Neuroscience 154 (2008) 677–689

RELATIONSHIP BETWEEN NEURONAL VULNERABILITY AND POTASSIUM-CHLORIDE COTRANSPORTER 2 IMMUNOREACTIVITY IN HIPPOCAMPUS FOLLOWING TRANSIENT FOREBRAIN ISCHEMIA

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Abstract—Cation chloride cotransporters have been reported to be expressed in neurons in the hippocampus and to regulate intracellular Cl^- concentration. The neuron-specific K-Cl cotransporter 2 (KCC2) is necessary for maintaining the low intracellular chloride concentration required for the hyperpolarizing actions of GABA. In this study we examined the vulnerability of KCC2-containing neurons as well as the changes in the pattern of KCC2 distribution in the rat hippocampus following 15 min ischemia induced by four-vessel occlusion. Immunostaining for the 72 kDa heat shock protein (HSP-72) was used to investigate the extent of damage in neuronal populations previously shown to be vulnerable to ischemia. At 6–24 h after ischemia, when the pyramidal cells in the CA1 (subfield of cornu Ammonis) region showed no morphological signs of damage, a small rise of KCC2 immunoreactivity was already observed. After 2 days, when the CA1 pyramidal cells started to degenerate, a progressive downregulation of the KCC2 protein was visible. Interestingly, in the same areas, the parvalbumin containing interneurons showed no signs of ischemic damage, and KCC2 immunoreactivity was retained on their membrane surface. In CA1 pyramidal cells, the reduction in KCC2 expression may lead to an elevation of intracellular Cl^- concentration, which causes a shift in equilibrium potential toward more positive levels. Consequently, the reduction of the inhibitory action of GABA through downregulation of KCC2 function may be involved in the pathomechanisms of delayed neuronal death in the CA1 subfield.

Key words: transient forebrain ischemia, GABA, KCC2, hippocampus, heat-shock protein-72.

GABA is the main inhibitory transmitter in the adult brain, and it exerts its fast hyperpolarizing effect through activation of predominantly Cl^- and to a lesser extent $HCO3^-$ -permeant $GABA_A$ receptors (Kaila, 1994; Barnard et al., 1998; Farrant and Kaila, 2007). A key requirement for the generation of hyperpolarizing inhibitory post synaptic potentials is a postsynaptic Cl^- extrusion mechanism, which maintains the Cl^- equilibrium potential (E_{Cl}) at values more negative than the resting membrane potential (Thompson and Gahwiler, 1989). Cl^- homeostasis in brain cells is mainly controlled by cation-chloride cotransporters, which mediate electrically neutral Cl^- uptake driven by extracellular Na^+ (the Na-K-2Cl cotransporter isoform 1, NKCC1) or Cl^- extrusion fueled by intracellular K^+ (the K-Cl cotransporters, KCC1-4) (Hiki et al., 1999; Race et al., 1999; Delpire and Mount, 2002; Payne et al., 2003; Mercado et al., 2004). These secondary active transporters do not directly consume ATP, but they derive their energy from the Na^+ and K^+ gradients generated by the Na-K ATPase.

The type 2 potassium-chloride cotransporter (KCC2) is a major regulator of Cl^- in cortical neurons and normally transports K^+ and Cl^- out of neurons in a 1:1 ratio (Payne et al., 2003). Several lines of evidence show that in native cortical neurons, KCC2 is functionally active right after its expression, leading to hyperpolarizing inhibition (Rivera et al., 1999; Khirug et al., 2005). In agreement with its essential role for inhibitory synapses (Rivera et al., 1999), KCC2 knockout mice die perinatally (Hubner et al., 2001). In immature neurons, GABA has a depolarizing, excitatory effect (Cherubini et al., 1990; Kakazu et al., 1999) because of a high intracellular Cl^- concentration maintained by NKCC1 (Yamada et al., 2004; Sipila et al., 2006). With maturation, the effect of this transmitter switches to inhibitory, resulting from a decrease of intracellular Cl^- concentration attributable to the developmental expression of KCC2 (Rivera et al., 1999).

