学 位 論 文

Ryanodine receptors contribute to the induction of ischemic tolerance.

香川大学大学院医学系研究科

機能構築医学専攻

丸山恵美

Brain Research Bulletin 122 (2016) 45–53

Contents lists available at ScienceDirect

Brain Research Bulletin

journal homepage: www.elsevier.com/locate/brainresbull

Research report

Ryanodine receptors contribute to the induction of ischemic tolerance

Emi Nakamura-Maruyama[a,b], Osamu Miyamoto[a], Naohiko Okabe[a], Naoyuki Himi[a], Lu Feng[a], Kazuhiko Narita[a], Richard F. Keep[c], Tohru Yamamoto[b], Takehiro Nakamura[b,*]

[a] Department of Physiology2, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 701-0192, Japan
[b] Department of Molecular Neurobiology, Kagawa University Faculty of Medicine, 1750-1 Ikenobe, Miki, Kita, Kagawa 761-0793, Japan
[c] Department of Neurosurgery, University of Michigan, 109 Zina Pitcher Place, Ann Arbor, MI 48109-2200, USA

ARTICLE INFO

Article history:
Received 24 August 2015
Received in revised form
14 December 2015
Accepted 24 February 2016
Available online 27 February 2016

Keywords:
Ischemic tolerance
Ryanodine receptor
Intracellular Ca^{2+}
CA1 hippocampal cells
Cell culture
Oxygen glucose deprivation

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

* Corresponding author at: Department of Neurobiology, Kagawa University Faculty of Medicine, 1750-1 Ikenobe, Miki, Kita, Kagawa 761-0793, Japan.
E-mail address: tanakamu@kms.ac.jp (T. Nakamura).

http://dx.doi.org/10.1016/j.brainresbull.2016.02.018
0361-9230/

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.

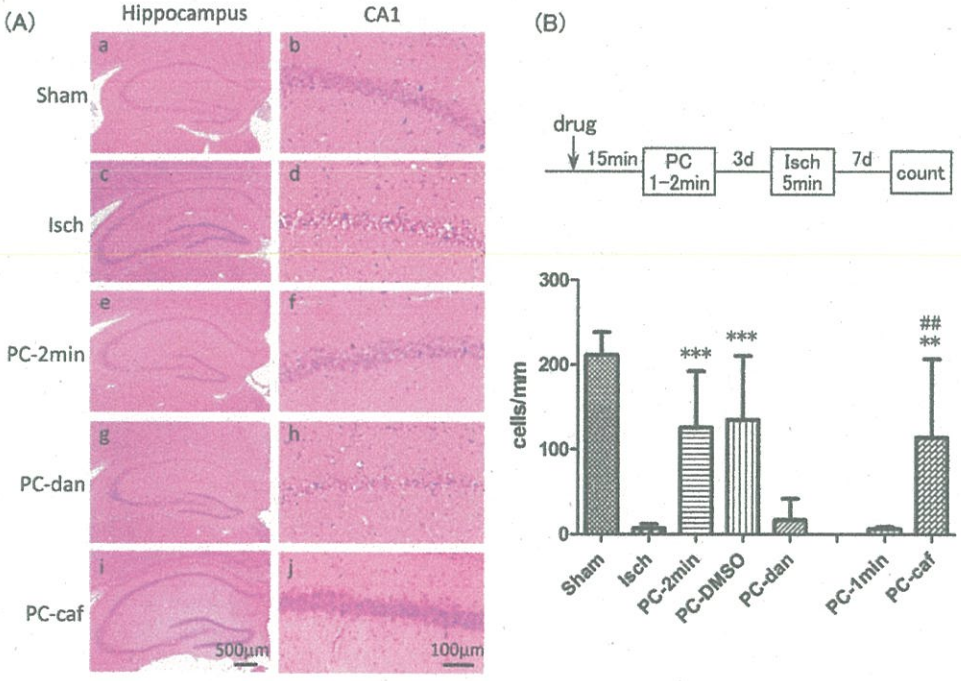

Fig. 1. The effect of bilateral common artery occlusion on CA1 pyramidal cells in the hippocampus. Gerbils underwent a sham operation or 5 min of occlusion (Isch) with or without different preconditioning (PC) stimuli. (A) Representative hematoxylin-eosin stained sections from the hippocampus with different 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.

