

Propofol Anesthesia Is Reduced in Phospholipase C–Related Inactive Protein Type-1 Knockout Mice[§]

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ABSTRACT

The GABA type A receptor (GABA_A-R) is a major target of intravenous anesthetics. Phospholipase C–related inactive protein type-1 (PRIP-1) is important in GABA_A-R phosphorylation and membrane trafficking. In this study, we investigated the role of PRIP-1 in general anesthetic action. The anesthetic effects of propofol, etomidate, and pentobarbital were evaluated in wild-type and PRIP-1 knockout (*PRIP-1 KO*) mice by measuring the latency and duration of loss of righting reflex (LORR) and loss of tail-pinch withdrawal response (LTWR). The effect of pretreatment with okadaic acid (OA), a protein phosphatase 1/2A inhibitor, on propofol- and etomidate-induced

LORR was also examined. PRIP-1 deficiency provided the reduction of LORR and LTWR induced by propofol but not by etomidate or pentobarbital, indicating that PRIP-1 could determine the potency of the anesthetic action of propofol. Pretreatment with OA recovered the anesthetic potency induced by propofol in *PRIP-1 KO* mice. OA injection enhanced phosphorylation of cortical the GABA_A-R $\beta 3$ subunit in *PRIP-1 KO* mice. These results suggest that PRIP-1–mediated GABA_A-R $\beta 3$ subunit phosphorylation might be involved in the general anesthetic action induced by propofol but not by etomidate or pentobarbital.

Introduction

Phospholipase C-related inactive protein type-1 (PRIP-1) is an inositol 1,4,5-trisphosphate binding protein, homologous to phospholipase C- $\delta 1$ but catalytically inactive (Kanematsu et al., 1992, 1996; Matsuda et al., 1998). PRIP-1 has various binding partners including GABA type A receptor (GABA_A-R) $\beta 1$ – $\beta 3$ subunits (Terunuma et al., 2004), GABA_A-R-associated protein (Kanematsu et al., 2002), protein phosphatases 1 and 2A (PP1 and PP2A) (Yoshimura et al., 2001; Kanematsu et al., 2006; Yanagihori et al., 2006), and Akt protein kinase (Fuji et al., 2010). PRIP-1 acts as a bridge between the $\gamma 2$ GABA_A-R subunit and GABA_A-R-associated protein, and facilitates GABA_A-R membrane trafficking (Kanematsu et al., 2002;

Mizokami et al., 2007). PRIP-1 binds to GABA_A-R β subunits as a scaffold protein of PP1/2A, which regulate the phosphorylation level of β subunits (Terunuma et al., 2004; Kanematsu et al., 2006, 2007; Yanagihori et al., 2006). PP1/2A dephosphorylate the β subunit and induce receptor endocytosis mediated by clathrin adaptor protein (AP2) and clathrin (Comenencia-Ortiz et al., 2014). PRIP-1 inactivates PP1 activity and reduces PP2A activity (Terunuma et al., 2004; Kittler et al., 2005; Kanematsu et al., 2007; Sugiyama et al., 2012). Phosphorylation of PRIP-1 itself and lack of PRIP-1 liberates the active form of PP1 and further activates PP2A (Terunuma et al., 2004; Yanagihori et al., 2006; Sugiyama et al., 2012).

Our previous studies revealed that PRIP-1 knockout (*PRIP-1 KO*) mice had reduced extrasynaptic GABAergic transmission (tonic inhibition) in the hippocampus, cerebral cortex, and spinal cord (Migita et al., 2011; Zhu et al., 2012). Furthermore, the effects of diazepam on GABAergic extrasynaptic transmission were markedly reduced in *PRIP-1 KO* mice compared with wild-type (WT) mice. Consequently, the anticonvulsant action of diazepam was suppressed in *PRIP-1 KO* mice

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ABBREVIATIONS: ANOVA, analysis of variance; AP2, clathrin adaptor protein; AUC, area under the curve; GABA_A-R, GABA type A receptor; KO, knockout; LORR, loss of righting reflex; LTWR, loss of tail-pinch withdrawal response; MPE, maximal possible effect; OA, okadaic acid; PP, protein phosphatase; PRIP-1, phospholipase C-related inactive protein type-1; TAS, total anesthesia score; WT, wild type.

