Dysfunction of Extrasynaptic GABAergic Transmission in Phospholipase C-Related, but Catalytically Inactive Protein 1 Knockout Mice Is Associated with an Epilepsy Phenotype

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ABSTRACT
Phospholipase C-related, but catalytically inactive protein (PRIP) was first identified as a novel inositol 1,4,5-triphosphate binding protein. The PRIP-1 subtype is expressed predominantly in the central nervous system and binds directly to the GABA type A receptor (GABAA-R) β-subunit and several other proteins involved in the trafficking of GABAA-Rs to the plasma membrane. We found that the PRIP-1 knockout mouse showed an epileptic phenotype, confirmed by electroencephalogram. These ictal seizures were completely suppressed by diazepam (DZP), but the interictal discharges could not be abolished. We studied the electrophysiological properties of GABAergic transmission in hippocampal CA1 pyramidal neurons, using a slice patch-clamp technique. There was no difference in the effect of up to 1 μM DZP on the amplitude and frequency of miniature inhibitory postsynaptic currents between PRIP-1 knockout neurons versus wild-type neurons. In contrast, the amplitude of the tonic GABA current in PRIP-1 knockout neurons was markedly reduced compared with that in wild-type neurons. Consequently, the effect of DZP on PRIP-1 knockout mice was reduced. Dysfunction of extrasynaptic GABAergic transmission probably is involved in the epileptic phenotype of PRIP-1 knockout mice.

Introduction
Phospholipase C-related, but catalytically inactive protein (PRIP) was first identified as a novel inositol 1,4,5-triphosphate binding protein (Kanematsu et al., 1992, 1996). PRIP has two subtypes: PRIP-1 and PRIP-2. PRIP-1 is expressed predominantly in the brain (Matsuda et al., 1998), whereas PRIP-2 is expressed ubiquitously (Uji et al., 2002). PRIP-1 binds directly to the GABA type A receptor (GABAA-R) β-subunit and the GABAA-R-associated protein (GABARAP), which is involved in the trafficking of GABAA-Rs to the plasma membrane (Kanematsu et al., 2002; Terunuma et al., 2004). It is noteworthy that both PRIP and the GABAA-R β2-subunit share the same binding site for GABARAP (Kanematsu et al., 2002). It seems that PRIP may competitively inhibit the binding of GABAA-Rs to GABARAP, which is involved in the trafficking of GABAA-Rs to the plasma membrane (Kanematsu et al., 2002; Terunuma et al., 2004). This work was supported in part by a Grant for Priority Research designated by the President of Hirosaki University; Grants-in-Aid for Scientific Research (S) [Grant 16109006]; Grants-in-Aid for Scientific Research (A) [Grant 18209038]; Grants-in-Aid for Scientific Research (B) [Grants 18300137, 18309316]; Grants-in-Aid for Exploratory Research [Grants 17590856, 20591361]; and Grants-in-Aid for Exploratory Research [Grants 1659272, 18659455], all provided by the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

ABBREVIATIONS: PRIP, phospholipase C-related, but catalytically inactive protein; DZP, diazepam; GABAA-R, GABA type A receptor; GABARAP, GABAA-R-associated protein; KO, knockout; BZP, benzodiazepine; BMI, bicuculline methiodide; EEG, electroencephalogram; MES, maximal electroshock; ACSF, artificial cerebrospinal fluid; miPSC, miniature inhibitory postsynaptic current; PCR, polymerase chain reaction; PBST, 0.05% Tween 20 in phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; WT, wild type; CGP55845, (2S)-3-[[1S]-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl][phenylmethyl]phosphinic acid hydrochloride.
transport of the GABA\textsubscript{A}-R \(\gamma_2\)-subunit; these mice also displayed a reduced sensitivity to benzodiazepine (BZP) (Kanematsu et al., 2002; Mizokami et al., 2007). PRIP-1 also modulates GABA\textsubscript{A}-R phosphorylation via inactivating protein phosphatase 1A and 2A activities (Yoshimura et al., 2001; Terunuma et al., 2004; Kanematsu et al., 2006), resulting in enhanced phosphorylation of GABA\textsubscript{A}-Rs. This modulation of GABA\textsubscript{A}-R phosphorylation may be involved in the internalization of GABA\textsubscript{A}-Rs (Kanematsu et al., 2006). Furthermore, it has been reported that PRIP directly binds to phosphorylated Akt and triggers the phosphorylation of its \(\beta\)-subunits through a direct association. This facilitates insertion of the GABA\textsubscript{A}-Rs into the plasma membrane (Fujii et al., 2010).

