ELSEVIER

Epilepsy Research 67 (2005) 153–162

Epilepsy Research

www.elsevier.com/locate/epilepsyres

Effects of zonisamide on neurotransmitter exocytosis associated with ryanodine receptors

Shukuko Yoshida [a,b], Motohiro Okada [a,*], Gang Zhu [a], Sunao Kaneko [a]

[a] *Department of Neuropsychiatry, Hirosaki University, Hirosaki 036-8562, Japan*
[b] *Institute of Neuroscience and Molecular Biology, Shibata IRIKA, Hirosaki 036-8084, Japan*

Received 25 July 2005; received in revised form 30 September 2005; accepted 2 October 2005

Abstract

To clarify the antiepileptic and neuroprotective actions of zonisamide (ZNS), we determined acute effects of ZNS on exocytosis of GABA and glutamate associated with ryanodine-receptor (Ryr) in rat hippocampus using microdialysis. ZNS increased basal GABA release concentration-dependently without affecting basal glutamate release; however, K^+-evoked glutamate and GABA releases were reduced by ZNS concentration-dependently. Inhibition of Ryr reduced K^+-evoked GABA and glutamate releases without affecting their basal releases. Ryanodine affected GABA and glutamate releases biphasic concentration-dependently: lower concentration of ryanodine increased both basal and K^+-evoked releases of GABA and glutamate, whereas higher concentration reduced them. The therapeutically relevant concentration of ZNS inhibited ryanodine-induced GABA and glutamate releases, and abolished the inflection point in concentration–response curve for ryanodine on neurotransmitter exocytosis. These data suggest that ZNS elevates seizure threshold via enhancement of GABAergic transmission during resting stage. ZNS inhibits propagation of epileptic hyperexcitability and Ryr-associated neuronal damage during neuronal hyperexcitable stage. These demonstrations indicate that the indirect inhibition of Ryr activities by ZNS during neuronal hyperexcitability appear to be involved in the mechanisms of action of antiepileptic and neuroprotective actions of ZNS.

Keywords: GABA; Glutamate; Epilepsy; Zonisamide; Ryanodine

1. Introduction

The novel antiepileptic drug (AED), zonisamide (ZNS) has a wide clinical spectrum of use in epileptic (Cramer et al., 2001; Bialer et al., 2004; Brodie, 2004; Seino, 2004; Willmore, 2004; Wilfong and Schultz, 2005), mood (Kanba et al., 1994; McElroy et al.,

* Corresponding author. Tel.: +81 172 395066; fax: +81 172 395067.
E-mail address: okadamot@cc.hirosaki-u.ac.jp (M. Okada).

0920-1211/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.eplepsyres.2005.10.001

2005) disorders and Parkinson's disease (Murata et al., 2001; Murata, 2004). Especially, ZNS has been well established as a highly effective first-line AED against both simple, complex partial and generalized seizures in Japan (Cramer et al., 2001; Bialer et al., 2004; Brodie, 2004; Seino, 2004; Willmore, 2004; Wilfong and Schultz, 2005). The antiepileptic mechanisms of ZNS were considered to be their inhibitory effects on voltage-gated Na^+ channel, voltage-sensitive Ca^{2+} channel (Suzuki et al., 1992; Kito et al., 1996) and other neurotransmission systems (Okada et al., 1995, 1998, 1999; Kawata et al., 1999). In spite of these efforts, the mechanism of clinical action of ZNS has remained to be clarified.

The intraneuronal Ca^{2+} mobilization plays an important role in numerous neuronal events, including excitability, transmitter exocytosis, synaptic plasticity, gene expression and neurotoxicity (Berridge et al., 1989; Berridge, 1998). The intraneuronal Ca^{2+} mobilization is composed of both Ca^{2+} influx via voltage-sensitive Ca^{2+} channels and ligand-gated ion channels, as well as output from intracellular Ca^{2+} store associated with endoplasmic reticulum, namely Ca^{2+}-induced Ca^{2+}-release system (CICR) (Berridge et al., 1989; Berridge, 1998). Recently, several studies have indicated that the functional abnormalities of CICR, which is comprised of the ryanodine receptor (Ryr) and inositol 1,4,5-trisphosphate receptors (IP3-R), contribute to the elevation of intraneuronal Ca^{2+} concentration associated with epileptic seizure (Matsumoto et al., 1996; Matsumoto and Nagata, 1999; Pal et al., 1999, 2000, 2001a; Raza et al., 2001; Mori et al., 2005). A Ryr inhibitor, dantrolene, protects neurons against kainate-induced apoptosis in vitro and in vivo (Berg et al., 1995; Popescu et al., 2002). Our resent studies have demonstrated that kainate administration produced transient up-regulation of gene expression of both c-Fos and Ryr-3 in hippocampus (Mori et al., 2005), and that a novel AED, topiramate, inhibited Ryr-associated neurotransmitter exocytosis (Okada et al., in press). On the basis of these previous evidences, Ryr-associated processes have been hypothesized to be involved in the induction of epilepsy and play a major role in seizure-triggered neuronal cell damage (Pal et al., 2001a; Mori et al., 2005). Therefore, to clarify the antiepileptic mechanisms of ZNS, the present study determined the effects of ZNS on Ryr-associated neurotransmitter exocytosis using in vivo microdialysis.

2. Materials and methods

All of the experiments described in this report conformed to the specifications of the Animal Research Committee and international guidelines on the ethical use of animals. Effort was made to minimize the number of animals used and their suffering. Male Wistar rats (Clea, Tokyo, Japan), weighing 250–300 g, were housed under conditions of constant temperature $22 \pm 2\,^{\circ}C$ with a 12 h light/dark cycle.

2.1. Chemical agents

The chemical agents used in this study included: the ryanodine receptor (Ryr) agonist, ryanodine (Calbiochem, San Diego, CA, USA); the Ryr antagonist, ruthenium red (RR: Tokyo Chemicals, Tokyo, Japan) and zonisamide (ZNS: Dainippon Pharmaceutical, Osaka, Japan).