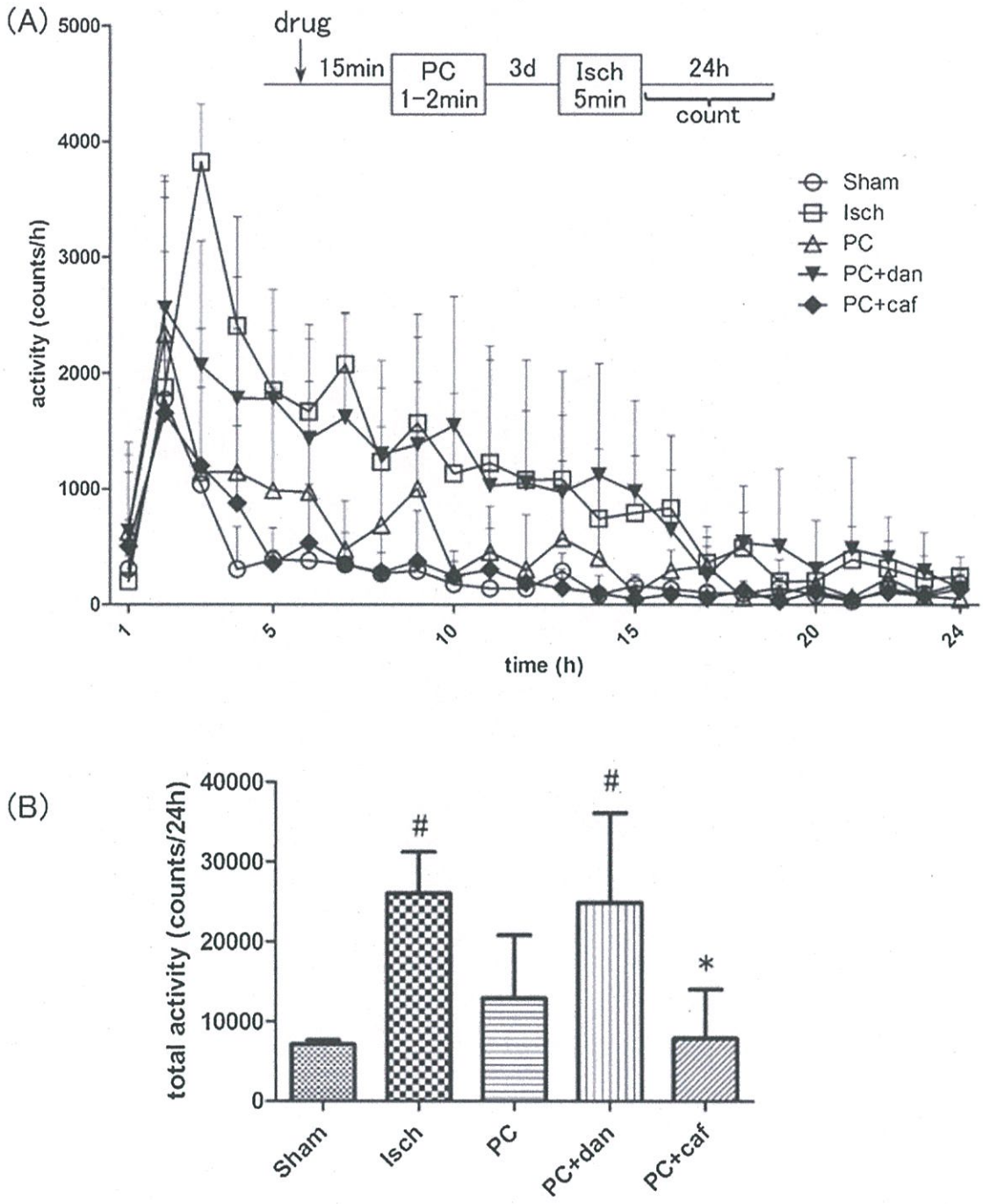

Fig. 2. The effect of bilateral common artery occlusion on locomotor activity of gerbils. (A) Time-dependent changes in measurement of locomotor activity with different treatments. (B) Total activity for 24 h. Under ischemic conditions (Isch), gerbils were hyperactivated compared with sham group, and pretreatment with 2 min of ischemia (PC) decreased hyperactivation. Administration of dantrolene (PC-dan) reduced the effect of 2 min of ischemia. 1 min ischemia combined with caffeine (PC-caf) significantly showed the decrease compared with the 5 min ischemia. *indicate $P < 0.05$ *vs.* Isch; # indicates $p < 0.05$ *vs.* Sham.

2.4. Immunocytochemistry

Cultured cells, plated on coverslip coated with PDL at 5×10^3 cells/cm^2, were fixed in 4% paraformaldehyde in phosphate buffer. For RyR straining, cells were incubated for 1 h with blocking buffer at room temperature, following overnight incubation at 4 °C with the anti-RyR1 (1:1000, Millipore), RyR2 (1:1000, Millipore), RyR3 (1:1000, Millipore), synaptophysin (1:2000, Millipore), or Tuj-1 (1:2000, Sigma-Aldrich) antibodies. Fluorescence images were acquired from each section using epifluorescence microscopy (BX61, Olympus, Tokyo, Japan).

2.5. Oxygen glucose deprivation

To reproduce the *in vivo* cerebral ischemia experiment, neuronal cultures were subjected to oxygen-glucose deprivation (OGD) as described previously (Grabb and Choi, 1999; Okabe et al., 2013). Briefly, cultures were washed twice with balanced salt solution lacking glucose (BSS: 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 1.8 mM $CaCl_2$, and 0.01 mM glycine) that was aerated with an anaerobic gas mix (90% N_2 and 10% CO_2) to remove residual oxygen. Culture media was replaced with BSS, and transferred to an anaerobic chamber (APM; ASTEC,