In the present study, we found that PRIP-1 KO mice showed spontaneous ictal seizure waveforms by electroencephalogram (EEG). The epileptic state or recurrent activity is mediated by excess glutamatergic excitation and balanced by GABAergic inhibition (Clark and Wilson, 1999). Disinhibition of GABA\textsubscript{A}-Rs, or enhancement of \(N\)-methyl-D-aspartic acid-mediated excitation, has been used to establish models of epileptic activity (Sperk, 1994). The epileptic phenotype in mutant animals is caused by dysfunction of inhibitory transmission and/or enhancement of excitatory transmission, resulting in a dramatic change in the balance between excitatory and inhibitory activity.

Because of the role of PRIP-1 in GABA\textsubscript{A}-R transport, it is likely that dysfunction of GABAergic transmission is involved in the epileptic phenotype in PRIP-1 KO mice. Therefore, we investigated the properties of GABA\textsubscript{A}ergic synaptic and extrasynaptic transmission and the effect of diazepam (DZP) on the synaptic and extrasynaptic currents, in PRIP-1 KO mice. We showed that the primary perturbation in PRIP-1 KO mice is caused by impaired extrasynaptic transmission.

### Materials and Methods

The experimental procedures used in this study complied with the guidelines for animal research issued by the Physiological Society of Japan and the Hirosaki University School of Medicine, and all efforts were made to minimize the number of animals used and their suffering.

**Electrocorticographic Recording.** We recorded electrocorticograms using a radiotelemetry system (Unimec, Tokyo, Japan). Each mouse was placed in a stereotaxic frame and kept under halothane anesthesia (1.5% halothane and \(O_2\) mixed with \(N_2O\)). The recording and reference electrodes were screwed onto the skull over the left temporal (3.2 mm posterior to bregma and 4.0 mm lateral to midline) and occipital (2 mm posterior to lambda) regions, respectively. The telemetry unit was placed subcutaneously in the flank behind the scapula. EEG recording was started 48 h after the mice had recovered from the anesthesia and injury.

**Maximal Electroshock Test.** The maximal electroshock (MES) test was carried out according to methods described previously (Yokoyama et al., 1992). The electroshocks were induced with an alternating current (100-Hz square waves with 30 mA for 0.1 s), delivered through ear-clips electrodes by an electric stimulator (Raud Shocker Type 221; Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany). Seizure susceptibility was evaluated as the duration of tonic extensor and clonic seizures. The tonic extensor phase was regarded as the period between the onset of hind limb extension and the beginning of the clonic seizure. The clonic phase was defined as the duration from the end of the tonic extensor seizure to complete relaxation. To study the effects of DZP on MES-induced seizures, DZP (0.5, 1, 5, and 10 mg/kg i.p.) was administered 30 min before MES testing.

**Brain Slice Preparation.** Transverse hippocampal slices were obtained from 8- to 10-week-old mice. Each mouse was deeply anesthetized with halothane and decapitated, and its brain was rapidly removed. Hippocampal slices of 300-\(\mu\)m thickness were cut on a brain slicer (Vibratome VIB1500; Intracel, Royston, UK) at 4°C in standard artificial cerebrospinal fluid (ACSF). The solution contained 126 mM NaCl, 2.5 mM KCl, 1.25 mM NaH\(_2\)PO\(_4\), 2 mM MgSO\(_4\), 2 mM CaCl\(_2\), 26 mM NaHCO\(_3\), and 20 mM glucose. Slices were incubated for at least 1 h at room temperature in standard ACSF with 95% \(O_2\) and 5% \(CO_2\) at room temperature before recording.