2.2. Microdialysis system preparation

Each rat was placed in a stereotaxic frame and kept under halothane anesthesia (1.5% mixture of halothane and O_2 with N_2O). A concentric I-type dialysis probe (0.22 mm diameter; 3 mm exposed membrane; Eicom, Kyoto, Japan) was implanted in the synaptic area in hippocampal Schaffer collateral/commissural pathway ($A = -5.8$ mm, $L = 4.8$ mm, $V = -4.0$ mm relative to bregma) (Okada et al., 1995, 1999), and the perfusion experiments were started 36 h after the rats had recovered from anesthesia (Okada et al., 2001). The perfusion rate was always 1 μl/min, using MRS composed of (in mM): 145 Na^+, 2.7 K^+, 1.2 Ca^{2+}, 1.0 Mg^{2+}, 154.4 Cl^- and buffered with 2 mM phosphate buffer and 1.1 mM Tris buffer to adjust the pH to 7.40 (Okada et al., 2001). To study the effects of an increase in the extracellular K^+ levels (K^+-evoked stimulation) on the extracellular levels of glutamate and GABA in rat hippocampus, MRS containing 50 mM K^+ (HKMRS) was perfused for 20 min (Okada et al., 1998, 2001). The ionic composition was modified and isotonicity was maintained by an equimolar decrease of Na^+ (Okada et al., 1998, 2001). Each hippocampal dialysate was injected every 20 min into a high performance liquid chromatograph (HPLC).

The concentrations of glutamate and GABA in hippocampal perfusate were determined by HPLC

with *o*-phthalaldehyde-derived fluorescence detection (Okada et al., 2003; Nakatsu et al., 2004). The analytical column (100 mm × 3.0 mm internal diameter) was packed with Mightysil RP-18 (particle size, 3 μm) (gift from Kanto Chemicals, Tokyo, Japan) by Masis Inc. (Hirosaki, Japan), and was maintained at 30 °C. The excitation and emission wavelengths of the fluorescence detector were set at 340 and 445 nm, respectively. A linear gradient elution program was performed over 30 min with mobile phase A (0.05 M phosphate buffer containing 25% methanol, pH 6.0) and B (0.05 M phosphate buffer containing 40% methanol, pH 3.5), at a flow rate of 400 μl/min.

2.3. Determination of diffusion rates of ZNS and ryanodine

In order to accurately measure the concentration of each agent in the extracellular fluid perfused from hippocampus, in vivo probe diffusion was determined according to the "reverse dialysis" procedure (Le Quellec et al., 1995; Okada et al., in press). Because solute diffusion occurs in both directions across the dialysis membrane, loss of solute from the perfusate occurs at the same rate as recovery of solute into the perfusate. During analysis the temperature was maintained at 37 °C with a perfusion warmer. In order to determine the concentration of ryanodine in the neuronal tissue, after the perfusion with ryanodine for 2 h, the brain tissue around the dialysis probe (2 mm diameter × 5 mm depth) (Dykstra et al., 1992) was punched out.

The concentrations of ZNS and ryanodine were measured by HPLC equipped with gradient pump system (PU-2089, Jusco, Tokyo, Japan), ultra-violet detector (UV-2070, Jusco) and analytical column (150 mm × 3 mm internal diameter) packed by Masis with Mightysil RP-18 (particle size 3 μm: Kanto Chemicals). The mobile phase for determination of ryanodine concentration consisted of 0.01 M phosphate buffer (pH 6.0) containing 50% methanol and monitored at 254 nm. The mobile phase for determination of ryanodine concentration consisted of 0.01 M phosphate buffer (pH 6.0) containing 50% methanol and monitored at 254 nm. The detailed method for determination of ZNS was according to the published methods (Juergens, 1987).

3. Results

3.1. Diffusion of ZNS and ryanodine

The mean rate at which ryanodine diffused from the dialysis probe (internal to external probes) was 11% (mean ± S.D., $N = 24$). Therefore, the estimated concentrations in extracellular space during perfusion with 1, 10, 100 and 1000 μM ryanodine were 110 nM, 1.1, 11 and 110 μM, respectively. On the other hand, the concentrations of ryanodine in brain tissue during perfusion with 1, 10, 100 and 1000 μM ryanodine were 5.1, 52 nmol/wet weight brain tissue (wwbt), 0.5 and 5.4 μmol/wwbt, respectively.

The minimum effective plasma concentration of ZNS against maximum electroshock seizure test in rats is 10 μg/ml (about 47 μM), while neurotoxic plasma concentrations are higher than 70 μg/ml (about 330 μM) (Masuda et al., 1979). The rate at which ZNS diffused from the dialysis probe (internal to external probes) was 20 ± 2 (mean ± S.D., $N = 24$), respectively. Therefore, during perfusion which ranged from 250 to 1000 μM ZNS, the estimated concentration of ZNS in hippocampus ranged from 50 to 200 μM (within therapeutically relevant concentration range); however, during perfusion with more than 2500 μM ZNS, the estimated concentration of ZNS in hippocampus was more than 500 μM (supratherapeutic range).

3.2. Effects of ZNS and Ryr agents on releases of GABA and glutamate in hippocampus

The concentration-dependent effects of ryanodine, RR and ZNS on basal hippocampal releases of glutamate and GABA are shown in Fig. 1a–c, respectively. The perfusion with the Ryr agonist, ryanodine (from 1 to 1000 μM, estimated concentrations in hippocampal tissue were ranged from 5.1 nmol/wwbt to 5.4 μmol/wwbt) increased hippocampal extracellular glutamate level concentration-dependently ($P < 0.01$) (Fig. 1a). The perfusion with ryanodine increased hippocampal extracellular GABA level concentration-dependently ($P < 0.01$) over the concentration range from 1 to 100 μM, whereas the perfusion with 1000 μM Ry did not affect extracellular GABA level (Fig. 1a). Neither hippocampal extracellular levels of glutamate nor GABA were affected by the Ryr inhibitor, RR, which ranged from 5 to 500 μM (Fig. 1b); however,