(Zhu et al., 2012). However, it remains unknown how PRIP-1 affects anesthetic action through the regulation of GABAergic transmission.

Most intravenous anesthetics enhance the inhibitory action of GABA_A-R (Rudolph and Antkowiak, 2004). Extrasynaptic GABAergic transmission is more likely to contribute to general anesthesia than synaptic transmission (Semyanov et al., 2004; Bieda and MacIver, 2004; Belelli et al., 2009; Bieda et al., 2009; Kubo et al., 2009; Herd et al., 2014). The phosphorylation state of GABA_A-R is thought to determine its localization and activity in the synaptic or extrasynaptic membrane (Terunuma et al., 2004; Abramian et al., 2010; Comenencia-Ortiz et al., 2014).

We investigated whether PRIP-1 deficiency influences the anesthetic action of three intravenous anesthetics—propofol, etomidate, and pentobarbital—and whether PRIP-1-mediated GABA_A-R phosphorylation states are involved in their anesthetic action.

Materials and Methods

Animals. We used adult male *PRIP-1 KO* ($n = 223$) and WT (C57BL/6; $n = 184$) mice, aged 12–18 weeks and weighing 25–35 g. Animals were group-housed at $24 \pm 2^\circ\text{C}$ under a 12-hour light/dark cycle (lights on at 8:00 AM), and food and water were available ad libitum. The experimental procedures used in this study complied with the guidelines for animal research issued by the Physiologic Society of Japan and Hirosaki University School of Medicine, and all efforts were made to minimize the number of animals used and their suffering.

Drugs. For behavioral studies, mice received propofol (Maruishi Pharmaceuticals, Osaka, Japan), etomidate (Tokyo Chemical Industry, Tokyo, Japan), or pentobarbital (Somunopentyl; Kyoritsu Seiyaku, Tokyo, Japan) intraperitoneally (i.p.) at a volume of $10 \mu\text{L/g}$ body weight. The vehicle for propofol and etomidate was 20% intralipid fluid solution (Fresenius Kabi, Tokyo, Japan). Pentobarbital was diluted with 0.9% saline (Otsuka Pharmaceuticals, Tokyo, Japan). PP1/2A inhibitor okadaic acid (OA) sodium salt (Wako Pure Chemical Industries, Tokyo, Japan) was dissolved in 0.9% saline (0.01 mg/ml) and injected ($10 \mu\text{L/g}$ body weight i.p.) 30 minutes before administration of propofol or etomidate. The dose of OA used in this study did not cause cyanosis, dyspnea, or diarrhea (Tubaro et al., 2003).

No hypnotic or analgesic effects were observed after intralipid or saline injection in either genotype ($n = 4$ per group; data not shown). For Western blotting, mice were injected ($10 \mu\text{L/g}$ body weight i.p.) with OA (0.01 mg/ml) or saline. Other animals received OA ($1 \text{ pg}/5 \mu\text{L}$) or vehicle (saline, $5 \mu\text{L}$) intracerebroventricularly (i.c.v.) under 1%–3% isoflurane (Escaïn; Pfizer, Tokyo, Japan) for general anesthesia and lidocaine (Xylocaine jelly 2%; AstraZeneca, Osaka, Japan) for local surface anesthesia, as described previously elsewhere (Maeda et al., 2005). Thirty minutes after the i.p. injection, or 25 minutes after the i.c.v. injection, the mice were euthanized with pentobarbital (150 mg/kg i.p.), and the brain tissue was collected ($n = 3$ per group).

Behavioral Analysis. Loss of righting reflex (LORR) was used to measure the hypnotic effect of the anesthetic agents. Immediately after drug injection, each animal was placed in a chamber ($20 \times 28 \times 15 \text{ cm}$) with an electric heat pad, and the righting reflex was assessed every 2 minutes for a maximum of 2 hours. Anesthesia was evaluated using the following scoring system (Irifune et al., 2003): 0, normal righting reflex; 1, righting within 2 seconds; 2, righting latency 2–10 seconds; 3, no righting within 10 seconds. Anesthetic scores were determined every 2 minutes as the median of three trials. Total anesthetic score (TAS) was the sum of all anesthetic scores used to evaluate anesthetic efficacy. Sensitivity to anesthetics was expressed as the percentage of animals to be given a score of 3 (% LORR). The time from drug administration to a score of 3 was considered the

latency to LORR, and the duration of LORR was measured as the time between a score of 3 and a subsequent score of 2. When a score of 1 was followed by two consecutive scores of 0, the mice were considered to have recovered from anesthesia.