**Whole-Cell Patch-Clamp Recordings.** Individual slices were transferred to a recording chamber, perfused (2.5 mL/min) with prewarmed standard ACSF, and maintained at 30°C. Hippocampal pyramidal neurons in the slices were viewed on a monitor via a 40× water-immersion objective lens with an infrared differential interference contrast filter and a charge-coupled device camera (ORCA-ER C4742-48; Hamamatsu Photonics, Shizuoka, Japan).

Patch electrodes were fabricated from thin-wall borosilicate glass tubing (GD-1.5; Narishige, Tokyo, Japan) using a pipette puller (PP-830; Narishige). The electrode resistance ranged from 3 to 5 \(M\Omega\) when filled with a high-\(Cl^-\) solution containing 130 mM CsCl, 2 mM MgCl\(_2\), 0.5 mM EGTA, 10 mM HEPES, 3 mM Mg\(\text{adenosine triphosphate}\), and 0.4 mM guanosine triphosphate, pH 7.3 with CsOH. Cells were voltage-clamped at \(-60\) mV in the presence of the \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (5 \(\mu\)M), the \(N\)-methyl-D-aspartic acid receptor antagonist \(k\)-\((2\)-aminophosphonopentanoic acid (5 \(\mu\)M), the GABA\textsubscript{A} receptor antagonist (2S)-3-[(1S)-(1S)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl]phenoxyethyl)methylphosphinic acid hydrochloride (CGP55845) (3 \(\mu\)M), and tetrodotoxin (0.3 \(\mu\)M) so that GABA\textsubscript{A}-R mediated currents were the inward current. To detect extrasynaptic GABA\textsubscript{A}ergic transmission, the specific GABA\textsubscript{A} receptor antagonist bicuculline methiodide (BMI) was applied. Membrane currents and membrane potentials were recorded by using an Axopatch multiclamp amplifier (Molecular Devices, Sunnyvale, CA) and digitized at 5 to 10 kHz by using a DigiData1322A and pCLAMP9.2 software (Molecular Devices). The data were acquired by using pClamp9.2 software (Molecular Devices) and stored for off-line analysis by using Igor Pro 5.03 software (WaveMetrics, Lake Oswego, OR). Series resistance compensation was not applied. Miniature inhibitory postsynaptic currents (mIPSCs) obtained from individual neurons were examined by constructing cumulative probability distributions for 1-min epochs, immediately before (control) and from 4 to 5 min of drug application, and compared by using the Kolmogorov-Smirnov test with the aid of NeuroMatic v.1.86 software (http://www.neuromatic.thinkrandom.com/). Numerical values are provided as the mean \pm S.E.M., with each value being normalized to the control. Possible differences were tested by using the Student’s two-tailed paired t test or the Steel-Dwass test for multiple comparisons. Values of \(p < 0.05\) were considered significant.

**Quantitative Reverse Transcriptase-Polymerase Chain Reaction.** Total RNA was extracted from the dissected hippocampus by using an RNAeasy lipid tissue mini-kit (QIAGEN GmbH, Hilden, Germany). Single-strand cDNA was synthesized from 1 \(\mu\)g of the total RNA by using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s protocol. Real-
time PCR was performed with a CFX96 real-time system (Bio-Rad Laboratories) using SsoFast EvaGreen Supermix (Bio-Rad Laboratories). Amplification conditions were as follows: 30 s at 95°C and then 40 cycles of 5 s at 95°C and 5 s at 57°C. Quantitative analysis of the data were performed by using CFX Manager software v.1.5 (Bio-Rad Laboratories). We designed PCR primers against the α1, α2, β1, β2, β3, γ2, and γ3 GABA<sub>A</sub>-R subunit genes by using Primer3Plus software (http://frodo.wi.mit.edu/primer3). Primer sequences are shown in Table 1.