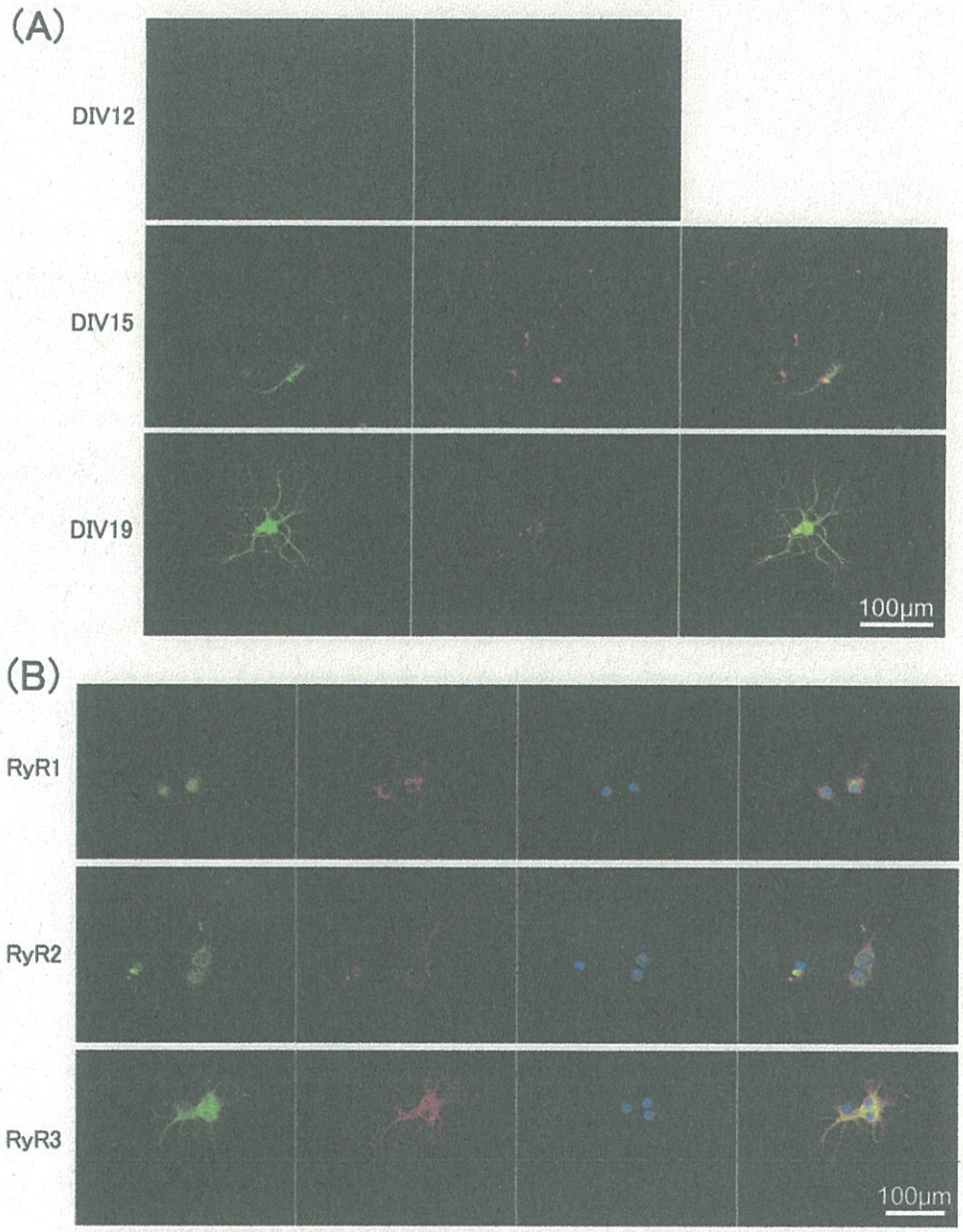

Fig. 3. Immunohistochemistry of rat hippocampal cells in culture. (A) The expression of RyR3 (green) and synaptophysin (red) increased with days *in vitro* (DIV), with marked RyR expression in cell cytoplasm and dendrites by DIV19. (B). All three subtypes of RyR were expressed in cultured neurons. RyR: green; Tuji-1: red; DAPI: blue.

Kasuya, Japan) containing a gas mixture of 95% N_2 and 5% CO_2. For preconditioning (PC), culture cells were incubated at 37 °C for 10 min (1st OGD). The cultures were incubated for 24 h with normal media, and then incubated with OGD for 2 h (2nd OGD). Control cell cultures were incubated with BSS containing 20 mM glucose in a normal chamber (5% CO_2 and atmospheric O_2) (Normal group). After incubation for 2 h, BSS was replaced with the normal media and further incubated for 24 h until neuronal injury assessment. As the inhibitors of RyR, dantrolene sodium hydorate (10–100 μM, Astellas Pharma, Tokyo, Japan) and ryanodine (50–200 μM, Abcam, Cambridge, UK) were used. The reagents were added the medium before 1st OGD and removed just before 2nd OGD.

Twenty-four hours after the 2nd OGD, neuronal injury was evaluated by measuring lactate dehydrogenase (LDH) in the media using a LDH Cytotoxicity Detection Kit (Takara Bio, Ootsu, Japan). Data were normalized to the LDH release from cells with no PC group (100%) as described previously (Singer et al., 1999). The percentage of live cells was also computed using ImageXpress^{MICRO} (Molecular Devices, Sunnyvale, CA) with Hoechst 33342 and propidium iodide (PI) dyes.

2.6. Time-course experiment

The time course of PC effect was examined using LDH release from cultured cells. After the hippocampal cells were cultured with 10 min OGD (1st OGD, PC), they were returned to the normal culture medium. Two hours OGD (2nd OGD) was performed at each time point (6 h, 1 day, 7 day, 14 day) after 1st OGD. Twenty-four hours following the 2nd OGD, neuronal injury was evaluated by medium LDH.

2.7. Western blot and Real-Time RT-PCR analyses

Changes in the protein expression of each RyR subtype were measured by Western blot analysis as described previously (Kurokawa et al., 2010). Briefly, at 1 day after the 1st OGD, cul-

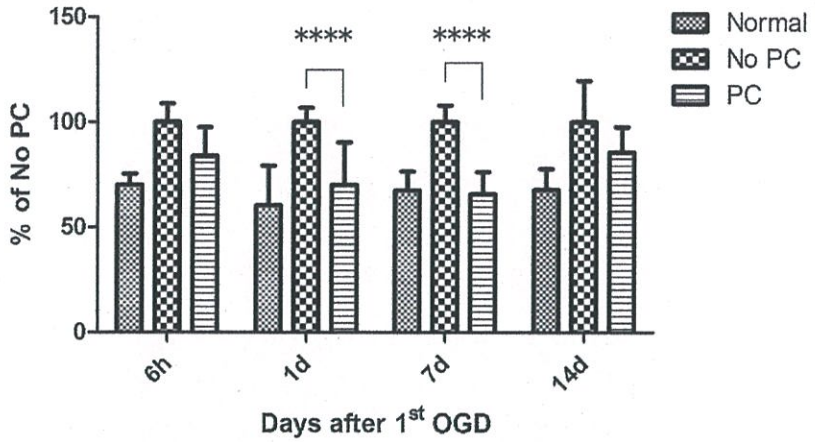

Fig. 4. Time dependency of preconditioning (PC)-induced neuroprotection in rat hippocampal cells in culture. Cells underwent no oxygen glucose deprivation (OGD; normal), 2 h OGD without (No PC) or with (PC) prior exposure to 10 min of OGD. Cell injury was assessed by LDH release into the media (expressed as a percentage of that in the no PC group). The duration between the PC stimulus and the injurious 2 h OGD was varied from 6 h to 14 day. PC induced significant protection with an interval of 1 and 7 day. **** $p < 0.0001$ PC *vs.* no PC (Two-way ANOVA).

tured cells were rinsed twice with 0.01 M PBS containing a protease inhibitor cocktail (Roche, Basel, Schweiz) and then collected by cell scraper. The collected cells were homogenized in ice-cold lysis buffer (10 mM Tris-HCl pH7.5, 150 mM NaCl, 0.5 mM EDTA, 10 mM NaF, and 0.5% Triton X-100) including protease inhibitors. The homogenate was centrifuged at 1000 g for 10 min at 4 °C and then supernatants were centrifuged at 100,000*g* for 60 min at 4 °C. The precipitate was re-suspended in lysis buffer.

