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Review

Calcium-permeable ion channels involved in glutamate receptor-independent ischemic brain injury

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Brain ischemia is a leading cause of death and long-term disabilities worldwide. Unfortunately, current treatment is limited to thrombolysis, which has limited success and a potential side effect of intracerebral hemorrhage. Searching for new cell injury mechanisms and therapeutic interventions has become a major challenge in the field. It has been recognized for many years that intracellular Ca^{2+} overload in neurons is essential for neuronal injury associated with brain ischemia. However, the exact pathway(s) underlying the toxic Ca^{2+} loading remained elusive. This review discusses the role of two Ca^{2+}-permeable cation channels, TRPM7 and acid-sensing channels, in glutamate-independent Ca^{2+} toxicity associated with brain ischemia.

Keywords: acid-sensing ion channel; TRPM7; brain ischemia; neurons

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Introduction

Stroke or cerebral ischemia is a leading cause of death and long-term disabilities worldwide. Although major advances have occurred in the past decades in the prevention of brain ischemia, treatment is limited to the use of tissue plasminogen activator (tPA), which has limited success and a major side effect of intracranial hemorrhage[1, 2]. Searching for new cell injury mechanisms and effective therapeutic strategies therefore constitutes a major challenge for stroke research.

It has been recognized for several decades that excessive Ca^{2+} entry and resultant cytosolic Ca^{2+} overload play an important role in neuronal injury associated with stroke/brain ischemia[3]. In the resting condition, free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is maintained at nanomolar levels. Following ischemia, $[Ca^{2+}]_i$ can reach as high as several micromoles. Excessive $[Ca^{2+}]_i$ loading can activate enzymes such as proteases, phospholipases, and endonucleases. Over-activation of these enzymes causes breakdown of proteins, lipids and nucleic acids, which leads to destruction of neurons[4–6]. In addition, overloading Ca^{2+} in mitochondria can cause opening of mitochondria permeability transition pore (PTP), a large conductance channel residing in mitochondrial membrane[7, 8], promoting apoptosis through release of cytochrome *c* and activation of caspases[9–11].

Ca^{2+} may enter neurons through various Ca^{2+}-permeable ion channels (*eg* voltage-gated or ligand-gated channels) or through ion exchange systems (*eg* reverse Na^+/Ca^{2+} exchanger). Accumulation of $[Ca^{2+}]_i$ can also occur through Ca^{2+} release from intracellular stores (*eg* endoplasmic reticulum, ER). The exact source(s) of Ca^{2+} loading responsible for ischemic brain injury, however, remains unclear. This review discusses the involvement of two novel Ca^{2+}-permeable cation channels, TRPM7 channels and acid-sensing ion channels, in ischemic brain injury.

Glutamate mediated Ca^{2+}-toxicity

Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS)[12–14]. Its receptors are widely expressed at soma and dendrites of the CNS neurons. Activation of these receptors is involved in a variety of physiological functions of neurons including synaptic transmission/plasticity, learning/memory, neuronal development and differentiation[12, 15]. Glutamate receptors are classified into two major categories: ionotropic receptors, which are ligand-gated cation channels; and metabotropic receptors, which are coupled through G proteins to second messenger systems[16].

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One subtype of ionotropic glutamate receptors, the *N*-methyl-*D*-aspartate (or NMDA) receptor, is highly permeable to Ca^{2+} ions. Activation of these receptors has been considered to play a critical role in Ca^{2+} toxicity associated with ischemic brain injury[3, 17–20]. Accordingly, blocking these receptors has been shown to be neuroprotective in cell culture and animal models of brain ischemia. Unfortunately, none of the human trials using the antagonists of glutamate receptors showed a satisfactory protection for stroke patients. Although multiple factors, including difficulty in early initiation of treatment and intolerance of severe side effects, may have contributed to the failure of the trials[4, 21–24], recent studies suggest that Ca^{2+} entry through glutamate-independent pathways, *eg* TRPM7 channels and Ca^{2+}-permeable acid-sensing ion channels (ASICs), may contribute to the injury of neurons associated with brain ischemia.

