. Interestingly, new studies in the last several years have suggested that blocking the glutamate receptors in the late stage of ischemia or in chronic neurodegenerative disorders may actually be detrimental to neuronal survival (46, 47). These studies suggest that glutamate toxicity is only involved in the acute neurodestructive phase, 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 trials may involve the inhibition of neuronal survival by NMDA receptor blockade (11, 12, 46).

Apart from these reasons, emerging new studies have also provided alternative explanations for the failure of clinical trials using the antagonists of glutamate receptors alone (48, 49, 50, 51). These studies clearly demonstrated that several glutamate-independent Ca^{2+} toxicity pathways, including TRPM7 channels and Ca^{2+} -permeable ASICs, are activated in ischemia and contribute to ischemic neuronal injury. This review focuses on the role of ASIC1a, in ischemic brain injury.

2.2. Acidosis in brain ischemia

In normal brain tissue, extracellular pH (pH_o) is maintained at ~7.3 while intracellular pH (pH_i) is at ~7.0 (52, 53, 54). Maintaining these pH values is critical for normal brain function, since various biochemical reactions and cellular metabolism depend on a stable acid-base balance (54). Similarly, the activities of various membrane receptors and/or ion channels are affected by pH. It has been recognized for several decades that acute neurological disorders such as brain ischemia are accompanied by marked reductions of tissue pH, or acidosis (52, 55, 56, 57, 58, 59). In brain ischemia, 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: (60, 61)). Accumulation of lactic acid, along with H^+ release from ATP hydrolysis, results in a dramatic decrease in tissue pH. At the same time, cessation of local circulation results in carbon dioxide accumulation and carbonic acid build up, which also contribute to the decrease of tissue pH (58). During ischemia, brain pH typically falls to ~6.5 under normoglycemic conditions, and to 6.0 or below during severe ischemia or under hyperglycemic conditions (52, 56, 59, 62, 63). The time course of this pH change during brain ischemia has been documented in several studies (52, 60, 64, 65, 66). In most experiments, tissue pH starts to decrease significantly within minutes following ischemia, and falls further or is maintained at a low level over the course of permanent ischemia in brain. For transient brain ischemia, tissue pH recovers rather slowly following reperfusion (67).

2.3. Acidosis and ischemic brain injury

For many years, acidosis has been considered as one of the main mechanisms responsible for ischemic brain injury (58, 60, 61). Large numbers of studies, performed both *in vitro* and *in vivo,* have demonstrated that acidosis aggravates neuronal injury (60, 68, 69, 70). A direct correlation of brain acidosis with infarct size has also been described (53, 58).

A number of mechanisms have been proposed over the last two decades for acidosis-mediated neuronal injury. For example, low tissue pH may cause a non-selective denaturation of proteins and nucleic acids (71), trigger cell swelling via stimulation of the Na^+/H^+ and Cl^-/HCO_3^- exchanger leading to cellular edema and osmolysis (72), hinder postischemic metabolic recovery by inhibiting mitochondrial energy metabolism and by impairing postischemic blood flow via vascular edema (73). The stimulation of pathologic free radical formation by acidosis has also been shown in some systems (74). At the neurotransmitter level, profound acidosis inhibits astrocytic glutamate uptake, which may contribute to excitatory neuronal injury (75). Marked acidosis, with tissue pH <5.5, may influence neuronal vulnerability indirectly by damaging glial cells (61, 70, 76). It has also been suggested that acidosis may potentiate AMPA/kinate receptor mediated neuronal injury (77).

In contrast to severe acidosis, mild acidosis has actually been shown to be neuroprotective against excitotoxic injury (78, 79, 80). This effect could be explained by the finding that a decrease in pH_o inhibits NMDA channel activity (81, 82). This finding may provide an additional explanation for the failure of NMDA antagonist in ischemia as Ca^{2+} entry through NMDA receptor-gated channels is largely inhibited by the ischemic acidosis.