### Quantitative Autoradiographic Analysis

Autoradiographic binding was performed as described previously (Skilbeck et al., 2007). In brief, sagittal cryostat sections (14-μm) from frozen, non-fixed mouse brains were thawed for 20 min at room temperature then preincubated twice for 15 min in 50 mM Tris-HCl, pH 7.4 at 4°C. Sections were then incubated at 4°C in 50 mM Tris-HCl, pH 7.4 containing 50 nM [3H]muscimol (PerkinElmer Life and Analytical Sciences, Waltham, MA) for 40 min. Using additional sections, the nonspecific binding of [3H]muscimol was determined by adding 100 μM bicuculline. After incubation, sections were washed four times in ice-cold buffer and then in distilled H<sub>2</sub>O. The sections were dried overnight under a stream of cold air and then exposed to Kodak Biomax MR film (PerkinElmer Life and Analytical Sciences) at −20°C for 6 weeks with tritium microscale standards. Films were developed and fixed. The films were quantitatively analyzed by using Image v.1.63 (National Institutes of Health, Bethesda, MD). Nonspecific binding values were subtracted from the mean intensity values to provide specific binding values. Statistical analysis was performed with the Student’s t test, and <i>p</i> < 0.05 was considered statistically significant.

### Western Blotting

Tissues were homogenized in lysis buffer containing 320 mM sucrose and 10 mM HEPES, pH 7.4, with protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). The mixture was centrifuged at 800g for 5 min at 4°C, and the protein concentration of the supernatant was determined by the Bradford method (Bradford, 1976). Aliquots containing 20 μg of total protein were electrophoresed on 5 or 12% SDS-polyacrylamide gels. Proteins were transferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore Corporation, Billerica, MA) using a Trans-Blot SD semidry system (Bio-Rad Laboratories) for 30 min at 8 V. The membranes were blocked by agitation in 5% nonfat dried milk and 0.05% Tween 20 in phosphate-buffered saline (PBS) for 1 h at room temperature. Blots were then incubated with the following antibodies (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA): anti-GABAA-R<sub>1</sub> (1:200), anti-GABAA-R<sub>2</sub> (1:200), anti-GABAA-R<sub>3</sub> (1:200), anti-GABAA-R<sub>8</sub> (1:200), or anti-GABAA-R<sub>9</sub> (1:200), diluted in PBST, for 1 h at room temperature. After washing, Cy<sub>3</sub>-conjugated secondary antibodies were added and incubated for 1 h at room temperature. Quantification of the signals was performed on an FX molecular image (Bio-Rad Laboratories). To determine which band or staining was specific, an immunizing peptide-blocking experiment was performed, using each blocking peptide according to the manufacturer’s protocol (Santa Cruz Biotechnology, Inc.).

### Results

#### The Epileptic Phenotype of PRIP-1 KO Mice.

All 8-week-old mice (<i>n</i> = 5) showed spike waves upon EEG monitoring. Four of the five showed spontaneous seizures with ictal discharges. Typical EEGs during spontaneous seizures in PRIP-1 KO mice are shown in Fig. 1. The spontaneous seizures initiated from single spikes and were followed by multiple spikes. The frequency and amplitude of the spikes became larger during the ictal discharges. During ictal discharges, mice showed akinesia followed by repetitive movements of extremities. Seizure-type EEG traces continued for approximately 30 s. After these ictal discharges, the amplitude of the EEG waves rapidly decreased and flattened (Fig. 1). Ictal discharges occurred three to four times per 48 h. Interictal discharges were detected more than 1000 times per 30 min in day in PRIP-1 KO mice.