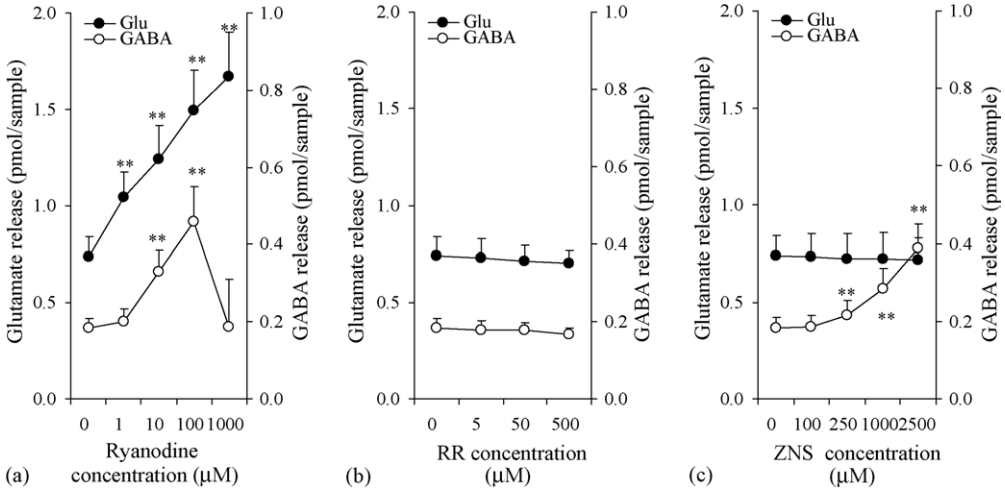

Fig. 1. Concentration-dependent effects of Ryr agents and ZNS on basal releases of glutamate and GABA in rat hippocampus. The concentration-dependent effects of ryanodine, RR and ZNS on basal releases of glutamate (Glu: ●) and GABA (○) are represented in (a)–(c), respectively. The right and left ordinates indicate the mean ± S.D. ($N = 6$) of extracellular levels of glutamate and GABA (pmol/sample), respectively. The abscissas show the concentration of ryanodine, RR and ZNS (μM). The concentration-dependent effects of Ryr agents and ZNS on basal releases were analyzed using one-way ANOVA with Dunnett's multiple comparison test ($^{*}P < 0.05$; $^{**}P < 0.01$).

the perfusion with 50 μM RR reduced the ryanodine-induced releases of GABA and glutamate (data not shown). The perfusion with ZNS (ranged from 100 to 2500 μM, estimated concentrations ranging from 20 and 500 μM) increased hippocampal extracellular GABA level concentration-dependently ($P < 0.01$) (Fig. 1c), whereas the hippocampal extracellular glutamate level was not affected by ZNS (Fig. 1c).

To clarify the effects of Ryr and ZNS on releases of glutamate and GABA induced by neuronal hyperexcitation, the present study determined the effects of ryanodine, RR and ZNS on 50 mM K^+-evoked hippocampal releases of glutamate and GABA. The perfusion medium was commenced with MRS with or without ryanodine, RR or ZNS. After confirming stabilization of extracellular levels of glutamate and GABA, the perfusion medium was switched to HK-MRS containing the same agent for 20 min. The concentration-dependent effects of ryanodine, RR and ZNS on 50 mM K^+-evoked hippocampal releases of glutamate and GABA are shown in Fig. 2a–c, respectively. The K^+-evoked release was calculated by subtracting the basal release level from extracellular level during K^+-evoked stimulation. The perfusion with ryanodine (range from 1 to 10 μM) increased K^+-evoked GABA release concentration-dependently ($P < 0.01$), whereas at concentrations higher than 10 μM, the stimulatory effect of ryanodine on K^+-evoked GABA release was attenuated concentration-dependently ($P < 0.05$) (Fig. 2a). Especially, K^+-evoked GABA release was reduced by perfusion with 1000 μM ryanodine ($P < 0.01$) (Fig. 2a). The perfusion with ryanodine (range from 1 to 100 μM) increased K^+-evoked glutamate release concentration-dependently ($P < 0.01$), whereas at concentrations 1000 μM, the stimulatory effect of ryanodine on K^+-evoked glutamate release was attenuated ($P < 0.05$) (Fig. 2a). Both K^+-evoked releases of GABA and glutamate were reduced by perfusion with RR in a concentration-dependent manner ($P < 0.01$) (Fig. 2b). The perfusion with ZNS also reduced the hippocampal K^+-evoked releases of GABA and glutamate concentration-dependently ($P < 0.01$) (Fig. 2c).

3.3. Interaction between ZNS and Ryr on releases of GABA and glutamate

To clarify the effects of therapeutically relevant concentration of ZNS on Ryr, the present study determined the interaction between ZNS and ryanodine on basal releases of glutamate and GABA (Fig. 3a and b). The perfusion medium was commenced with MRS with (add-ZNS) or without (non-ZNS) 1000 μM ZNS