3. ACID-SENSING ION CHANNELS IN GLUTAMATE-INDEPENDENT NEURONAL INJURY

Changes in pH_o can modulate the activity of a variety of membrane receptors and ion channels (81, 81, 82, 83, 84, 85, 86, 87, 88). In general, decreased pH_o inhibits while increased pH_o potentiates the activities of the majority of voltage-gated and ligand-gated ion channels. For example, NMDA receptor-gated channels are strongly inhibited by decreases in pH_o (81, 81, 82, 89).

In contrast to it's modulating effect on other ion channels, recent studies have demonstrated that decreasing pH_o can activate a distinct family of ligand-gated channels, the ASICs, in both peripheral sensory neurons and in neurons of the CNS (90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100). This finding has dramatically changed the landscape of brain ischemia and provided a new mechanism for acidosis-mediated, glutamate-independent neuronal injury (48, 49, 50, 101).

3.1. Structure and function of acid-sensing ion channels

ASICs belong to the amiloride-sensitive degenerin/epithelial Na^+-channel (Deg/ENaC) superfamily, but are uniquely gated by the proton. Similar to other channels in the ENaC/Deg family, ASICs are believed to assemble as a tetramer with either identical or different subunits. Each subunit is composed of two transmembrane spanning regions (TM I and TM II) flanked by a large cysteine-rich extracellular loop and short N and C termini facing the intracellular space (Figure 1)(90, 91, 102, 103, 104, 105, 106, 107). To date, six ASIC subunits encoded by four genes have been cloned and characterized. ASIC1a (also named ASIC or BNaC2) is enriched in peripheral sensory neurons and in most brain regions (91, 95, 108). These channels can be activated by moderate decreases of pH_o to below 7.0, with a pH for half maximal activation ($pH_{0.5}$) at ~6.2 (91, 109). In addition to Na^+, homomeric ASIC1a channels are also permeable to Ca^{2+} ions (91, 101, 110). ASIC1b (or ASICβ) is a splice variant of ASIC1a, which is expressed only in sensory neurons (111, 112). Similar to ASIC1a, homomeric ASIC1b channels have high sensitivity to H^+ with a $pH_{0.5}$ at ~5.9 (112). Different from

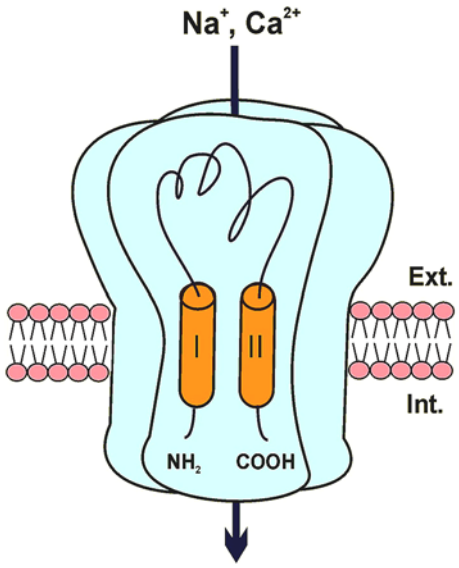

Figure 1. Schematc drawing represents the structure of acid-sensing ion channels (ASICs). Each functional ASIC is formed by four identical or different subunits. A single ASIC subunit contains two hydrophobic transmembrane dmains (I & II), a large extracellular loop, and short intracellular NH_2 and COOH termini. Although most ASICs only conduct Na^+ ions, homomeric ASIC1a is also permable to Ca^{2+} ions. Ext. - extracellular; Int. - intracellular.