Protein samples were mixed with NuPAGE LDS Sample Buffer and NuPAGE Sample Reducing Agent (Life Technologies) and incubated for 10 min at 70 °C to denature proteins. The denatured protein sample (40 μg/lane) was loaded and separated by 3–8% Tris-acetate gel electrophoresis and transferred electrophoretically to nitrocellulose membranes. Membranes were blocked by 0.5% skim milk dissolved in Tris-buffered saline plus 0.1% Tween-20 (TBST) and then incubated overnight at 4 °C with the primary antibody against anti-RyR1 (1:1000, Millipore), RyR2 (1:1000, Sigma- Aldrich), RyR3 (1:1000, Millipore), in blocking buffer with gentle shaking. The membranes were washed with TBST and incubated for 2 h at room temperature with horseradish peroxidase conjugated secondary antibodies (1:5000) in blocking buffer. Membranes were again washed with TBST, followed by incubation with enhanced chemiluminescence substrate (Western Lightning ECL Pro, PerkinElmer, Waltham, MA). Chemiluminescence was detected using a charge-coupled device camera image system (LAS4000 mini; GE Healthcare Japan, Tokyo, Japan). Protein expression was quantified by Image J and normalized to β-actin expression.

Changes in RyR subtype mRNA expression were measured by Real-Time RT-PCR analysis. Cultured cells were collected using RNAprotect® Cell Reagent (Qiagen, Vento, NL) at 1 day after the 1st OGD. Total RNA was isolated using RNeasy® Plus Mini Kit (Qiagen) and converted to cDNA by PrimeScript® RT reagent Kit (Takara Bio). The quantity of mRNA was determined on a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Asheville, NC). The amplification procedure was consisted of incubation at 95 °C for 10 s to activate the polymerase, and then 45 cycles of PCR (95 °C for 5 s, and 60 °C for 30 s). Primer sequences for RyR1 and RyR3 were designed using Primer3 and obtained from Life Technologies (Japan). The primer sequences were; RyR1 (Forward: TTCTGGGGCATCTTTGATTC, Reverse: CCTGTGGAGACCCTCAATGT), RyR3 (Forward: ACATTGCCTTCCCAAAGATG, Reverse: CCTACTTGGCAGGTTGTGGT). The primers for RyR2 and GAPDH were obtained from TAKARA Bio. The detection of the RyRs and GAPDH domain of the cDNA by SYBR® *Premix Ex Taq* II (Takara Bio) was carried out using Quantitative PCR System (Mx3000P; Stratagene, Santa Clara, CA). All data were normalized to GAPDH expression.

2.8. Statistical analyses

All data were reported as mean ± SD. Multiple comparisons were performed One-Way ANOVA with post hoc Tukey's test (Figs. 1, 2B and 5) or Two-Way ANOVA with Bonferroni post-test (Figs. 4, 6 and 7). A statistically significant difference was defined as a P value less than 0.05. All statistical analyses were performed using GraphPad Prism 5 (GraphPad, La Jolla, CA).

3. Results

3.1. In vivo *experiments*

Fig. 1 shows the effects preconditioning (PC) on neuronal injury after bilateral common carotid artery (CCA) occlusion in the gerbil. The pyramidal cells of the hippocampal CA1 area formed a thick line in the Sham group (Fig. 1A a, b). Five minutes of occlusion resulted in almost complete death of the pyramidal cells (Isch, Fig. 1A c, d). In contrast, almost 60% of CA1 neurons survived if 2 min ischemia (PC induction) was given ahead of the lethal ischemia (PC-2 min, Fig. 1A e, f). However, administration of dantrolene, a RyR antagonist, before PC abolished the protective effect of PC (PC-dan, Fig. 1A g, h).

Unlike 2 min of ischemia, 1 min of prior ischemia (PC-1 min) was insufficient to induce neuroprotection. However, combining that length of ischemia with a RyR agonist, caffeine, induced neuroprotection (PC-caf, Fig. 1A i, j, B). Fig. 1B quantifies the neuronal density in the CA1 region in each group.

Results of locomotor assay were showed indicated in Fig. 2. Gerbils with 5 min ischemia (Isch) were significantly hyperactivated. The effect of 5 min ischemia on locomotor activity of gerbils were hyperactivated compared with Sham group ($P < 0.05$). The preconditioning with 2 min ischemia (PC) decreased hyperactivation induced by 5 min ischemia. However, this effect was abolished by the pretreatment of dantrolene (PC-dan), on the other hand, the locomotor activity in the pretreatment of caffeine group (PC-caf) was attenuated compared with Isch group ($P < 0.05$).