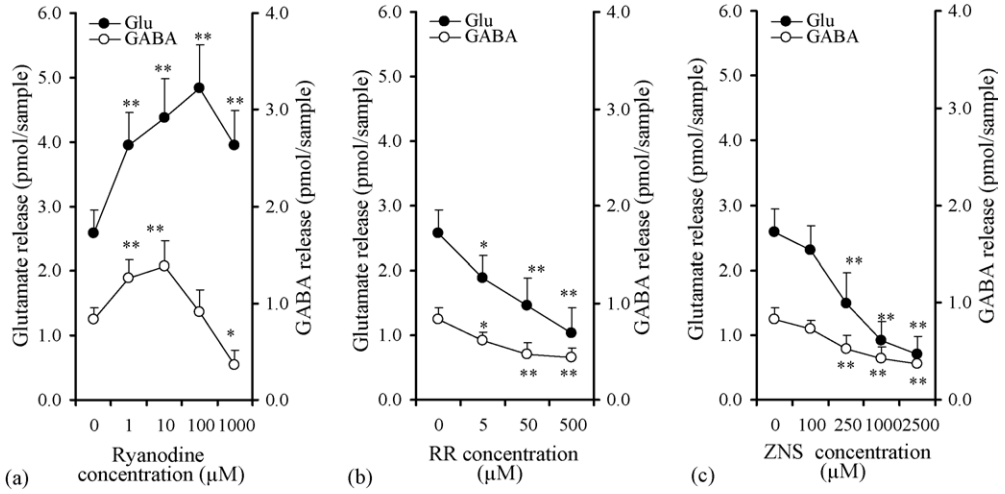

Fig. 2. Concentration-dependent effects of Ryr agents and ZNS on K^+-evoked releases of glutamate and GABA in rat hippocampus. The concentration-dependent effects of ryanodine, RR and ZNS on K^+-evoked releases of glutamate (Glu: ●) and GABA (○) are represented in (a)–(c), respectively. The right and left ordinates indicate the mean ± S.D. ($N = 6$) of K^+-evoked releases of glutamate and GABA (pmol/sample), respectively. The level of K^+-evoked release was defined as the basal extracellular level subtracted from the maximal extracellular level during K^+-evoked stimulation (Okada et al., 2001). The abscissas show the concentration of ryanodine, RR and ZNS (μM). The concentration-dependent effects of Ryr agents and ZNS on K^+-evoked releases were analyzed using one-way ANOVA with Dunnett's multiple comparison test ($^*P < 0.05$; $^{**}P < 0.01$).

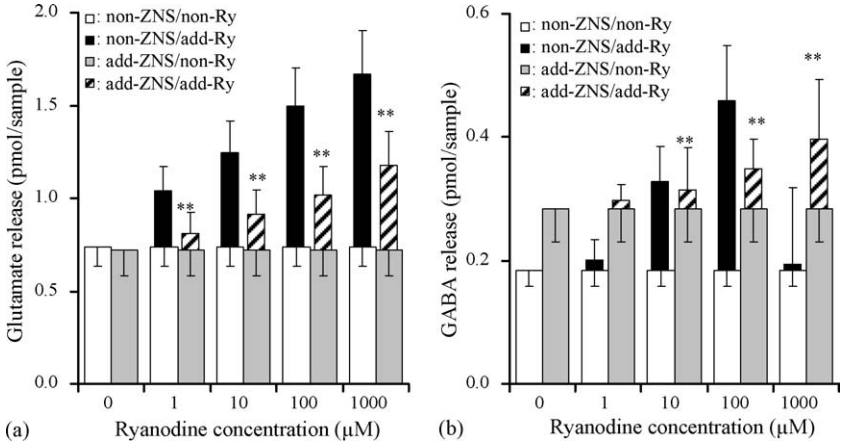

Fig. 3. Effects of ZNS on ryanodine-induced elevation of basal releases of glutamate and GABA in rat hippocampus. The effects of pre-perfusion with therapeutically relevant concentration of ZNS (1000 μM: estimated concentration is 200 μM) on ryanodine -induced elevation of basal releases of glutamate and GABA are represented in (a) and (b), respectively. The perfusion medium was commenced with MRS with (add-ZNS) or without (non-ZNS) 1000 μM ZNS. After confirming stabilization of extracellular levels of glutamate and GABA, the perfusion medium was switched to the same perfusion medium containing ryanodine (add-Ry: from 1 to 1000 μM). The ordinates indicate the mean ± S.D. ($N = 6$) of extracellular levels of glutamate and GABA (pmol/sample). The abscissas show the concentration of ryanodine (μM). The concentration-dependent effects of ryanodine on basal releases were analyzed using two-way ANOVA with Tukey's multiple comparison test (non-ZNS/add-Ry vs. add-ZNS/add-Ry) ($^*P < 0.05$; $^{**}P < 0.01$).

(estimated concentration of ZNS in hippocampus was 200 μM). After confirming stabilization of extracellular levels of glutamate and GABA, the perfusion medium was switched to the same perfusion medium containing ryanodine (add-Ry: from 1 to 1000 μM). The stimulatory effects of ryanodine on basal glutamate release were reduced by pre-treatment with 1000 μM ZNS ($P < 0.01$) (Fig. 3a). The stimulatory effects of ryanodine, ranged from 1 to 100 μM, on basal GABA release were also reduced by pre-treatment with 1000 μM ZNS ($P < 0.05$) (Fig. 3b); however surprisingly, the perfusion with 1000 μM ZNS produced 1000 μM ryanodine-induced elevation of basal GABA release ($P < 0.01$) (Fig. 3b). Thus, the pre-perfusion with 1000 μM ZNS abolished the inflection point in the concentration–response curve for ryanodine on basal GABA release. In other words, under the condition of perfusion with therapeutically relevant concentration of ZNS, ryanodine can enhance the basal GABA release concentration-dependently rather than glutamate release.

To clarify the stimulatory effects of ZNS on basal GABA release, the present study determined the interaction between ZNS and RR on basal GABA release (Fig. 4). The perfusion medium was commenced with MRS with (add-RR) or without (non-RR) 50 μM RR. After confirming stabilization of extracellular GABA level, the perfusion medium was switched to the same perfusion medium containing ZNS (add-ZNS: from 100 to 2500 μM). The stimulatory effect of ZNS on basal GABA release was not affected by pre-treatment with 50 μM RR (Fig. 4).