TRPM channels and ischemic neuronal injury

Transient receptor potential (TRP) channels belong to a novel family of cation channels that are highly expressed in various tissues including the brain[25, 26]. Several members of TRP family can be activated by oxidative stress and oxygen free radicals, both of which play important roles in neuronal injury associated with stroke/brain ischemia. Recent work has indicated that members of the melastatin subfamily (TRPM) of the TRP channels, particularly the TRPM7, play a key role in neuronal cell death associated with brain ischemia[27–31].

The TRP superfamily is a diverse group of voltage-independent calcium-permeable cation channels expressed in mammalian cells[25, 26]. These channels have been divided into six subfamilies, and two of them, TRPC and TRPM, have members that are widely expressed and activated by oxidative stress. TRPC3 and TRPC4 are activated by oxidants, which induce Na^+ and Ca^{2+} entry into cells through phospholipase C-dependent mechanisms. TRPM2 is activated by oxidative stress or TNFalpha, and the mechanism involves production of ADP-ribose, which binds to an ADP-ribose binding cleft in the TRPM2 C-terminus. Treatment of neurons or HEK 293T cells expressing TRPM2 with H_2O_2 resulted in Ca^{2+} influx and increased susceptibility to cell death[27]. Inhibition of endogenous TRPM2 function, in contrast, protected cell viability[27, 32]. Nevertheless, the exact role of TRPM2 in Ca^{2+} toxicity associated with ischemic brain injury remains to be explored.

The potential role of TRPM7 channels in ischemic neuronal death has been described recently[30, 31]. Aarts and colleagues first examined the mechanism of neuronal cell death in ischemic conditions in the presence of glutamate antagonists. Cultured mouse cortical neurons were exposed to oxygen-glucose deprivation (OGD), an *in vitro* model of ischemia reported to mediate neuronal death through NMDA receptor activation[33, 34]. Blocking the glutamate excitotoxicity in these cultures, however, unmasked a potent, previously unappreciated mechanism of non-excitotoxic neuronal cell death, which became increasingly responsible for neurodegeneration as the duration of OGD was prolonged[30]. Further studies demonstrated that the mechanism of cell death involved activation of a non-selective cation current with high permeability to Ca^{2+}. The current showed outward rectifying properties, was potentiated by reactive oxygen/nitrogen species (ROS), and was blocked by Gd^{3+}. The electrophysiological characteristics and pharmacological properties of the current suggested the involvement of TRPM7 channels. Indeed, molecular biological approaches (*eg* siRNA) confirmed the involvement of TRPM7 channels in glutamate-independent anoxic neuronal injury[30]. Although a specific agonist remains to be determined, these studies suggest that, in ischemic conditions, TRPM7 channels could be activated by ROS. Ca^{2+} entry through these channels participates in neuronal injury. A lethal positive feedback loop is established when Ca^{2+} influx through TRPM7 channels stimulates additional ROS production, causing further TRPM7 activation[30]. Blocking TRPM7 channels or suppressing its expression by RNA interference was effective in preventing the death of neurons by OGD.

Very recent studies by Sun and colleagues also demonstrated involvement of TRPM7 channels in the injury of hippocampal neurons *in vivo* in rat model of global ischemia[31]. Suppressing TRPM7 expression in CA1 neurons by intrahippocampal injections of viral vectors bearing shRNA specific for TRPM7 channels had no ill effect on animal survival, neuronal and dendritic morphology, neuronal excitability, or synaptic plasticity. However, TRPM7 suppression made neurons resistant to ischemic injury and preserved neuronal morphology and function. Also, it prevented ischemia-induced deficits in long-term potentiation and preserved performance in fear-associated and spatial-navigational memory tasks. Thus, regional suppression of TRPM7 is feasible, well tolerated and inhibits delayed neuronal death *in vivo*. In addition to Ca^{2+} toxicity mediated by TRPM7 channels, studies by Inoue and colleagues have suggested that Zn^{2+} permeability of these channels also plays a role in ischemic brain injury[35].