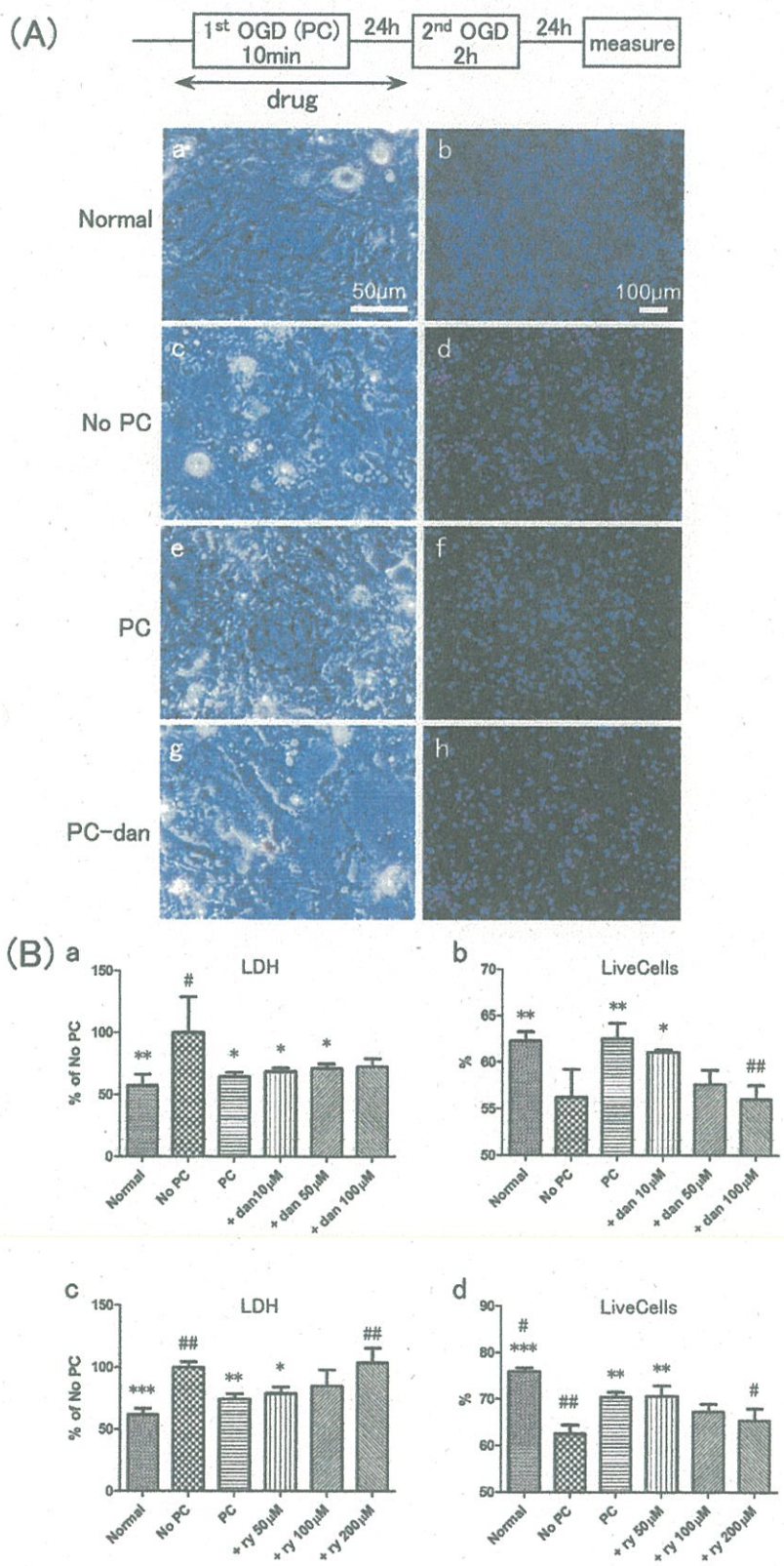

Fig. 5. Effects of RyR inhibition on preconditioning (PC)-induced neuroprotection in rat hippocampal cells (DIV21) in culture. Cells underwent no oxygen glucose deprivation (OGD; normal), 2 h OGD without (No PC) or with (PC) prior exposure to 10 min of OGD. PC was performed in the presence or absence of the RyR inhibitors dantrolene (dan) and ryanodine (ry) at different concentrations. (A) The effects of PC were examined 24 h after the 2 h of OGD by phase microscopy (a, c, e, g) or the stains Hoechst 33342 (b, d, f, h; blue: all cells) and propdium iodide (b, d, f, h; red: dead cells). (B) Quantification of the effects of PC with and without RyR inhibition on neuroprotection. Cell injury was assessed by media LDH (a, c), normalized to the release in the No PC group, and the % of live cells in the cultures (b, d). The neuroprotective effects of PC were dose-dependently attenuated by administration of dantrolene and ryanodine. *, ** and *** $p<0.05$, $p<0.01$ and $p<0.001$ *vs.* No PC group; # and ## $p<0.05$ and $p<0.01$ *vs.* PC group.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. In vitro *experiments*

By immunocytochemistry, RyR started to be expressed from DIV 15 and were fully observed at DIV 19 (Fig. 3A). Each subtype of RyR was expressed in Tuj-1 positive cells (Fig. 3B).

The release of LDH from culture cells was increased about 1.5-fold by 2 h OGD (2nd OGD) compared to control cells (Fig. 4). Preconditioning with 10 min OGD (1st OGD) attenuated that LDH release. With an interval of 1 day and 7 day between the 1st and 2nd OGD, the PC group had significantly alleviated cell damage compared to the No PC group (Fig. 4).

Based on the results above, further experiments examining the effects of RyR inhibition on PC used hippocampal neurons at DIV19 with an interval in OGD of 1 day (Fig. 5). In those experiments, PC with 10 min of OGD again protected from the cell death induced by the 2nd OGD as assessed by LDH release and LiveCell assay (Fig. 5A a-f, Fig. 5B). Two inhibitors of RyRs, dantrolene or ryanodine, dose-dependently blocked the protective effects of PC as assessed by LDH release (Fig. 5B a, c) and Live Cell assay (Fig. 5A g, h, Fig. 5B b, d).

3.3. *Expression of RyRs protein and mRNA*

After the 1st OGD, neuronal cultures were collected at time intervals and RyR protein and mRNA levels measured by Western blot (Fig. 6) and real time RT-PCR (Fig. 7), respectively. Protein and mRNA levels were normalized to β-actin and GAPDH, respectively, and compared to control (non-OGD) treated cells (100%) at each time point. At the protein level, RyR3 was significantly decreased by 57% at 1 day after 1st OGD ($p < 0.01$; Fig. 6), but largely recovered by 7 day and 14 day. For RyR1 and RyR2, there was no significant effect of the 1st OGD at any time point, although there was a tendency to decrease at day 1 (23 and 25% reduction, respectively, Fig. 6). Again, by 14 day, protein levels were similar to the non-OGD group.