ASIC1a, ASIC1b has no detectable Ca^{2+} permeability (111, 112). ASIC2a subunits (also named MDEG, or BNaC1) have widespread distribution in both peripheral sensory and central neurons (93, 108, 113). Homomeric ASIC2a channels have very low sensitivity to H^+ with a $pH_{0.5}$ of 4.4 (93, 113, 114). ASIC2b subunits (or MDEG2) are expressed both in peripheral sensory and in central neurons (114). They do not form functional homomeric channels, but may associate with other ASIC subunits (e.g. ASIC3) to form heteromultimeric channels (114). ASIC3 (also named DRASIC) is predominantly expressed in neurons of dorsal root ganglia (115, 116). Homomeric ASIC3 responds to pH drops biphasically with a fast desensitizing current followed by a sustained component (115, 116, 117). ASIC4 subunits are highly expressed in the pituitary gland (118, 119). Similar to ASIC2b, they do not seem to form functional homomeric channels (119).

Although it is difficult to tell the exact subunit combination of ASICs in native neurons, the electrophysiological properties and pharmacological profiles of recombinant homomeric and heteromeric ASICs in heterologous expression systems have been extensively investigated (91, 109, 120, 121, 122). These studies offered important information for evaluating the subunit composition of ASICs in native neurons. For example, the findings that the tarantula toxin PcTX1 specifically blocks homomeric ASIC1a channels (123), while sea anemone peptide APETx2 blocks the ASIC3 channels (124), have provided invaluable pharmacological tools for detecting the presence of ASIC1a or ASIC3 channels in native neurons, and for evaluating channel functions. Other important findings, including ASIC1a-specific Ca^{2+}-permeability (91, 101, 110), high affinity Zn^{2+} inhibition (109), and ASIC2a-specific Zn^{2+} potentiation (122), are also helpful for defining the presence of specific ASIC subunits in native neurons. More significant studies related to the subunit composition and the functions of specific ASIC subunits in the brain have been made by gene knockout approaches, combined with ectrophysiological recordings and behavioral testing (48, 94, 125).

The main function of ASICs in peripheral sensory neurons include nociception (126, 127, 128, 129, 130, 131, 132) and mechanosensation (133, 134, 135). It is also suggested that activation of ASICs is involved in taste transduction (136, 137, 138). The presence of ASICs in the brain, which lacks nociceptors, suggests that these channels have functions beyond nociception (139, 140). Indeed, recent studies have indicated that ASIC1a is involved in synaptic plasticity, learning and memory (94, 141), while ASIC2a may be required for the maintenance of retinal integrity (142) and survival of neurons following global ischemia (140). Studies by our laboratories and others have demonstrated that activation of Ca^{2+}-permeable ASIC1a is also responsible for glutamate-independent, acidosis mediated, ischemic brain injury (48, 101).

3.2. Electrophysiology and pharmacology of ASICs in CNS neurons

The presence of acid-activated conductance in CNS neurons was described over 2 decades ago (106). These findings were then confirmed by molecular cloning of ASICs about 10 years later (91). The electrophysiological properties of ASICs have been characterized in neurons from different regions of brain including hippocampus (92, 94, 125, 143, 144), cerebellum (123, 139), and cortex (48, 100, 109).

A detailed characterization of the ASIC, including its electrophysiological properties, pharmacological profiles, its modulation by endogenous signaling molecules, and its role in acidosis-mediated neuronal injury have been performed in cultured mouse cortical neurons, a preparation commonly used for cell toxicity studies (48, 100, 109, 145, 146, 147). In these neurons, lowering pH_o from 7.4 to below 7.0 evokes large transient inward currents (48, 109). The amplitude of peak ASIC current increases in a sigmoidal fashion in response to decreased pH_o, with a $pH_{0.5}$ of ~6.1. This pH sensitivity suggests that the acid-activated current in mouse cortical neurons is largely mediated by homomeric ASIC1a and heteromeric ASIC1a/2a channels (48, 109).