It has also been shown that, in addition to the upregulation of KCC2, NKCC1 is downregulated in CNS neurons during development (Plotkin et al., 1997).

Under some pathophysiological conditions (e.g. trauma (Katchman et al., 1994; van den Pol et al., 1996; Kohling et al., 1998; Cohen et al., 2002; Huberfeld et al., 2007), $GABA_A$ receptors can mediate a depolarizing Cl^- efflux. The elevation of intracellular Cl^- concentration and the resulting depolarizing action of $GABA_A$ receptors appear to be mainly caused by alterations in KCC2 expression (Nabekura et al., 2002; Rivera et al., 2004). In addition to the developmental regulation of the intracellular Cl^- concentration, extensive evidence from studies measuring the efficacy of Cl^- extrusion indicates that neuronal activity and the brain-derived neurotrophic factor (BDNF) regulate the functional expression of KCC2 (Rivera et al., 2002, 2004).

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Abbreviations: BDNF, brain-derived neurotrophic factor; CA1, CA3, subfields of cornu Ammonis according to Lorente de No; DAB, 3,3′-diaminobenzidine; KCC2, K-Cl cotransporter 2; NKCC1, Na-K-2Cl cotransporter 1; PB, phosphate buffer; TBS, Tris-buffered saline; 4VO, four-vessel occlusion.

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doi:10.1016/j.neuroscience.2008.03.072

Cerebral ischemia, produced by impaired blood flow to the brain, leads to neuronal death in vulnerable brain regions (Schmidt-Kastner and Freund, 1991). Neurons of the hippocampal subfields of cornu Ammonis (according to Lorente de No, 1934; CA1) region are among those that are particularly susceptible to ischemic insult. Ischemic episodes are characterized by rapid energy depletion and major disturbances in neuronal ionic homeostasis, including significant rises in intracellular Na^+, Ca^{2+} and Cl^-, and extracellular K^+ (Hansen, 1985). The decrease in ATP has many consequences that might activate cell death, the most dramatic is the breakdown of Na-K ATPase. In the hippocampus a rapid and gradual loss of neuronal Na-K ATPase, which is responsible for maintaining a high intracellular and a low extracellular K^+ concentration, occurs selectively in the CA1 neurons immediately after ischemia and reperfusion (Pylova et al., 1989). The increase in extracellular K^+ concentration has many consequences including a decrease in the net outward flux of Cl^-, or even a reversal of Cl^- transport by KCC2. This could lead to depolarizing GABA actions that may contribute to excitotoxic cell death of the delayed type in CA1.

However, generating an inwardly-directed Cl^- electrochemical gradient under normal conditions is not the only function of KCC2. This transporter appears to selectively accompany excitatory glutamatergic synapses, closely following their distribution in space and time (e.g. during development) (Gulyas et al., 2001). For example, KCC2 is expressed in most pyramidal cell spines (that rarely receive GABAergic inputs), it appears in the most distal dendrites at the same time when the perforant path input arrives, and the KCC2 expression of various interneuron types correlates with the density of their glutamate rather than GABAergic input (Gulyas et al., 2001). One of the roles of KCC2 in nearby excitatory synapses is likely to be volume control, since K^+ and Cl^- efflux will also take the osmotically obliged water out of the cells (Payne et al., 2003). Thus, a reversal of KCC2 transport upon the increase of extracellular K^+ is likely to enhance rather than prevent the swelling (and often irreversible edema) that takes place in several neurons during, or immediately after ischemia. Furthermore, KCC2 has been shown to have an important role in the formation of mature dendritic spines (Li et al., 2007).

To investigate whether the distribution or redistribution of KCC2—playing a role in the control of Cl^- gradients for GABAergic inhibition, as well as in volume control (Payne et al., 2003)—is causally related to the characteristic pattern of ischemic cell death, we studied the KCC2 immunoreactivity of various hippocampal cell types (vulnerable and resistant) at different time points following 15 min complete forebrain ischemia induced by four-vessel occlusion (4VO), at the light and electron microscopic levels.