Similarly, RyR mRNA expression tended to decrease at 1 day after 1st OGD, but only the change in RyR2 reached significance ($p < 0.05$; Fig. 7). By day 7, mRNA levels for each RyR were very similar the non-OGD group.

4. Discussion

In this experiment, acquisition of ischemic tolerance (IT) was inhibited when Ca^{2+} release from RyRs was blocked by dantrolene both *in vivo* and *in vitro*, and the acquisition of IT was stimulated by caffeine *in vivo*. Moreover, IT acquisition was inhibited by dantrolene or ryanodine dose-dependently.

In the present study, cerebral ischemia increased locomotor activity and its influence was disappeared by preconditioning as previous report (Duszczyk et al., 2006). Hyperactivity in the PC-dan group with same level of Isch group indicates the abolishment of the effect of preconditioning. Then, locomotor activity of PC-caf group was showed the same level of both PC and Sham group. Thus, we surmised that the effect of PC was attenuated by dantrolene, and potentiated by caffeine. These supplement the results of the cell density in the hippocampal CA1 (Fig. 1).

There are several reports that Ca^{2+} release in non-lethal ischemia or OGD is involved in the acquisition of IT (Ohta et al., 1996; Kirino, 2002; Bickler et al., 2005; Tauskela et al., 2003). Taken together with previous studies, the present results suggest that calcium release through RyRs in endoplasmic reticulum is an important factor for inducing IT.

The current study examined the effects of two RyR inhibitors, dantrolene and ryanodine, and one RyR agonist, caffeine, on ischemic PC. Although the inhibitors and the agonist had opposite effects on PC, as expected from their opposite actions on RyRs, caffeine has effects other than through RyR. Notably, it is an adenosine receptor antagonist. Caffeine was chosen for this study because of its high blood-brain barrier permeability so it can access neurons. In the systemic vasculature, caffeine has been reported to block ischemic preconditioning (Riksen et al., 2006) and this may be related to the importance of adenosine receptors in the endothelial effects of ischemic PC (Rubino and Yellon, 2000). More experiments are needed to evaluate the relative effects of RyRs and adenosine receptors in neuronal protection by ischemic PC.

While calcium release through RyRs appears important for inducing PC, there was evidence that RyR protein and mRNA levels were decreased compared with the normal group one day after PC-inducing OGD (Figs. 6 and 7). By 14 day, proteins tended to return to normal level (Figs. 6 and 7). Because the changes of protein level resemble these of neuronal injury (Figs. 4 and 6), Ca^{2+} release from endoplasmic reticulum during ischemic insult may be reduced by the decrease of RyRs and resulted in IT.

Bardo et al. (2006) found that RyRs are expressed throughout the cell, including axon, dendrites and dendritic spines in the hippocampus. In contrast, IP_3Rs are also expressed throughout the cell but not in dendritic spines. Dahl et al. (2000) reported that [^{3}H]IP_3 binding was reduced in the hippocampus by tolerance-inducing ischemic insults, although both IP_3R mRNA and protein levels did not change. They suggested that a decrease in functional IP_3Rs protected neurons by a reduction in excessive Ca^{2+} release from endoplasmic reticulum. Different from IP_3R, our results suggest that RyRs are themselves decreased dynamically by PC. This may participate in neuroprotection in severe ischemia by suppressing Ca^{2+}-induced Ca^{2+} release. Thus, RyRs might be contributed to maintain IT in a manner different from IP_3R.

The distribution of RyR subtypes within the rat hippocampus differs (Hertle and Yeckel, 2007; Sharp et al., 1993). For example, in the hippocampal CA1 area, RyR1 was detected strongly, but not RyR2 and RyR3, whereas in dentate gyrus region, RyR2 was detected strongly, and RyR1 and RyR3 were expressed weakly (Hertle and Yeckel, 2007). With respect to the distribution of RyRs in hippocampal cells, RyR1 was observed in the somata of pyramidal cells, RyR2 was primarily detected in mossy fiber axons of dentate gyrus granule neurons, and RyR3 was detected in the entire CA1 subfield and pyramidal cells within the stratum pyramidale of the CA3 subfield (Hertle and Yeckel, 2007). From these reports, each subtype of RyR functions differently in the brain, and they may differ in their involvement in the acquisition and maintenance of IT. The participation of each RyR subtype in IT should be investigated in future studies.

In conclusion, these results suggest that RyRs have an important role in acquiring IT. A decrease in RyRs might play the role in the preservation of IT.

Acknowledgment

This work was partially supported by Research Project Grant (21-607) from Kawasaki Medical School.

References

Bardo, S.B., et al., 2006. The role of the endoplasmic reticulum Ca^{2+} store in the plasticity of central neurons. Trends Pharmacol. Sci. 27, 78–84.
Bickler, P.E., Fahlman, C.S., 2010. Enhanced hypoxic preconditioning by isoflurane: signaling gene expression and requirement of intracellular Ca^{2+} and inositol triphosphate receptors. Brain Res. 1340, 86–95.
Bickler, P.E., Zhan, X., Fahlman, C.S., 2005. Isoflurane preconditions hippocampal neurons against oxygen-glucose deprivation: role of intracellular Ca^{2+} and mitogen-activated protein kinase signaling. Anesthesiology 103, 532–539.
Bickler, P.E., et al., 2009. Inositol 1,4,5-triphosphate receptors and NAD(P)H mediate Ca^{2+} signaling required for hypoxic preconditioning of hippocampal neurons. Neuroscience 160, 51–60.
Bouchard, R., Pattarini, R., Geiger, J.D., 2003. Presence and functional significance of presynaptic ryanodine receptors. Prog. Neurobiol. 69, 391–418.

3.2. In vitro *experiments*

By immunocytochemistry, RyR started to be expressed from DIV 15 and were fully observed at DIV 19 (Fig. 3A). Each subtype of RyR was expressed in Tuj-1 positive cells (Fig. 3B).