Similar to ASICs in peripheral sensory neurons and in heterologous expression systems, ASIC currents in CNS neurons are inhibited by amiloride at low micromolar concentrations (IC_{50}: 10-20 μM). The concentration of amiloride for blocking ASICs is however, not different from that required to block other ion channels or ion transporter systems such as Na^+-H^+ and Na^+-Ca^{2+} exchangers (148). Therefore, amiloride alone is not very useful in characterizing the involvement of ASICs in either physiological or pathological processes. Recent studies by

Escoubas and colleagues have demonstrated that *Psalmotoxin-1* or PcTX1 isolated from the venom of tarantula *Psalmopoeus cambridgei* specifically blocks homomeric ASIC1a channels with little effect on other configurations of ASICs (123). Further studies also demonstrate that PcTX1 has no effect on other voltage-gated and ligand-gated ion channels (48). This toxin is therefore very useful in defining the function of ASIC1a channels in CNS neurons. In the majority of mouse cerebellar granule neurons and cortical neurons, ASICs are highly sensitive to PcTX1 blockade, indicating the presence of homomeric ASIC1a channels in native neurons (48, 123).

3.3. Activation of ASICs induces increased $[Ca^{2+}]_i$ in CNS neurons

Overloading neurons with Ca^{2+} is an important mechanism of neuronal injury. To determine whether activation of ASICs in CNS neurons can induce an increase of $[Ca^{2+}]_i$, fluorescent Ca^{2+} imaging was performed in mouse cortical and hippocampal neurons (48, 101). In both neurons, decreased pH_o induced a dramatic increase of $[Ca^{2+}]_i$. This increase of $[Ca^{2+}]_i$ is eliminated in neurons cultured from ASIC1a knockout mice (48, 101), suggesting involvement of ASIC1a in acid-induced, increased $[Ca^{2+}]_i$. This acid-induced $[Ca^{2+}]_i$ increase was not blocked by the antagonists of voltage-gated Ca^{2+} channels and glutamate receptors, but was eliminated by amiloride and PcTX1, indicating an entry of Ca^{2+} directly through the ASIC1a channels. Additional evidence supporting the Ca^{2+}-permeability of ASICs in CNS neurons came from the finding that acid-induced currents were recorded with Ca^{2+} as the only conducting ion in the extracellular solution (48).

3.4. Involvement of ASIC1a activation in acidosis induced neuronal injury

Acidosis has long been known to aggravate ischemic brain injury (60, 61). However, the mechanism(s) underlying acid-mediated neuronal injury remained unclear, although a number of possibilities were suggested (60, 75, 77, 149). The presence of ASIC1a in the brain, its activation by pH drop to a level commonly seen in ischemia, and its permeability to Ca^{2+} all make it likely that activation of ASIC1a is involved in acidosis-mediated injury. A series of recent studies, performed in neuronal cell culture (cortical and hippocampal neurons) and in whole animal models, have provided strong evidence supporting this hypothesis (48, 101, 159). In cultured mouse cortical neurons, for example, brief (1 h) acid incubation at pH 6.0 induced significant neuronal injury, as indicated by increased LDH release and fluorescent staining of alive/dead cells. This acid-induced neuronal injury was glutamate-independent, but was inhibited by either amiloride or PcTX1, indicating the involvement of ASIC1a activation. In contrast to the neurons from $ASIC1^{+/+}$ mice, neurons cultured from the $ASIC1^{-/-}$ mice did not show increased cell death following a brief acid incubation. Reducing the concentration of extracellular Ca^{2+}, which lowers the driving force for Ca^{2+} entry, also decreased acid-induced injury of mouse cortical neurons (48).

3.5. Involvement of ASIC1a activation in ischemic brain injury *in vivo*

Further evidence supporting a role of ASIC1a activation in ischemic brain injury was provided by *in vivo* studies using both rat and mouse models of focal ischemia (48). In rats, intracerebral ventricular injection of either amiloride or PcTX1 significantly reduced the infarct volume by up to 60%. Similarly, in a mouse model of ischemia, ASIC1a knockout provided similar protection against ischemic brain injury. Furthermore, ASIC1a blockade and ASIC1 gene knockout provided additional protection in the presence of the glutamate receptor antagonist memantine (48).