To clarify the effects of therapeutically relevant concentration of ZNS on ryanodine-associated K^+-evoked releases of glutamate and GABA, the present study determined the interaction between ZNS and ryanodine on K^+-evoked releases of glutamate and GABA (Fig. 5a and b). The perfusion medium was commenced with MRS with (add-ZNS) or without (non-ZNS) 1000 μM ZNS. After confirming stabilization of extracellular levels of glutamate and GABA, the perfusion medium was switched to the same perfusion medium containing ryanodine (from 1 to 1000 μM). After confirming stabilization of extracellular levels of glutamate and GABA, the perfusion medium was switched to HK-MRS containing the same agents. The stimulatory effects of ryanodine on K^+-evoked glutamate release were reduced by pre-treatment with 1000 μM ZNS ($P < 0.05$) (Fig. 5a). Especially, the inflection point in the concentration–response curve for ryanodine on K^+-evoked glutamate release was abolished by the pre-treatment of 1000 μM ZNS. The stimulatory effects of ryanodine, ranged lower than 100 μM, on K^+-evoked glutamate release were reduced by pre-treatment with 1000 μM ZNS ($P < 0.05$) (Fig. 5a); however, under the condition of perfusion with 1000 μM ZNS, ryanodine, ranged from 1 to 1000 μM, could enhance the K^+-evoked GABA release concentration-dependently. Similar to glutamate, the inflection point in the concentration–response curve for ryanodine on K^+-evoked GABA release (10 μM) was abolished by the pre-treatment of 1000 μM ZNS. Therefore, under the condition of perfusion with therapeutically relevant concentration of ZNS, ryanodine could enhance

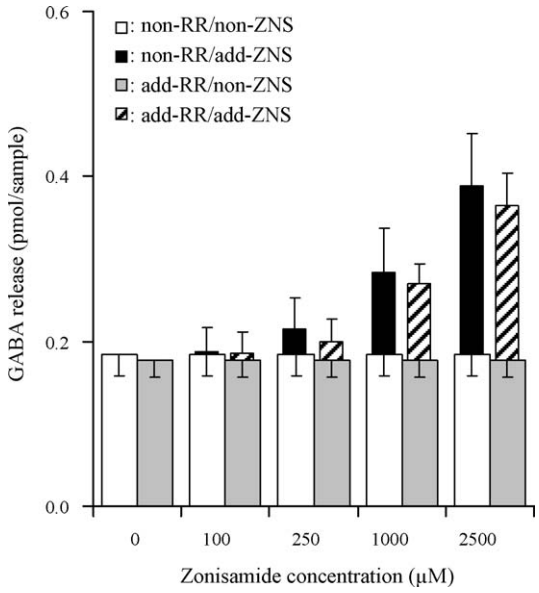

Fig. 4. Effects of Ryr antagonist on ZNS-induced elevation of basal GABA release in rat hippocampus. The effects of pre-perfusion with Ryr antagonist, 50 μM RR, on concentration-dependent elevation of basal GABA release induced by ZNS (ranged from 100 to 2500 μM: estimated concentrations are 20–500 μM) are represented. The perfusion medium was commenced with MRS with (add-RR) or without (non-RR) 50 μM RRS. After confirming stabilization of extracellular GABA level, the perfusion medium was switched to the same perfusion medium containing ZNS (add-ZNS). The ordinates indicate the mean ± S.D. ($N = 6$) of extracellular GABA level (pmol/sample). The abscissas show the concentration of ZNS (μM). The concentration-dependent effects of RR on ZNS-induced elevation of basal GABA release were analyzed using two-way ANOVA with Tukey's multiple comparison test.

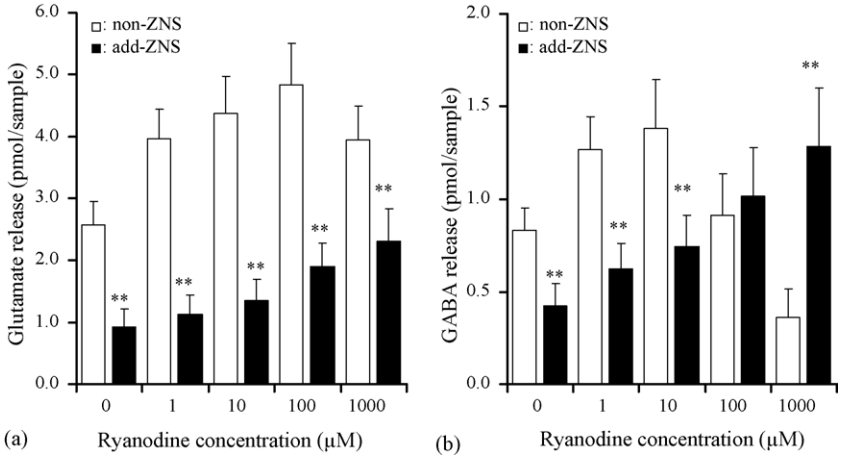

Fig. 5. Effects of ZNS on ryanodine-induced elevation of K^+-evoked releases of glutamate and GABA in rat hippocampus. The effects of pre-perfusion with therapeutically relevant concentration of ZNS (1000 μM: estimated concentration is 200 μM) on ryanodine-induced elevation of K^+-evoked releases of glutamate and GABA are represented in (a) and (b), respectively. The perfusion medium was commenced with MRS with (add-ZNS) or without (non-ZNS) 1000 μM ZNS. After confirming stabilization of extracellular levels of glutamate and GABA, the perfusion medium was switched to the same perfusion medium containing ryanodine (from 1 to 1000 μM). After confirming stabilization of extracellular levels of glutamate and GABA, the perfusion medium was switched to HK-MRS containing the same agents. The level of K^+-evoked release was defined as the basal extracellular level subtracted from the maximal extracellular level during K^+-evoked stimulation (Okada et al., 2001). The ordinates indicate the mean ± S.D. ($N = 6$) of K^+-evoked releases of glutamate and GABA (pmol/sample). The abscissas show the concentration of ryanodine (μM). The concentration-dependent effects of ryanodine on basal releases were analyzed using two-way ANOVA with Tukey's multiple comparison test (non-ZNS vs. add-ZNS) ($^*P < 0.05$; $^{**}P < 0.01$).

the K^+-evoked releases of glutamate and GABA concentration-dependently.