The release of LDH from culture cells was increased about 1.5-fold by 2 h OGD (2nd OGD) compared to control cells (Fig. 4). Preconditioning with 10 min OGD (1st OGD) attenuated that LDH release. With an interval of 1 day and 7 day between the 1st and 2nd OGD, the PC group had significantly alleviated cell damage compared to the No PC group (Fig. 4).

Based on the results above, further experiments examining the effects of RyR inhibition on PC used hippocampal neurons at DIV19 with an interval in OGD of 1 day (Fig. 5). In those experiments, PC with 10 min of OGD again protected from the cell death induced by the 2nd OGD as assessed by LDH release and LiveCell assay (Fig. 5A a-f, Fig. 5B). Two inhibitors of RyRs, dantrolene or ryanodine, dose-dependently blocked the protective effects of PC as assessed by LDH release (Fig. 5B a, c) and Live Cell assay (Fig. 5A g, h, Fig. 5B b, d).

3.3. *Expression of RyRs protein and mRNA*

After the 1st OGD, neuronal cultures were collected at time intervals and RyR protein and mRNA levels measured by Western blot (Fig. 6) and real time RT-PCR (Fig. 7), respectively. Protein and mRNA levels were normalized to β-actin and GAPDH, respectively, and compared to control (non-OGD) treated cells (100%) at each time point. At the protein level, RyR3 was significantly decreased by 57% at 1 day after 1st OGD ($p < 0.01$; Fig. 6), but largely recovered by 7 day and 14 day. For RyR1 and RyR2, there was no significant effect of the 1st OGD at any time point, although there was a tendency to decrease at day 1 (23 and 25% reduction, respectively, Fig. 6). Again, by 14 day, protein levels were similar to the non-OGD group.

Similarly, RyR mRNA expression tended to decrease at 1 day after 1st OGD, but only the change in RyR2 reached significance ($p < 0.05$; Fig. 7). By day 7, mRNA levels for each RyR were very similar the non-OGD group.

4. Discussion

In this experiment, acquisition of ischemic tolerance (IT) was inhibited when Ca^{2+} release from RyRs was blocked by dantrolene both *in vivo* and *in vitro*, and the acquisition of IT was stimulated by caffeine *in vivo*. Moreover, IT acquisition was inhibited by dantrolene or ryanodine dose-dependently.

In the present study, cerebral ischemia increased locomotor activity and its influence was disappeared by preconditioning as previous report (Duszczyk et al., 2006). Hyperactivity in the PC-dan group with same level of Isch group indicates the abolishment of the effect of preconditioning. Then, locomotor activity of PC-caf group was showed the same level of both PC and Sham group. Thus, we surmised that the effect of PC was attenuated by dantrolene, and potentiated by caffeine. These supplement the results of the cell density in the hippocampal CA1 (Fig. 1).

There are several reports that Ca^{2+} release in non-lethal ischemia or OGD is involved in the acquisition of IT (Ohta et al., 1996; Kirino, 2002; Bickler et al., 2005; Tauskela et al., 2003). Taken together with previous studies, the present results suggest that calcium release through RyRs in endoplasmic reticulum is an important factor for inducing IT.

The current study examined the effects of two RyR inhibitors, dantrolene and ryanodine, and one RyR agonist, caffeine, on ischemic PC. Although the inhibitors and the agonist had opposite effects on PC, as expected from their opposite actions on RyRs, caffeine has effects other than through RyR. Notably, it is an adenosine receptor antagonist. Caffeine was chosen for this study because of its high blood-brain barrier permeability so it can access neurons. In the systemic vasculature, caffeine has been reported to block ischemic preconditioning (Riksen et al., 2006) and this may be related to the importance of adenosine receptors in the endothelial effects of ischemic PC (Rubino and Yellon, 2000). More experiments are needed to evaluate the relative effects of RyRs and adenosine receptors in neuronal protection by ischemic PC.

While calcium release through RyRs appears important for inducing PC, there was evidence that RyR protein and mRNA levels were decreased compared with the normal group one day after PC-inducing OGD (Figs. 6 and 7). By 14 day, proteins tended to return to normal level (Figs. 6 and 7). Because the changes of protein level resemble these of neuronal injury (Figs. 4 and 6), Ca^{2+} release from endoplasmic reticulum during ischemic insult may be reduced by the decrease of RyRs and resulted in IT.

Bardo et al. (2006) found that RyRs are expressed throughout the cell, including axon, dendrites and dendritic spines in the hippocampus. In contrast, IP_3Rs are also expressed throughout the cell but not in dendritic spines. Dahl et al. (2000) reported that [^{3}H]IP_3 binding was reduced in the hippocampus by tolerance-inducing ischemic insults, although both IP_3R mRNA and protein levels did not change. They suggested that a decrease in functional IP_3Rs protected neurons by a reduction in excessive Ca^{2+} release from endoplasmic reticulum. Different from IP_3R, our results suggest that RyRs are themselves decreased dynamically by PC. This may participate in neuroprotection in severe ischemia by suppressing Ca^{2+}-induced Ca^{2+} release. Thus, RyRs might be contributed to maintain IT in a manner different from IP_3R.

The distribution of RyR subtypes within the rat hippocampus differs (Hertle and Yeckel, 2007; Sharp et al., 1993). For example, in the hippocampal CA1 area, RyR1 was detected strongly, but not RyR2 and RyR3, whereas in dentate gyrus region, RyR2 was detected strongly, and RyR1 and RyR3 were expressed weakly (Hertle and Yeckel, 2007). With respect to the distribution of RyRs in hippocampal cells, RyR1 was observed in the somata of pyramidal cells, RyR2 was primarily detected in mossy fiber axons of dentate gyrus granule neurons, and RyR3 was detected in the entire CA1 subfield and pyramidal cells within the stratum pyramidale of the CA3 subfield (Hertle and Yeckel, 2007). From these reports, each subtype of RyR functions differently in the brain, and they may differ in their involvement in the acquisition and maintenance of IT. The participation of each RyR subtype in IT should be investigated in future studies.