#### DZP Abolishes Spontaneous Ictal Discharges, but Not Interictal Discharges.

EEG analysis revealed that PRIP-1 KO mice demonstrated an epileptic phenotype. Because of the previously observed lower sensitivity of BZP in PRIP-KO mice, we next examined the effect of a BZP derivative, DZP, on epileptic discharges in PRIP-1 KO mice. In naive mice, the mean frequency of ictal discharges was 4 ±

### Table 1

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shorter latencies until the first clonic convolution and tonic extensor after injection of pentyl benztrazol, the latencies of MES-induced seizure were not significantly different between KO and wild-type mice (Yamaguchi et al., 2004). In the present study, there was no significant difference in the duration of tonic and clonic convulsions between wild-type and PRIP-1 KO mice in the absence of DZP (n = 6) (Fig. 3). Next, we compared the effect of DZP on electrical stimulation-induced seizure between PRIP-1 KO and wild-type mice. Similar to its effects on spontaneous seizures in PRIP-1 KO mice, DZP suppressed the duration of both tonic and clonic convulsions in a concentration-dependent manner in both wild-type and PRIP-1 KO mice. In wild-type mice, DZP exhibited significant suppressive effects at more than 0.3 mg/kg i.p. In PRIP-1 KO mice, DZP at more than 0.3 mg/kg i.p. significantly decreased the duration of tonic convulsions, but its effects were weaker than those in wild-type mice. DZP at 10 mg/kg i.p. abolished most MES-induced convulsions in wild-type mice, but not in PRIP-1 KO mice (Fig. 3).

**Knockout of PRIP-1 Does Not Alter Current Properties or the Sensitivity to DZP of GABAergic IPSCs.** To characterize the GABAA-R-mediated synaptic events, we compared the frequency, amplitude, and current kinetics of GABAergic mIPSCs between wild-type and PRIP-1 KO mice (Fig. 4B) and examined the effects of DZP on mIPSCs (Figs. 4C and 5). mIPSCs were pharmacologically isolated by using tetrodotoxin, glutamate receptor antagonists, and a GABAB receptor antagonist.

The frequency, amplitude, and kinetics of the mIPSCs in hippocampal neurons of PRIP-1 KO mice displayed features not significantly different from those of wild-type mice (Fig. 4B). mIPSCs from neurons of wild-type mice showed an interevent interval of 139 ± 11 ms (n = 8), a mean amplitude of 24.0 ± 1.7 pA (n = 8), a rise time (10–90%) of 1.17 ± 0.08 ms (n = 8), a decay time constant of 7.61 ± 0.54 ms (n = 8), and an area of −247 ± 12 pA ms (n = 8), whereas mIPSCs from neurons of PRIP-1 KO mice showed an interevent interval of 138 ± 14 ms (n = 8), a mean amplitude of −23.7 ± 1.6 pA (n = 8), and a decay time constant of 7.80 ± 0.50 ms (n = 8), whereas mIPSCs from neurons of PRIP-1 KO mice showed an interevent interval of 138 ± 14 ms (n = 8), a mean amplitude of −23.7 ± 1.6 pA (n = 8), and a decay time constant of 7.80 ± 0.50 ms (n = 8).
1.9 pA \((n = 8)\), a rise time \((10–90\%)\) of 1.34 \pm 0.08 ms \((n = 8)\), a decay time constant of 8.40 \pm 0.55 ms \((n = 8)\), and an area of \(-251 \pm 3\) pA ms \((n = 8)\).