4. Discussion

4.1. Ryr-associated transmission

Neuronal Ca^{2+} mobilization, including the voltage-sensitive Ca^{2+} channel, ligand-gated ion channels, Mg^{2+}/Ca^{2+} ATPase and CICR, plays an important role in a wide variety of neuronal events including the Ca^{2+}-dependent neurotransmitter exocytosis (Berridge et al., 1989; Berridge, 1998; Rettig and Neher, 2002). Furthermore, the intraneuronal Ca^{2+} regulating system, CICR and Mg^{2+}/Ca^{2+} ATPase, are affected by neuronal hyperexcitability associated with epileptic seizure (Pal et al., 1999, 2000, 2001a; Raza et al., 2001; Mori et al., 2005). The study using hippocampal neuronal culture model of epilepsy has demonstrated that activities of Mg^{2+}/Ca^{2+} ATPase and IP3-R were chronically reduced and increased, respectively; however, Ryr activity was not changed (Pal et al., 2001a,b). Ryr activity plays an important role in kainate-induced apoptosis and seizure-triggered neuronal cell damage (Berg et al., 1995; Popescu et al., 2002). We have recently demonstrated the up-regulation of mRNA expression of Ryr-3 and c-Fos in rat hippocampus following kainate-induced seizures (Mori et al., 2005). Indeed, dantrolene, a Ryr inhibitor, protects neurons against kainate-induced apoptosis in vitro and in vivo (Berg et al., 1995; Popescu et al., 2002). Therefore, the inhibition of Ryr-3 activity contributes to the inhibition of neuronal cell damage associated with epileptic seizures.

Although the present study suggests that Ryr tends to be nonfunctional during resting stage; however, the potential of the Ryr system in neurotransmitter exocytosis could conceivably be promoted during the neuronal hyperexcitable stage. Ryanodine enhanced releases of hippocampal glutamate and GABA, although the enhancement of GABA and glutamate releases by ryanodine was biphasic in a concentration-dependent manner. The inflection points in the concentration–response curves for ryanodine on releases of GABA and glutamate were shifted to

the left by K^+-evoked neuronal hyperactivation. This shift is similar to that observed for frontal and striatal monoamine release (Zhu et al., 2004b; Okada et al., in press). The noteworthy result is that the inflection point in the concentration–response curves for ryanodine on GABA release is lower than that on glutamate release in both resting and K^+-evoked conditions. Thus, these observations suggest that hyperactivation of ryanodine-sensitive CICR produces an imbalance between GABAergic and glutamatergic transmission, with a relative predominance of glutamatergic transmission rather than GABAergic transmission.

The overload response of Ca^{2+} mobilization can lead to a process that results in neuronal cell damage directly (Pelletier et al., 1999a; Paschen and Frandsen, 2001; Zhu et al., 2004a,b; Mori et al., 2005; Okada et al., in press). In particular, the abnormal activation of Ryr-sensitive CICR contributes to the elevation of intracellular Ca^{2+} level associated with neuronal cell damage following epileptic seizures (Pelletier et al., 1999a; Mori et al., 2005). Therefore, the inflection point in the concentration–response curves for ryanodine on releases of GABA and glutamate indicates a dysfunction of the exocytosis mechanism induced by overload response of Ca^{2+} mobilization via hyperactivation of Ryr.

4.2. Effects of ZNS on Ryr-associated neurotransmitter exocytosis

The present study demonstrated that during resting stage, ZNS increased basal GABA release concentration-dependently without affecting basal glutamate release. Contrary to basal release, ZNS inhibited K^+-evoked releases of glutamate and GABA concentration-dependently. These demonstrations suggest that ZNS elevates the seizure threshold during resting stage, as well as inhibits propagation of epileptic hyperactivation.

We have already provided a hypothesis that ZNS possibly affects the activity two functional complexes of neurotransmitter exocytosis (Okada et al., 2001), inhibition of P-type voltage-sensitive Ca^{2+} channel/synaptobrevin/protein kinase A, which regulates depolarization-induced release predominantly, and enhancement of N-type voltage-sensitive Ca^{2+} channel/syntaxin/protein kinase C, which regulates basal release predominantly (Okada et al., 1995, 1998, 1999; Murakami et al., 2001; Zhu et al., 2002). The mechanisms of enhancement of basal GABA release by therapeutically relevant concentration of ZNS might be explained by our functional complex hypothesis. The concentration-dependent inhibitory action of ZNS on K^+-evoked releases of glutamate and GABA are also explained by the functional complex hypothesis with inhibition of voltage-gated Na^+ channel activity.

ZNS has been characterized as a broad-spectrum antiepileptic and neuroprotective agent in Japan (Seino, 2004). We have already demonstrated that the elevation of intracellular free Ca^{2+} induced by neuronal hyperactivation was inhibited by therapeutically relevant concentration of ZNS (Kawata et al., 1999). Based on both clinical and experimental evidence, to clarify the antiepileptic and neuroprotective mechanisms of ZNS, the present study determined the effects of Ryr-associated neurotransmitter exocytosis of GABA and glutamate. The ZNS-induced elevation of basal GABA release was not affected by inhibition of Ryr; however, ZNS inhibited the ryanodine-induced elevation of both basal releases of glutamate and GABA. These results suggest that therapeutically relevant concentration of ZNS reduces the Ryr-associated transmission system.

In the present study, therapeutically relevant concentration of ZNS, which reduced ryanodine-induced release, abolished the inflection point in the concentration–response curve for ryanodine on releases of GABA and glutamate. This result indicates that therapeutically relevant concentration of ZNS prevents the overload response of CICR induced by hyperactivation of Ryr. The Ryr antagonist was without effect on the induction or the persistence of epileptiform discharges, but prevented seizure-induced cell damage (Pelletier et al., 1999b; Pal et al., 2001b). On the basis of these previous evidences, the present results provide a hypothesis, that the abolishment of dysfunction of Ryr-associated neurotransmitter exocytosis by therapeutically relevant concentration of ZNS during both resting and neuronal hyperactivation stages, at least partially, may be involved in the neuroprotective action of ZNS via inhibition of overload response of Ryr-associated Ca^{2+} mobilization.

In conclusion, the present study demonstrated three important pharmacological actions of ZNS. The first is that ZNS concentration-dependently increased GABA release without affecting glutamate release during resting stage; however, during neuronal hyperexcitable

stage, ZNS inhibited both releases of glutamate and GABA concentration-dependently. The second is that therapeutically relevant concentration of ZNS inhibited Ryr-associated exocytosis mechanisms. The third is that therapeutically relevant concentration of ZNS prevented overload response induced by hyperactivation of Ryr. These data suggest that during resting stage, ZNS elevates the seizure-threshold via enhancement of GABAergic transmission without affecting glutamatergic transmission. By contrast, during neuronal hyperexcitable stage, ZNS inhibits propagation of epileptic heperexcitation and the overload response of Ryr-associated CICR. Therefore, these present demonstrations indicate that the inhibition of Ryr-associated CICR during neuronal hyperexcitability appears to be involved in the mechanisms of action of neuroprotective action of ZNS.