In conclusion, these results suggest that RyRs have an important role in acquiring IT. A decrease in RyRs might play the role in the preservation of IT.

Acknowledgment

This work was partially supported by Research Project Grant (21-607) from Kawasaki Medical School.

References

Bardo, S.B., et al., 2006. The role of the endoplasmic reticulum Ca^{2+} store in the plasticity of central neurons. Trends Pharmacol. Sci. 27, 78–84.
Bickler, P.E., Fahlman, C.S., 2010. Enhanced hypoxic preconditioning by isoflurane: signaling gene expression and requirement of intracellular Ca^{2+} and inositol triphosphate receptors. Brain Res. 1340, 86–95.
Bickler, P.E., Zhan, X., Fahlman, C.S., 2005. Isoflurane preconditions hippocampal neurons against oxygen-glucose deprivation: role of intracellular Ca^{2+} and mitogen-activated protein kinase signaling. Anesthesiology 103, 532–539.
Bickler, P.E., et al., 2009. Inositol 1,4,5-triphosphate receptors and NAD(P)H mediate Ca^{2+} signaling required for hypoxic preconditioning of hippocampal neurons. Neuroscience 160, 51–60.
Bouchard, R., Pattarini, R., Geiger, J.D., 2003. Presence and functional significance of presynaptic ryanodine receptors. Prog. Neurobiol. 69, 391–418.

Dahl, C.D., et al., 2000. Reduced [^{3}H]IP_3 binding but unchanged IP_3 receptor levels in the rat hippocampus CA1 region following transient global ischemia and tolerance induction. Neurochem. Int. 36, 379–388.
Duszczyk, M., et al., 2006. Behavioral evaluation of ischemic damage to CA1 hippocampal neurons: effects of preconditioning. Acta Neurobiol. Exp. 66, 311–319.
Furuichi, T., et al., 1994. Multiple types of ryanodine receptor/Ca^{2+} release channels are differentially expressed in rabbit brain. J. Neurosci. 14, 4794–4805.
Gidday, J.M., 2006. Cerebral preconditioning and ischaemic tolerance. Nat. Rev. Neurosci. 7, 437–448.
Grabb, M.C., Choi, D.W., 1999. Ischemic tolerance in murine cortical cell culture: critical role for NMDA receptors. J. Neurosci. 19, 1657–1662.
Grabb, M.C., et al., 2002. Preconditioned resistance to oxygen-glucose deprivation-induced cortical neuronal death: alterations in vesicular GABA and glutamate release. Neuroscience 115, 173–183.
Gray, J.J., et al., 2005. Isoflurane neuroprotection in hypoxic hippocampal slice cultures involves increases in intracellular Ca^{2+} and mitogen-activated protein kinases. Anesthesiology 102, 606–615.
Hertle, D.N., Yeckel, M.F., 2007. Distribution of inositol-1,4,5-trisphosphate receptor isotypes and ryanodine receptor isotypes during maturation of the rat hippocampus. Neuroscience 150, 625–638.
Inan, S., Wei, H., 2010. The cytoprotective effects of dantrolene: a ryanodine receptor antagonist. Anesth. Analg. 111, 1400–1410.
Kirino, T., 2002. Ischemic tolerance. J. Cereb. Blood Flow Metab. 22, 1283–1296.
Kurokawa, K., et al., 2010. Regulation of ryanodine receptors by dopamine D_1 receptors during methamphetamine-induced place conditioning. J. Neurochem. 115, 1206–1214.
Kurokawa, K., et al., 2011. Gabapentin blocks methamphetamine-induced sensitization and conditioned place preference via inhibition of α_2/δ-1 subunits of the voltage-gated calcium channels. Neuroscience 176, 328–335.
Kushnir, A., Betzenhauser, M.J., Marks, A.R., 2010. Ryanodine receptor studies using genetically engineered mice. FEBS Lett. 584, 1956–1965.
Liu, Y.X., et al., 2012. The role of glutamate transporter-1a in the induction of brain ischemic tolerance in rats. Glia 60, 112–124.
Ohta, S., et al., 1996. Calcium movement in ischemia-tolerant hippocampal CA1 neurons after transient forebrain ischemia in gerbils. J. Cereb. Blood Flow Metab. 16, 915–922.
Okabe, N., et al., 2013. Delayed administration of the nucleic acid analog 2Cl-C: OXT-A attenuates brain damage and enhances functional recovery after ischemic stroke. Brain Res. 1506, 115–131.
Riksen, N.P., et al., 2006. Caffeine prevents protection in two human models of ischemic preconditioning. J. Am. Coll. Cardiol. 48, 700–707.
Rubino, A., Yellon, D.M., 2000. Ischemic preconditioning of the vasculature: an overlooked phenomenon for protecting the heart? TiPS 21, 225–230.
Sharp, A.H., et al., 1993. Differential immunohistochemical localization of inositol 1,4,5-trisphosphate- and ryanodine-sensitive Ca^{2+} release channels in rat brain. J. Neurosci. 13, 3051–3063.
Singer, C.A., et al., 1999. The Mitogen-activated protein kinase pathway mediates estrogen neuroprotection after glutamate toxicity in primary cortical neurons. J. Neurosci. 19, 2455–2463.
Tauskela, J.S., Morley, P., 2004. On the role of Ca^{2+} in cerebral ischemic preconditioning. Cell Calcium 36, 313–322.
Tauskela, J.S., et al., 2003. Preconditioning of cortical neurons by oxygen-glucose deprivation: tolerance induction through abbreviated neurotoxic signaling. Am. J. Physiol. Cell Physiol. 285, C899–911.
Verkhratsky, A., Toescu, E.C., 2003. Endoplasmic reticulum Ca($^{2+}$) homeostasis and neuronal death. J. Cell. Mol. Med. 7, 351–361.
Zhang, L., et al., 1993. Dantrolene protects against ischemic: delayed neuronal death in gerbil brain. Neurosci. Lett. 158, 105–108.