EXPERIMENTAL PROCEDURES

Animals and surgical procedures

Experiments were carried out according to the guidelines of the institutional ethical code and the Hungarian Act of Animal Care and Experimentation (1998, XXVIII, Section 243/1998), which are in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (2302/003). All efforts were made to restrict the number of animals and to minimize their suffering. The 4VO experiments were carried out on 30 adult (200–250 g) (four to six per each survival time point) male Sprague–Dawley rats (Charles River Laboratories, Budapest, Hungary) (Pulsinelli and Brierley, 1979; Pulsinelli and Buchan, 1988). The animals were anesthetized with Equitesin (chlornembutal, 0.3 ml/kg, i.p.; CEVA-Phylaxia Rt. Budapest, Hungary), and the vertebral arteries were exposed by drilling the alar foramina bilaterally, then cauterized and split under direct visual control (Todd et al., 1986). One day later the animals were anesthetized again using ether, and both carotid arteries were prepared and clamped by small aneurism clips for 15 min, while the anesthesia was discontinued. During ischemia, skull temperature was maintained between 37 °C and 38 °C by a manually controlled heating lamp, and the completeness of forebrain ischemia was confirmed by testing the loss of righting and pupil reflexes (Pulsinelli and Brierley, 1979). After the termination of ischemia the animals were sutured and allowed to recover for survival periods ranging from 6 h to 2 weeks.

Perfusion and preparation of tissue sections

The animals were deeply anesthetized again using Equitesin, and perfused through the heart first with saline (2–3 min) followed by a fixative (30 min) containing 0.1% glutaraldehyde, 4% paraformaldehyde, and 0.2% picric acid in 0.1 M phosphate buffer (PB) (pH 7.4). Brains were removed from the skull. After slicing and extensive washing in 0.1 M PB, the 60 μm-thick sections were incubated in 30% sucrose overnight, followed by freeze thawing over liquid nitrogen four times.

Immunocytochemistry

The sections were processed for immunoperoxidase, immunofluorescent, or preembedding immunogold staining. Subsequently, all washing steps and dilutions of the antibodies were done in 0.05 M Tris-buffered saline (TBS), pH 7.4. After extensive washing in TBS, the sections were blocked in 10% normal goat serum for 45 min and then incubated in rabbit anti-KCC2 (1:6000–1:10,000) (Williams et al., 1999) or mouse anti-HSP-72 (1:600) or mouse anti-parvalbumin (1:6000) antibodies a minimum of 48 h at 4 °C. Following the primary antisera, the sections were treated with biotinylated anti-rabbit IgG (1:300) or with biotinylated anti-mouse IgG (1:300), both raised in goat, for 2 h and then with avidin biotinylated horseradish peroxidase complex (1:500; Elite ABC; Vector Laboratories) for 1.5 h. The immunoperoxidase reaction was developed using 3,3′-diaminobenzidine (DAB) as the chromogen. In the immunogold staining procedure, the sections were incubated in 0.8 nm gold-conjugated goat anti-rabbit antibody for KCC2 respectively (1:50 dilution; Aurion, Wageningen, The Netherlands), overnight at 4 °C. Then the sections were silver intensified using the silver enhancement system R-GENT SE-EM according to the kit protocol (Aurion). In the double-immunostaining experiments, the sections were first developed for immunogold and then for immunoperoxidase staining. Lack of cross-reactivity of the secondary antibodies in the sequential detection scheme was verified by omission of either primary antibody, which eliminated labeling by the irrelevant secondary antibody.

After development of the immunostaining, the sections were treated with 1% OsO_4 in 0.1 M PB for 20 min, dehydrated in an ascending series of ethanol and propylene oxide, and embedded in Durcupan (ACM; Fluka, Buchs, Switzerland). During dehydration, the sections were treated with 1% uranyl acetate in 70% ethanol for 20 min. From sections embedded in Durcupan, areas of interest were reembedded and resectioned for electron microscopy. Sections were collected on Formvar-coated single-slot grids, stained with lead citrate, and examined with a Hitachi (Yokohama, Japan) 7100 electron microscope.