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Research report

Ryanodine receptors contribute to the induction of ischemic tolerance

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ABSTRACT

Ischemic tolerance (IT) is induced by a variety of insults to the brain (*e.g.*, nonfatal ischemia, heat and hypoxia) and it provides a strong neuroprotective effect. Although the mechanisms are still not fully elucidated, Ca^{2+} is regarded as a key mediator of IT. Ryanodine receptors (RyRs) are located in the sarcoplasmic/endoplasmic reticulum membrane and are responsible for the release of Ca^{2+} from intracellular stores. In brain, neuronal RyRs are thought to play a role in various neuropathological conditions, including ischemia. The purpose of the present study was to investigate the involvement of RyRs in IT. Pretreatment with a RyR antagonist, dantrolene (25 mg/kg, i.p), blocked IT in a gerbil global ischemia model, while a RyR agonist, caffeine (100 mg/kg, i.p), stimulated the production of IT. *In vitro*, using rat hippocampal cells, short-term oxygen/glucose deprivation induced preconditioning and RyR antagonists, dantrolene (50 and 100 μM) and ryanodine (100 and 200 μM) prevented it. RyR protein and mRNA levels were transiently decreased after induction of IT. These results suggest that RyRs are involved in the induction of ischemic tolerance.

1. Introduction

Ischemic tolerance (IT) is transiently acquired after a variety of insults to the brain such as non-lethal hypoxia/ischemia (ischemic preconditioning, IPC), and provides strong neuroprotective effect against a subsequent lethal ischemic event (Gidday, 2006; Kirino, 2002). Although the mechanisms are not fully understood, factors affecting intra-ischemic glutamate/Ca^{2+} toxicity are considered as one key mediator of IPC (Gidday, 2006). Ischemia-induced glutamate release was reduced by IPC in global rat ischemia (Grabb et al., 2002; Liu et al., 2012) and in mouse cortical cell cultures with oxygen glucose deprivation (OGD) (Grabb et al., 2002). Ohta et al. (1996) reported that 2-min ischemia (non-lethal ischemia) enhanced activities of mitochondrial Ca^{2+} sequestration and plasma membrane Ca^{2+}-ATPase, resulting in reduced Ca^{2+} toxicity following 5-min ischemia in gerbils. Bickler et al. (2005) demonstrated that OGD-induced increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in CA1 neurons of rat hippocampal slices were reduced about 5-fold by IPC. While inhibition of Ca^{2+} toxicity is involved in IT, the induction of IT may demand a moderate increase in [Ca] (Tauskela and Morley, 2004). Thus, preconditioning by isoflurane in hypoxic hippocampal slices required inositol triphosphate receptors (IP_3Rs)-dependent increases in $[Ca^{2+}]_i$ (Bickler et al., 2009; Bickler and Fahlman, 2010; Gray et al., 2005). Tauskela et al. (2003) also pointed out cytosolic and mitochondrial Ca^{2+}-dependent cellular signaling is involved in the induction of IT.

Besides IP_3Rs, ryanodine receptors (RyRs) are expressed in endoplasmic reticulum, which are composed of three isoforms (RyR1, RyR2, and RyR3), and also regulate $[Ca^{2+}]_i$ in brain through Ca^{2+}-induced Ca^{2+} release (Furuichi et al., 1994). Although RyR1 and 2 are expressed at high levels in skeletal and cardiac muscle, respectively, they are also present in brain. In contrast, RyR3 is found most abundantly in brain, especially in the hippocampus, caudate putamen and dorsal thalamus (Furuichi et al., 1994). Although the function of RyRs in both brain physiology and pathophysiology are still obscure (Bouchard et al., 2003; Kushnir et al., 2010), an involvement of RyRs and IP_3R in ischemic neuronal cell death is suggested due to intra-ischemic disruption of $[Ca^{2+}]_i$ homeostasis (Inan and Wei, 2010; Verkhratsky and Toescu, 2003; Zhang et al., 1993).

The aim of the present study was to investigate the contribution of RyRs both during and after IPC in IT. Both *in vivo* and *in vitro* ischemia models were used, global ischemia in gerbils and OGD in rat hippocampal cell cultures. The contribution of RyRs to

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IPC was examined using RyR antagonists/agonists, and the association of both RyR protein and mRNA levels with tolerance was also investigated.

2. Materials and methods

2.1. Animal experiments for histological analysis

The animal studies were approved by the Animal Committee of the Kawasaki Medical School. Male Mongolian gerbils (wt. 60–70 g) were anesthetized with pentobarbital sodium solution (50 mg/kg, i.p). Both common carotid arteries (CCA) were exposed, and bilateral cerebral ischemia was produced by occluding these arteries with miniature aneurismal clips. During the operation, body temperature was controlled at 36.5–37.5 °C using a warming blanket. IPC was induced by a nonfatal occlusion of bilateral CCA. Dantrolene (RyR antagonist, 25 mg/kg as 10 mg/ml in dimethyl sulfoxide) or caffeine (RyR agonist, 100 mg/kg as 25 mg/ml in saline) was administered intraperitoneally 15 min before IPC induction. Animals were divided into 3 groups by the duration of IPC induction; 5 min ischemia 3 day after 1 min ischemia, 5 min ischemia 3 day after 2 min ischemia, and 5 min ischemia 3 day after sham operation (PC-1 min, PC-2 min, and Isch, respectively). Further 3 groups were added to PC groups; dantrolene or dimethyl sulfoxide (DMSO) was administered to the PC-2 min group (PC-dan and PC-DMSO, respectively), and caffeine was administered to the PC-1 min group (PC-caf). Sham operated (without CCA occlusion) group was also produced as a normal control (Sham). One week after the second ischemia, animals were perfused and fixed with 4% phosphate-buffered paraformaldehyde. Frozen sections (10 μm thickness) were made for hematoxylin-eosin (HE) staining and immunohistochemistry. Neuronal density in the hippocampal CA1 area in three HE sections (corresponding to around 1.7 mm posterior to the bregma) was counted for each animal. The mean neuronal density from the left and right CA1 was used as the outcome in each single animal.