Next, we investigated the effect of DZP application \((0.3 \mu\text{M})\) on mIPSCs of wild-type and KO mice. The data were normalized to the value before DZP application in wild-type or PRIP-1 KO mice and are shown in Fig. 4C as the percentage of control values. mIPSCs of wild-type mice in the presence of DZP showed a mean amplitude of 95.3 \pm 4.8\% \((n = 8)\), an interevent interval of 104.0 \pm 6.7\% \((n = 8)\), a rise time \((10–90\%)\) of 110.5 \pm 8.7\% \((n = 8)\), a decay time constant of 114.0 \pm 5.5\% \((n = 8)\), and an area of 119 \pm 4.7\% \((n = 8)\). mIPSCs of PRIP-1 KO mice in the presence of DZP showed a mean amplitude of 105.0 \pm 2.9\% \((n = 7)\), an interevent interval of 88.3 \pm 4.2\% \((n = 7)\), a rise time \((10–90\%)\) of 101.7 \pm 1.4\% \((n = 7)\), a decay time constant of 115.5 \pm 2.5\% \((n = 7)\), and an area of 138.3 \pm 4.0\% \((n = 7)\). DZP significantly prolonged the decay time constant and significantly augmented the IPSC current area in both wild-type and KO mice versus the relevant control \((p < 0.05\) for wild-type versus PRIP-1 KO mice (Student’s \(t\) test)).

DZP at the higher concentration of 1 \(\mu\text{M}\) significantly increased the amplitude of mIPSCs in both wild-type and PRIP-1 KO mice, but not the frequency (Fig. 5). However, there was no significant difference in the extent of DZP \((1 \mu\text{M})\)-induced enhancement of synaptic currents between PRIP-1 KO neurons and wild-type neurons (Fig. 5). In other words, deficiency of the PRIP-1 protein had no clear effect on synaptic GABAergic currents.

**Extrasynaptic GABAergic Transmission Is Dysfunction in PRIP-1 KO Mice.** We next examined the properties of tonic GABA\(_A\)-R-mediated currents in wild-type and PRIP-1 KO mice. Drugs were applied to the cells by using a bath perfusion system. To measure the tonic current amplitude, the zero level of the holding current was determined by applying BMI \((50 \mu\text{M})\) at the end of the experiment. In these experiments, the GABAergic tonic component was measured as the change in holding current from this zero current level (Fig. 6). First, we analyzed the amplitude of the tonic current in the absence of DZP. The amplitude of the tonic current in PRIP-1 KO mice \((4.7 \pm 0.5; n = 7)\) was significantly decreased compared with that in wild-type mice \((24.1 \pm 5.3; n = 5)\) (Fig. 6). We also confirmed that the amplitude of the tonic current of cerebral cortex pyramidal neurons in PRIP-1 KO.
KO mice (2.9 ± 0.9; n = 4) was less than that in wild types (10.0 ± 1.6; n = 4). Second, we examined the effect of DZP (1 μM) on tonic currents to examine whether the GABA\(_A\)-Rs in the extrasynaptic region contained functional γ-subunits. The inward shift evoked by application of DZP from the initial holding current level (the dashed line in Fig. 6) indicated the enhanced component of the tonic current. The amplitude of the DZP-enhanced tonic current in PRIP-1 KO mice (7.4 ± 1.5 pA; n = 7) was significantly less than that in wild-type mice (35.5 ± 5.2 pA; n = 11). Although DZP augmented the tonic current in PRIP-1 KO mice, this enhanced amplitude was still considerably smaller than the non-DZP-enhanced tonic current in wild-type mice.

We also analyzed the effect of DZP on holding currents in PRIP-1 KO and wild-type mice, to examine whether the absence of PRIP-1 affects the DZP sensitivity of GABA\(_A\)-Rs in the extrasynaptic regions. In this case, the relative values of the DZP-induced component were obtained by comparing the amplitude of the tonic current in the absence and presence of DZP. There was no significant difference between the relative value for wild-type mice (66.2 ± 5.3% of the tonic current in the absence of DZP; n = 5) and that for PRIP-1 KO mice (58.7 ± 6.7%; n = 5).