Acid-sensing ion channels and ischemic brain injury

In acute neurological conditions such as brain ischemia, marked reduction of tissue pH takes place[36–41]. Following ischemia, shortage of oxygen supply promotes anaerobic glycolysis, leading to lactic acid accumulation and resultant decrease in brain pH[42, 43]. Increased ATP hydrolysis and release of H^+ also contributes to pH drop. At the same time, cessation of local circulation results in carbon dioxide accumulation and carbonic acid build up, which may participate in the decrease of tissue pH[39]. During ischemia, decreases of brain pH to ~6.5 are commonly observed. It can also fall to 6.0 or below during severe ischemia or under hyperglycemic conditions[37, 40, 41, 44, 45].

Decrease of brain pH or acidosis has long been known to play an important role in ischemic brain injury[39, 42, 43, 46–48]. A direct correlation between the degree of brain acidosis and infarct size has also been described[39, 49]. However, the exact mechanism underlying acidosis-mediated neuronal injury remained vague. Acidosis may cause non-selective denaturation of proteins and nucleic acids[50]; trigger cell swelling and osmolysis via stimulation of Na^+/H^+ and Cl^-/HCO_3^- exchang-

ers[51]; hinder postischemic metabolic recovery by inhibiting mitochondrial energy metabolism and impairing postischemic blood flow via vascular edema[52]; or stimulate pathologic free radical formation[53]. At the neurotransmitter level, profound acidosis inhibits astrocytic glutamate uptake, which may contribute to excitatory neuronal injury[54].

Interestingly, mild acidosis has been considered to be beneficial in protecting neurons from excitotoxic injury[55–57]. This may be explained by proton inhibition of NMDA channel activity[58, 59]. In contrast to its modulating effect on other ion channels, protons can activate a distinct family of ligand-gated channels, the acid-sensing ion channels (ASICs)[60–71]. ASICs belong to the amiloride-sensitive epithelial Na^+-channel/degenerin (ENaC/Deg) superfamily[60, 72], which are formed with homomultimeric or heteromultimeric subunits. Each subunit contains two transmembrane spanning regions (TM1 and TM2) flanked by a large cysteine-rich extracellular loop and short intracellular N and C termini[60, 61, 73–78]. To date, four genes encoding seven ASIC subunits have been cloned and characterized. ASIC1a subunits (originally named ASIC or BNaC2) are widely expressed in peripheral sensory neurons and in CNS neurons[61, 65, 79, 80]. Pertinent to brain ischemia, these channels are activated by moderate decreases of pH_o with a threshold pH of ~7.0 and a pH for half maximal activation ($pH_{0.5}$) at ~6.2[61, 81]. In addition to Na^+, homomeric ASIC1a channels are permeable to Ca^{2+} ions[61, 82, 83]. ASIC1β and its longer form variant ASIC1b are expressed only in sensory neurons[84, 85]. Similar to ASIC1a, homomeric ASIC1β channels have high sensitivity to H^+ with a $pH_{0.5}$ at ~5.9[85]. Unlike ASIC1a, however, ASIC1b or ASIC1β has no detectable Ca^{2+} permeability[84, 85]. ASIC2a subunits (originally named MDEG, or BNaC1) have widespread distribution in both peripheral sensory and central neurons[63, 79, 86]. However, homomeric ASIC2a channels have very low sensitivity to H^+ with a $pH_{0.5}$ of 4.4[63, 86, 87]. It is unlikely that homomeric ASIC2a channels can be activated in any physiological or pathological conditions in the brain. Similarly, ASIC2b subunits (originally named MDEG2) are expressed in peripheral sensory and central neurons[87]. However, they do not form functional homomeric channels, but may associate with other ASIC subunits (*eg* ASIC3) to form heteromultimeric channels[87]. ASIC3 subunits (originally also named DRASIC) are predominantly expressed in neurons of dorsal root ganglia[88, 89], though its expression in the brain has been reported[90]. Homomeric ASIC3 channels respond to pH drops biphasically, with a fast desensitizing current followed by a sustained component[88, 89, 91]. ASIC4 subunits are highly expressed in the pituitary gland[92, 93]. Similar to ASIC2b, they do not seem to form functional homomeric channels[93]. ASICs were initially believed to be assembled as tetramers[61, 77]. However, recent analysis of crystal structure suggested that ASICs existed as trimers[94].