2.2. Locomotor assay

To investigate neurological functions, locomotor activity of gerbils was measured by an animal-movement analysis system (Actimo-100 system, Shintechno Ltd., Fukuoka, Japan) as described previously (Kurokawa et al., 2011; Duszczyk et al., 2006). Animal locomotor activity of 5 groups (Sham, Isch, PC, PC-dan, PC-caf) were measured in every 1 h period for 24 h from arousal after 5 min ischemia as previously reported (Kurokawa et al., 2011).

2.3. Primary hippocampal neuron cultures

Primary hippocampal neurons were prepared from Sprague-Dawley rats embryos at 18 days of gestation (Shimizu Laboratory Supplies, Kyoto, Japan). The meningeal tissue, brain stem, cortex cerebri, and basal ganglia were removed from the cerebral hemispheres. Collected hippocampi were dissociated by Nerve-Cells Dispersion Solutions (Sumitomo Bakelite, Tokyo, Japan) and plated into 24 Well Cell Culture Microplates (Corning Inc., Corning, NY) coated with poly-D-lysine (PDL; 10 μg/ml; Sigma-Aldrich, St. Louis, MO) at 5×10^4 cells/cm^2 in plating medium (Dulbecco's Modified Eagle Medium Nutrient Mixture F12 [Life Technologies, Carlsbad, CA] containing 10% fetal bovine serum [Nichirei, Tokyo, Japan], 1% penicillin/streptomycin [Life Technologies], and penicillin/streptomycin 50 U/ml [Life Technologies]). Plating medium was replaced after 4 h with neuronal culture medium consisting of Neurobasal medium (Life Technologies) containing 2% B27 (Life Technologies), penicillin/streptomycin 50 U/ml (Life Technologies), and 2 mM L-alanyl-L-glutamine (GlutaMAX-I; Life Technologies). Half of the medium was replaced weekly, and experiments were begun using cells *in vitro* days (DIV) 19–21.

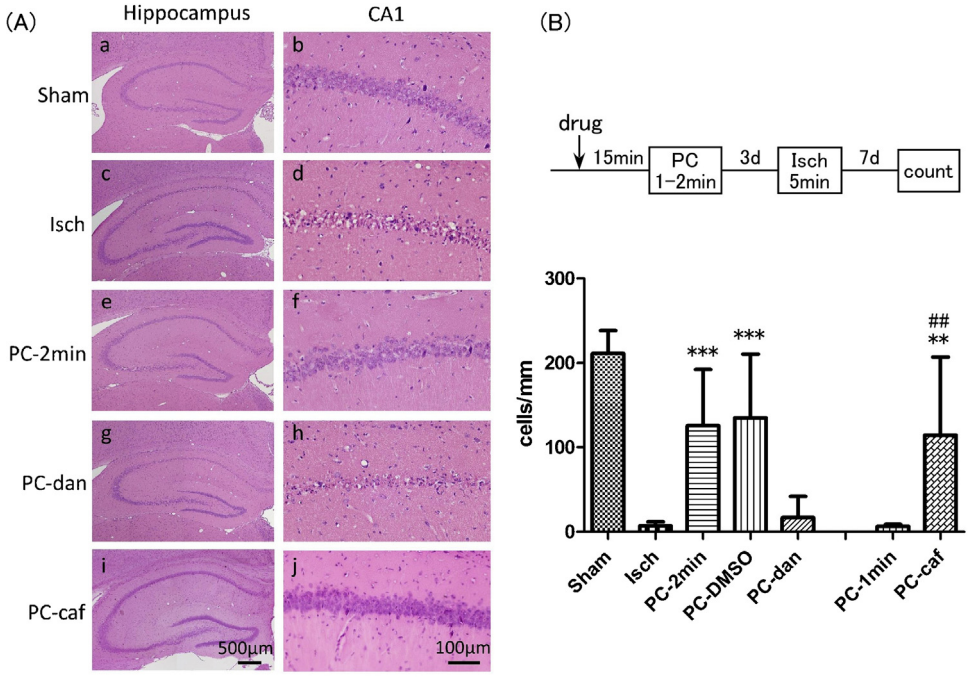

Fig. 1. The effect of bilateral common artery occlusion on CA1 pyramidal cells in the hippocampus. Gerbils underwent a sham operation or 5 min of occlusion (Isch) with or without different preconditioning (PC) stimuli. (A) Representative hematoxylin-eosin stained sections from the hippocampus with different treatments. (B) Experimental format and neuronal density in the CA-1 region. Almost all CA1 neurons died by after exposure to 5-min ischemia (Ac, d; B) and prior exposure to 2 min of ischemia (PC) significantly protected those neurons (Ae, f; B). Administration of dantrolene (dan), inhibited the protective effect of the 2 min of ischemia (Ag, h; B). Although 1 min of ischemia was insufficient to induce PC, 1 min ischemia combined with caffeine (caf) showed neuroprotection comparable to the 2 min ischemia PC (Ai, j; B). ** and *** indicate $P < 0.01$ and $P < 0.001$ *vs.* Isch; ## indicates $p < 0.01$ *vs.* PC-1 min.