Expression Analysis of GABA\(_A\) Receptors in PRIP-1 KO Mice. The PRIP-1 protein is involved in the trafficking of GABA\(_A\)-R subunits. We therefore compared the expression of each GABA\(_A\)-R, at the mRNA and protein levels, in PRIP-1 KO and wild-type mice. Quantitative reverse transcription-PCR and Western blotting of hippocampal samples showed that there were no significant differences in GABA\(_A\)-R expression at the mRNA or protein level for whole tissue samples, respectively, between PRIP-1 KO and wild-type mice (Fig. 7, A and B). As for protein levels of membrane fraction, expression of β3 and γ3-subunits significantly decreased in PRIP-1 KO (p < 0.05). To examine expression of functional GABA\(_A\)-Rs, autoradiography was performed with \(^{3}H\)muscimol, which specifically binds GABA\(_A\)-Rs. There were no significant differences in signal intensity between PRIP-1 KO and wild-type animals in each hippocampal region (Fig. 7D).

Discussion

In this study, we demonstrated that PRIP-1 KO mice characteristically demonstrate epileptogenesis with spontaneous seizures. There are several animal models of epilepsy gener-
dentate gyrus regions, respectively. Mol. CA1, CA2–3, and DG indicate the hippocampal CA1, CA2/CA3, and DG subunits at the protein level in whole tissue (B) or the membrane fraction (C). D, autoradiographic analysis of GABA-A-R-binding using [3H]muscimol at ASPET Journals on July 8, 2017 jpet.aspetjournals.org Downloaded from

Because the PRIP-1 protein is involved in the trafficking of GABA-A receptors (Kanematsu et al., 2002), it is likely that dysfunction of GABAergic transmission is responsible for the spontaneous seizures in PRIP-1 KO mice.

In a previous study, PRIP-1 KO mice showed hypersensitivity to pentylenetetrazol-induced convulsion, possibly because of a loss of inhibitory effect on excessive excitation via GABAergic transmission (Yamaguchi et al., 2004). Here, we demonstrated that DZP was less effective in suppressing MES-induced seizure in PRIP-1 KO mice than in wild-type mice. Our results are consistent with those of a previous paper that showed a reduced effect of DZP in PRIP-1 KO mice (Kanematsu et al., 2002). However, in the present study, spontaneous seizures detected by EEG were abolished by the application of DZP at clinically relevant concentrations. In contrast, interictal discharges were not completely suppressed by DZP, even at high concentrations in these mice. These results suggest that the epileptic activity in PRIP-1 KO mice cannot be explained simply by a dysfunction in GABA-A-R γ-subunits.

To examine other dysfunctions in GABAergic transmission, we investigated synaptic and tonic GABAergic responses in PRIP-1 KO and wild-type mice. The features of synaptic GABAergic transmission, such as mIPSC amplitude and frequency, were not affected by knockout of the PRIP-1 gene. The sensitivity of mIPSC events to DZP in PRIP-1 KO mice was also similar to that in wild types. In contrast, functional changes in tonic GABAergic responses were detected in PRIP-1 KO mice. Tonic GABAergic transmission itself was profoundly attenuated in PRIP-1 KO mice. Even in the presence of DZP, the amplitude of the tonic current in PRIP-1 KO mice was not enough to reach the normal, non-DZP-enhanced levels of wild-type mice. Consequently, the effect of DZP on MES-evoked seizures in PRIP-1 KO mice was reduced.

In other studies, tonic GABAergic responses were recorded in the presence of a low concentration of GABA (approximately 1 μM), under physiological conditions (Semyanov et al., 2004; Möhler, 2006), because 1 μM GABA is near to the concentration of ambient GABA (approximately 0.8 μM) (Lerm et al., 1986). The composition of subunits of extrasynaptic GABA-A-Rs is thought to be α6β6, α4β6, and α5β2γ2 in the cerebellar granule layer and the hippocampal dentate gyrus granule cells. These extrasynaptic GABA-A-Rs possess a high affinity for GABA and demonstrate the slow inactivation current kinetics of a tonic GABA response (Semyanov et al., 2004; Möhler, 2006). It has been previously reported that DZP failed to enhance GABA (3 μM)-induced currents using dissociated hippocampal CA1 pyramidal neurons in PRIP-1 KO mice, and the amplitude of the GABA-induced current and the dose-response relationship of GABA were not different between PRIP-1 KO and wild-type mice (Kanematsu et al., 2002). These previous data are consistent with the present notion that extremely attenuated extrasynaptic GABAergic transmission is involved in the reduced effect of DZP in PRIP-1 KO mice. Further pharmacological analysis is required to determine the precise combination of GABA-A-R subunits in the extrasynaptic regions in PRIP-1 KO mice.