ASICs in peripheral sensory neurons are implicated in nociception, mechanosensation, and taste transduction[95–107]. The presence of ASICs in the brain, which lacks nociceptors, suggests that these channels have functions beyond nociception. Indeed, ASIC1a has been shown to be involved in synaptic plasticity, learning and memory[64, 108]. Both ASIC1a and ASIC2a are implicated in the maintenance of retinal integrity[109–111]. In pathological conditions, activation of Ca^{2+}-permeable ASIC1a is involved in glutamate-independent, acidosis mediated, ischemic brain injury[71, 82, 112], and in axon degeneration associated with multiple sclerosis[113]. In contrast, increased expression of ASIC2a is associated with neuronal survival following global ischemia[114], while reduced expression of ASIC1a is associated with neuroprotection elicited by ischemic pre- and post-conditioning[115].

The presence of ASIC1a in the brain, its activation by pH drops to the levels commonly seen during brain ischemia, and its permeability to Ca^{2+} make it a potential player in ischemic brain injury. A series of recent studies, performed *in vitro* in neuronal cell culture and *in vivo* in whole animal models of ischemia, have provided strong evidence supporting this hypothesis[71, 82, 112]. In cultured neurons, for example, brief acid incubations induced significant neuronal injury. This acid-induced neuronal injury was glutamate-independent, but was inhibited by amiloride, a non-specific ASIC blocker, or PcTX1, a specific ASIC1a inhibitor. In contrast to the neurons from $ASIC1^{+/+}$ mice, neurons cultured from $ASIC1^{-/-}$ mice were resistant to acid injury. Reducing the concentration of extracellular Ca^{2+}, which lowers the driving force for Ca^{2+} entry through ASICs, also decreased acid-induced injury of CNS neurons[71, 82]. Thus, activation of ASICs, and subsequent Ca^{2+} entry, participates in acidosis-mediated injury of neurons.

While homomeric ASIC1a can conduct Ca^{2+}, some studies have suggested that, a significant portion of acid-evoked increases of intracellular Ca^{2+} is not due to Ca^{2+} entry directly through ASIC1a homomers. Rather, acidosis might induce Ca^{2+} accumulation through secondary activation of voltage-gated Ca^{2+} channels due to ASIC-mediated membrane depolarization and/or Ca^{2+} release from intracellular stores[116–118].

In vivo studies also support a role for ASIC1a activation in acidosis-mediated, ischemic brain injury[71, 112, 119]. In rats and mice, intracerebral ventricular injection of ASIC1a inhibitors reduced the infarct volume by up to 60%. Similarly, ASIC1a gene knockout protected the mouse brain from ischemic injury. Furthermore, ASIC1a blockade and ASIC1 gene knockout provided additional protection in the presence of glutamate receptor antagonist[71]. The protection by ASIC1a blockade has an effective time window of >5 h, and the protection persists for at least 7 d[119]. Attenuating brain acidosis by intracerebroventricular administration of $NaHCO_3$ is also protective, further suggesting that acidosis is a mediator of ischemic brain injury.

Interactions between ASIC1a and hypoxia/ischemia related signals contribute to ischemic brain injury

In the normal condition, ASIC1a current desensitizes rapidly in the continuous presence of acidosis. This property of ASIC1a argues against its role in brain ischemia in which acidosis is, in general, long-lasting. Recent findings showing that the properties of ASICs, particularly the ASIC1a channels, can be dramatically modulated by ischemia per se and/