3.6. Hypoxia/ischemia modulates the electrophysiological properties of ASICs

Activation of various ion channels and/or membrane receptors (e.g. NMDA channels) is known to be involved in the pathology of brain ischemia. On the other hand, ischemia itself may induce dramatic changes to the properties of certain ion channels. For example, following anoxia, NMDA channel activity decreases dramatically (by ~80%) in turtle brain, a phenomena termed "channel arrest" (150, 151). Similarly, in rat brain and cultured neurons, K^+ channel gene expression and channel activity decrease substantially following a sub-lethal ischemia, a process partially responsible for ischemic tolerance (152). These findings suggest that the properties of various ion channels may be subjected to modulation by ischemic signals. Although ischemic acidosis is known to activate ASICs in neurons under non-ischemic conditions, it does not guarantee that the same acidosis can activate these channels in neurons under ischemic conditions. To better understand the role of ASICs in brain ischemia, it is important to know how these channels behave in ischemic conditions. Thus, ASIC currents were also recorded in neurons in the setting of oxygen-glucose deprivation (OGD), an *in vitro* model of ischemia (153, 154). Unlike NMDA channels in anoxic turtle brain, the activity of ASICs in cultured mouse cortical neurons was enhanced by the OGD treatment (48). Brief OGD treatment not only increased the peak amplitude of acid-induced currents but also reduced the current desensitization. These changes of ASICs are expected to dramatically increase the amount of Ca^{2+} entry through these channels. Accordingly, OGD treatment enhanced acidosis-mediated injury of cultured mouse cortical neurons (48). The exact cellular and molecular mechanisms underlying the ischemia-induced increase of ASIC activity remain unclear. One explanation is that OGD treatment induces an increase in the affinity of ASICs to the proton. This is demonstrated by a shift in the H^+-dose response relationship towards less acidic pH (155). A recent study by Allen and Attwell demonstrated that arachidonic acid, a lipid metabolite released in ischemia, can increase the amplitude of the ASIC current in rat cerebellar Purkinje neurons (139). Gao and colleagues demonstrated that an increased CaMKII phosphorylation of the ASIC1a subunits by NMDAR activation may be involved in ischemia-induced enhancement of the ASIC responses (156). These findings, together with the reports that chelation of the extracellular Ca^{2+} by lactate enhances the activation of ASIC3 (157, 158), further suggest that ASICs are actively involved in the pathology of brain ischemia.

4. PERSPECTIVES

Acidosis, to the degree commonly seen in brain ischemia, activates ASICs. One subunit of ASICs, the ASIC1a, is permeable to Ca^{2+} in addition to Na^{+}. Therefore, activation of ASIC1a in CNS neurons induces membrane depolarization and an increase of $[Ca^{2+}]_i$, thus contributing to Ca^{2+} toxicity. Accordingly, pharmacological blockade of ASIC1a or genetic manipulation of the ASIC1 gene has proven to be effective in reducing acidosis-mediated and ischemic neuronal injury in both cell culture and whole animal studies. Furthermore, neuronal injury by acid incubation and neuroprotection by ASIC1a blockade persists in the presence of glutamate antagonists. Therefore, activation of ASIC1a represents a new, glutamate-independent, biological mechanism responsible for ischemic brain injury.

Ongoing studies will determine whether ASIC1a blockade and gene knockout provide neuroprotection in other models of brain injury, for example, in global ischemia and traumatic brain injury. The therapeutic time window for ASIC1a blockade will also be studied in detail, given the painful lesson learned from the failure of glutamate antagonists in clinical trials. In this regard, a preliminary study has provided rather encouraging data: the neuroprtoective effect of the ASIC1a blockade has an effective time window of up to 5 hours in a mouse model of ischemia (159). Additional studies should also define the down stream signal transduction pathways involved in ASIC1a mediated neuronal injury, and the detailed modulation/regulation of the channel activity by endogenous signaling molecules associated with both physiological and pathological conditions. These studies will be helpful in understanding exactly how ASICs behave in *in vivo* conditions, and how this biological system can best be modulated for the development of novel therapeutic intervention.

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