Acknowledgements

This study was supported by a Grant for Epilepsy from the Ministry of Health and Welfare of Japan, Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (15300146, 15390329, 15659267, 16109006, 17590856), grants from Hirosaki Research Institute for Neurosciences, Mitsubishi Pharma Research Foundation, Pharmacopsychiatry Research Foundation and Epilepsy Research Foundation.

References

Berg, M., Bruhn, T., Frandsen, A., Schousboe, A., Diemer, N.H., 1995. Kainic acid-induced seizures and brain damage in the rat: role of calcium homeostasis. J. Neurosci. Res. 40, 641–646.

Berridge, M.J., 1998. Neuronal calcium signaling. Neuron 21, 13–26.

Berridge, M.J., Downes, C.P., Hanley, M.R., 1989. Neural and developmental actions of lithium: a unifying hypothesis. Cell 59, 411–419.

Bialer, M., Johannessen, S.I., Kupferberg, H.J., Levy, R.H., Perucca, E., Tomson, T., 2004. Progress report on new antiepileptic drugs: a summary of the Seventh Eilat Conference (EILAT VII). Epilepsy Res. 61, 1–48.

Brodie, M.J., 2004. Zonisamide clinical trials: European experience. Seizure 13 (Suppl. 1), S66–S70 (discussion S71–S62).

Cramer, J.A., Ben Menachem, E., French, J., 2001. Review of treatment options for refractory epilepsy: new medications and vagal nerve stimulation. Epilepsy Res. 47, 17–25.

Dykstra, K.H., Hsiao, J.K., Morrison, P.F., Bungay, P.M., Mefford, I.N., Scully, M.M., Dedrick, R.L., 1992. Quantitative examination of tissue concentration profiles associated with microdialysis. J. Neurochem. 58, 931–940.

Juergens, U., 1987. Simultaneous determination of zonisamide and nine other anti-epileptic drugs and metabolites in serum. A comparison of microbore and conventional high-performance liquid chromatography. J. Chromatogr. 385, 233–240.

Kanba, S., Yagi, G., Kamijima, K., Suzuki, T., Tajima, O., Otaki, J., Arata, E., Koshikawa, H., Nibuya, M., Kinoshita, N., et al., 1994. The first open study of zonisamide, a novel anticonvulsant, shows efficacy in mania. Prog. Neuropsychopharmacol. Biol. Psychiatry 18, 707–715.

Kawata, Y., Okada, M., Murakami, T., Mizuno, K., Wada, K., Kondo, T., Kaneko, S., 1999. Effects of zonisamide on K^+ and Ca^{2+} evoked release of monoamine as well as K^+ evoked intracellular Ca^{2+} mobilization in rat hippocampus. Epilepsy Res. 35, 173–182.

Kito, M., Maehara, M., Watanabe, K., 1996. Mechanisms of T-type calcium channel blockade by zonisamide. Seizure 5, 115–119.

Le Quellec, A., Dupin, S., Genissel, P., Saivin, S., Marchand, B., Houin, G., 1995. Microdialysis probes calibration: gradient and tissue dependent changes in no net flux and reverse dialysis methods. J. Pharmacol. Toxicol. Meth. 33, 11–16.

Masuda, Y., Utsui, Y., Shiraishi, Y., Karasawa, T., Yoshida, K., Shimizu, M., 1979. Relationships between plasma concentrations of diphenylhydantoin, phenobarbital, carbamazepine, and 3-sulfamoylmethyl-1,2-benzisoxazole (AD-810), a new anticonvulsant agent, and their anticonvulsant or neurotoxic effects in experimental animals. Epilepsia 20, 623–633.

Matsumoto, M., Nagata, E., 1999. Type 1 inositol 1,4,5-trisphosphate receptor knock-out mice: their phenotypes and their meaning in neuroscience and clinical practice. J. Mol. Med. 77, 406–411.

Matsumoto, M., Nakagawa, T., Inoue, T., Nagata, E., Tanaka, K., Takano, H., Minowa, O., Kuno, J., Sakakibara, S., Yamada, M., Yoneshima, H., Miyawaki, A., Fukuuchi, Y., Furuichi, T., Okano, H., Mikoshiba, K., Noda, T., 1996. Ataxia and epileptic seizures in mice lacking type 1 inositol 1,4,5-trisphosphate receptor. Nature 379, 168–171.

McElroy, S.L., Suppes, T., Keck Jr., P.E., Black, D., Frye, M.A., Altshuler, L.L., Nolen, W.A., Kupka, R.W., Leverich, G.S., Walden, J., Grunze, H., Post, R.M., 2005. Open-label adjunctive zonisamide in the treatment of bipolar disorders: a prospective trial. J. Clin. Psychiatry 66, 617–624.

Mori, F., Okada, M., Tomiyama, M., Kaneko, S., Wakabayashi, K., 2005. Effects of ryanodine receptor activation on neurotransmitter release and neuronal cell death following kainic acid-induced status epilepticus. Epilepsy Res..

Murakami, T., Okada, M., Kawata, Y., Zhu, G., Kamata, A., Kaneko, S., 2001. Determination of effects of antiepileptic drugs on SNAREs-mediated hippocampal monoamine release using in vivo microdialysis. Br. J. Pharmacol. 134, 507–520.

Murata, M., 2004. Novel therapeutic effects of the anti-convulsant, zonisamide, on Parkinson's disease. Curr. Pharm. Des. 10, 687–693.

Murata, M., Horiuchi, E., Kanazawa, I., 2001. Zonisamide has beneficial effects on Parkinson's disease patients. Neurosci. Res. 41, 397–399.