or ischemia-related signals have provided good explanation supporting the role of ASIC1a channels in ischemic brain injury[71, 112, 120–122]. In cultured mouse cortical neurons, for example, brief OGD not only increased the amplitude but also reduced the desensitization of the ASIC current. Accordingly, OGD treatment enhanced acidosis-mediated neuronal injury[71]. The cellular and molecular mechanisms underlying ischemia-induced increase of ASIC activity has been investigated extensively by several studies. Allen and Attwell demonstrated that arachidonic acid, a lipid metabolite released in ischemia, increased the amplitude of the ASIC current in rat cerebellar Purkinje neurons[122]. Gao and colleagues demonstrated that an increased phosphorylation of ASIC1a channels by CaMKII, mediated by NMDA receptor activation, was involved in ischemia-induced enhancement of the ASIC responses[112]. Sherwood and Askwith demonstrated that dynorphins, the most basic neuropeptides abundantly expressed in the central nervous system, could increase the activities of ASIC1a channels and enhance neuronal damage following ischemia[120]. They do so by reducing the steady-state desensitization of the ASIC1a channels. Very recent studies by Duan and colleagues also showed that, spermine, one of the endogenous polyamines, exacerbated ischemic neuronal injury through sensitization of ASIC1a channels to extracellular acidosis[121]. Spermine slows down the desensitization of these channels in the open state, shifting steady-state desensitization to more acidic pH, and accelerating recovery of the channels between repeated periods of acid stimulation. Thus, therapeutic interventions for brain ischemia may target ASICs directly by using ASIC blockers/inhibitors or indirectly by blocking the ischemia-related signals which enhance the activation of ASICs.

Perspectives

Stroke/brain ischemia is a leading health problem worldwide. Although in recent years enormous progresses have been made in the prevention of stroke, unfortunately, there is still no effective treatment for stroke patients. Searching for new cell injury mechanisms and effective therapeutic strategies is therefore a major challenge in the field. Brain ischemia initiates various biochemical changes such as increased glutamate release, production of oxygen free radicals, lactic acidosis, and reduced ATP synthesis, *etc*. These changes may facilitate the opening of various Ca^{2+}-permeable ion channels such as glutamate-receptor-gated channels, voltage-gated Ca^{2+} channels, TRPM7 channels, acid-sensing ion channels, *etc*. Activation of these channels induces entry of Ca^{2+} and accumulation of intracellular Ca^{2+}. Intracellular Ca^{2+} accumulation can also occur through other pathways, *eg* release of Ca^{2+} from intracellular stores, or entry of Ca^{2+} through reversed Na^+/Ca^{2+} exchange system (Figure 1). Overload of neurons with Ca^{2+} activates a panel of enzymes including proteases, phospholipases and endonucleases, leading to destruction of neurons either through necrotic or apoptotic process. Targeting the pathways responsible for Ca^{2+} overload may lead to effective neuroprotective interventions for stroke patients. The recent failure of clinical trials using the antagonists of glutamate receptors, however, suggests that future effort should also consider glutamate-independent Ca^{2+} toxicity in ischemia, *eg* through activation of TRPM7 channels or ASICs.

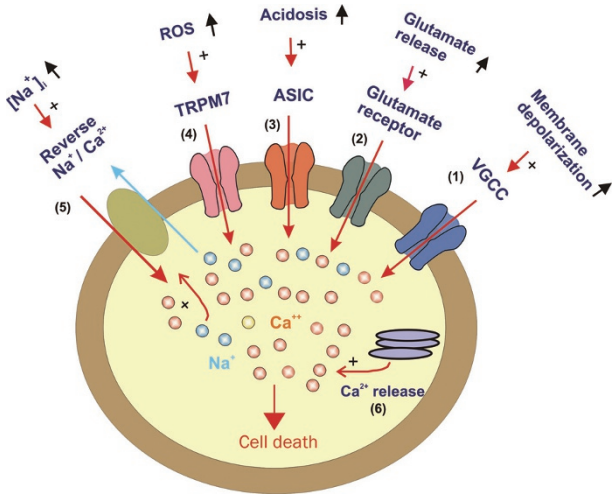

Figure 1. Potential pathways responsible for intracellular Ca^{2+} accumulation in neurons in ischemic condition. VGCC: voltage-gated Ca^{2+} channel; ASIC: acid-sensing ion channel; ROS: reactive oxygen species; TRPM7: transient receptor potential melastatin 7; Na^+/Ca^{2+}: sodium-calcium exchanger.

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