The present study suggests that PRIP-1 plays an important role in the regulation of extrasynaptic GABA-A-R function, probably via GABA-A-R β-subunits. GABA-A-R β-subunits are involved in the trafficking of GABA-A-Rs and have a
phosphorylation site within the cytoplasmic loop. Studies have revealed that PRIP-1 directly interacts with β-subunits of GABA<sub>A</sub>-Rs and is involved in the regulation of GABA<sub>A</sub>-R phosphorylation (Kanematsu et al., 2007; Fuji et al., 2010). Furthermore, PRIP-1 could modulate internalization of GABA<sub>A</sub>-Rs via β-subunit phosphorylation (Kanematsu et al., 2007; Fuji et al., 2010). In this study, expression analysis of GABA<sub>A</sub>-Rs at the protein level revealed that the expression of membrane fraction β3- and γ3-subunits was significantly decreased in PRIP-1 KO mice. However, expression analysis of the membrane fraction cannot discriminate between synaptic and extrasynaptic regions. Therefore, the contribution of β3 and γ3 expression alteration is still unknown.

With regard to functional receptor expression, autoradiography using [³H]muscimol and Western blotting analysis with whole tissue samples showed that the hippocampal signals were not significantly different between PRIP-1 KO and wild-type mice (Fig. 7, B and D). This implies that there is no difference in the amount of functional GABA<sub>A</sub>-R subunits in the hippocampus between wild-type and KO mice. As for the current amplitude, the tonic GABAergic current in PRIP-1 KO mice was significantly reduced, whereas the phasic current showed no significant changes between PRIP-1 KO mice and wild-type mice. In contrast, DZP evoked the same percentage of enhancement of tonic current in both PRIP-1 KO and wild-type mice. These expression and electrophysiological studies support the idea that GABA<sub>A</sub>-R expression levels in the extrasynaptic region were markedly attenuated in PRIP-1 KO mice, and GABA<sub>A</sub>-Rs in the extrasynaptic region contain the γ-subunit that confers DZP sensitivity.

Compared with PRIP-DKO mice (Mizokami et al., 2007), PRIP-1 KO mice showed smaller changes in GABA<sub>A</sub>-R expression at the mRNA and protein levels and ligand-binding patterns. This is probably because of differences in the expression patterns of the PRIP-1 and PRIP-2 proteins. PRIP-2 is expressed ubiquitously, whereas PRIP-1 is downregulated through the pathway of the interictal discharges. Epilepsy or seizure activity upregulated tonic currents in PRIP-1 KO mice, whereas the phasic current showed no significant changes in GABA<sub>A</sub>-R expression. Consequently, the dysfunction in tonic GABAergic transmission is mediated by clathrin and AP2 adaptor complex. J Neurochem 101:988–905. In this study, the expression of PRIP-2 was not significantly different between PRIP-1 KO and wild-type mice. These expression and electrophysiological studies support the idea that GABA<sub>A</sub>-R expression levels in the extrasynaptic region were markedly attenuated in PRIP-1 KO mice, and GABA<sub>A</sub>-Rs in the extrasynaptic region contain the γ-subunit that confers DZP sensitivity.

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Authorship Contributions

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Wrote or contributed to the writing of the manuscript: Zhu, Yoshida, Migita, Yamada, Mori, Tomiyama, Watanabe, Kaneko, Ueno, and Okada.

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