Nakatsu, F., Okada, M., Mori, F., Kumazawa, N., Iwasa, H., Zhu, G., Kasagi, Y., Kamiya, H., Harada, A., Nishimura, K., Takeuchi, A., Miyazaki, T., Watanabe, M., Yuasa, S., Manabe, T., Wakabayashi, K., Kaneko, S., Saito, T., Ohno, H., 2004. Defective function of GABA-containing synaptic vesicles in mice lacking the AP-3B clathrin adaptor. J. Cell. Biol. 167, 293–302.

Okada, M., Hirano, T., Kawata, Y., Murakami, T., Wada, K., Mizuno, K., Kondo, T., Kaneko, S., 1999. Biphasic effects of zonisamide on serotonergic system in rat hippocampus. Epilepsy Res. 34, 187–197.

Okada, M., Kaneko, S., Hirano, T., Mizuno, K., Kondo, T., Otani, K., Fukushima, Y., 1995. Effects of zonisamide on dopaminergic system. Epilepsy Res. 22, 193–205.

Okada, M., Kawata, Y., Mizuno, K., Wada, K., Kondo, T., Kaneko, S., 1998. Interaction between Ca^{2+}, K^{+}, carbamazepine and zonisamide on hippocampal extracellular glutamate monitored with a microdialysis electrode. Br. J. Pharmacol. 124, 1277–1285.

Okada, M., Nutt, D.J., Murakami, T., Zhu, G., Kamata, A., Kawata, Y., Kaneko, S., 2001. Adenosine receptor subtypes modulate two major functional pathways for hippocampal serotonin release. J. Neurosci. 21, 628–640.

Okada, M., Yoshida, S., Zhu, G., Hirose, S., Kaneko, S., in press. Biphasic actions of topiramate on monoamine exocytosis associated with both SNARE and CICR systems. Neurosciences.

Okada, M., Zhu, G., Hirose, S., Ito, K.I., Murakami, T., Wakui, M., Kaneko, S., 2003. Age-dependent modulation of hippocampal excitability by KCNQ-channels. Epilepsy Res. 53, 81–94.

Pal, S., Limbrick Jr., D.D., Rafiq, A., DeLorenzo, R.J., 2000. Induction of spontaneous recurrent epileptiform discharges causes long-term changes in intracellular calcium homeostatic mechanisms. Cell Calcium 28, 181–193.

Pal, S., Sombati, S., Limbrick Jr., D.D., DeLorenzo, R.J., 1999. In vitro status epilepticus causes sustained elevation of intracellular calcium levels in hippocampal neurons. Brain Res. 851, 20–31.

Pal, S., Sun, D., Limbrick, D., Rafiq, A., DeLorenzo, R.J., 2001a. Epileptogenesis induces long-term alterations in intracellular calcium release and sequestration mechanisms in the hippocampal neuronal culture model of epilepsy. Cell Calcium 30, 285–296.

Pal, S., Sun, D., Limbrick, D., Rafiq, A., DeLorenzo, R.J., 2001b. Epileptogenesis induces long-term alterations in intracellular calcium release and sequestration mechanisms in the hippocampal neuronal culture model of epilepsy. Cell Calcium 30, 285–296.

Paschen, W., Frandsen, A., 2001. Endoplasmic reticulum dysfunction—a common denominator for cell injury in acute and degenerative diseases of the brain? J. Neurochem. 79, 719–725.

Pelletier, M.R., Wadia, J.S., Mills, L.R., Carlen, P.L., 1999a. Seizure-induced cell death produced by repeated tetanic stimulation in vitro: possible role of endoplasmic reticulum calcium stores. J. Neurophysiol. 81, 3054–3064.

Pelletier, M.R., Wadia, J.S., Mills, L.R., Carlen, P.L., 1999b. Seizure-induced cell death produced by repeated tetanic stimulation in vitro: possible role of endoplasmic reticulum calcium stores. J. Neurophysiol. 81, 3054–3064.

Popescu, B.O., Oprica, M., Sajin, M., Stanciu, C.L., Bajenaru, O., Predescu, A., Vidulescu, C., Popescu, L.M., 2002. Dantrolene protects neurons against kainic acid induced apoptosis in vitro and in vivo. J. Cell. Mol. Med. 6, 555–569.

Raza, M., Pal, S., Rafiq, A., DeLorenzo, R.J., 2001. Long-term alteration of calcium homeostatic mechanisms in the pilocarpine model of temporal lobe epilepsy. Brain Res. 903, 1–12.

Rettig, J., Neher, E., 2002. Emerging roles of presynaptic proteins in Ca^{2+}-triggered exocytosis. Science 298, 781–785.

Seino, M., 2004. Review of zonisamide development in Japan. Seizure 13 (Suppl. 1), S2–S4.

Suzuki, S., Kawakami, K., Nishimura, S., Watanabe, Y., Yagi, K., Seino, M., Miyamoto, K., 1992. Zonisamide blocks T-type calcium channel in cultured neurons of rat cerebral cortex. Epilepsy Res. 12, 21–27.

Wilfong, A., Schultz, R., 2005. Zonisamide for absence seizures. Epilepsy Res. 64, 31–34.

Willmore, L.J., 2004. Zonisamide overview of the United States experience. Seizure 13 (Suppl. 1), S57–S58.

Zhu, G., Okada, M., Murakami, T., Kawata, Y., Kamata, A., Kaneko, S., 2002. Interaction between carbamazepine, zonisamide and voltage-sensitive Ca^{2+} channel on acetylcholine release in rat frontal cortex. Epilepsy Res. 49, 49–60.

Zhu, G., Okada, M., Uchiyama, D., Ohkubo, T., Yoshida, S., Kaneko, S., 2004a. Hyperactivity of endoplasmic reticulum associated exocytosis mechanism contributes to acute phencyclidine intoxication. J. Pharmacol. Sci. 95, 214–227.

Zhu, G., Okada, M., Yoshida, S., Hirose, S., Kaneko, S., 2004b. Both 3,4-dihydroxyphenylalanine and dopamine releases are regulated by Ca^{2+}-induced Ca^{2+} releasing system in rat striatum. Neurosci. Lett. 362, 244–248.