Loss of tail-pinch withdrawal response (LTWR) assay was performed to evaluate immobilization, which is known to be the anesthetic-induced ablation of the supraspinal nociceptive response to noxious stimuli. A surgical clip (125 g/cm^2 , micro-serrefine no. 18055-04; Fine Science Tools, Foster City, CA) was placed at the base of an animal's tail for 10 seconds (cutoff time) at 15 minutes after anesthetic administration. The nociceptive response was measured by the time (test latency) when the mouse showed any response to the tail-pinch.

Baseline nociceptive response (baseline latency) was measured 30 minutes before drug injection. To estimate LTWR, the percentage of maximal possible effect (% MPE) was evaluated using this formula: $\% \text{ MPE} = 100 \times (\text{Test latency} - \text{Baseline latency}) / (\text{Cutoff time} - \text{Baseline latency})$. The magnitude of LTWR was assessed by the area under the % MPE versus time curve (area under the curve [AUC]) using the trapezoidal method (Maeda et al., 2005).

Western Blotting. Immediately after euthanasia, each mouse was perfused with ice-cold phosphate-buffered saline and the brain removed (Maeda et al., 2005; Zhu et al., 2012). Thick coronal sections of the frontal cortex were made at the level of the striatum (Paxinos and Franklin, 2001). Total protein extracts and Western blotting were performed as described elsewhere (Ozaki et al., 2012). The crude membrane fraction was prepared using a previous method with modification (Goebel-Goody et al., 2009; Garcão et al., 2014). Tissue was homogenized in ice-cold homogenization buffer (pH 6.0) containing the following (in mM): 320 sucrose, 20 Tris-HCl, 0.1 CaCl_2 , 1 MgCl_2 , cOmplete Protease Inhibitor Cocktail EDTA-free (Roche Diagnostics, Mannheim, Germany), and PhosSTOP Phosphatase Inhibitor Cocktail (Roche Diagnostics). The homogenate was centrifuged at $1000g$ for 10 minutes, and then supernatant was further centrifuged at $10,000g$ for 20 minutes to obtain the crude membrane fraction. The precipitate was dissolved in homogenization buffer containing 1% SDS and saved as the crude membrane fraction. Solubilized proteins were isolated from the frontal cortex and equal amounts of protein was separated by SDS-PAGE and transferred electrophoretically to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA).

Membranes were incubated with the following primary antibodies: anti-GABA_A-R $\beta 2,3$ subunit antibody (Millipore, Temecula, CA); anti-GABA_A-R $\beta 3$ subunit antibody (Millipore); anti-GABA_A-R $\beta 3$ phospho-Ser408/409 antibody (PhosphoSolutions, Aurora, CO); anti- β -actin antibody (Abcam, Cambridge, MA); or anti-gephyrin antibody (Synaptic Systems, Göttingen, Germany). Membranes were washed with Tween 20–phosphate-buffered saline and incubated with horseradish peroxidase-conjugated goat anti-rabbit or rabbit anti-mouse IgG (Dako, Cambridge, UK). Immunoreactive signals were developed with an enhanced chemiluminescence Western blotting detection kit (GE Healthcare, Princeton, NJ) and quantified using a luminescent image analyzer (LAS-4000; Fujifilm, Tokyo, Japan). Band intensities were measured using ImageJ software (<http://rsbweb.nih.gov/ij/>).

Statistics. To estimate the median effective dose (ED_{50}) with 95% confidence, probit analysis was performed on the LORR dose–response data for each anesthetic using the generalized linear model function (glm) with probit link function and dose predict function (dose.p) in the R-package MASS (Venables and Ripley, 2002; R Core Team, 2016). TAS, expressed as the median (range), was analyzed using the Mann-Whitney U test. Data for the latency and duration of LORR and percentage with MPE of LTWR are expressed as mean \pm S.E.M.

To compare the effects of anesthetics on LORR and LTWR between genotypes, unpaired t tests were performed. One-way analysis of variance (ANOVA) with a post hoc Tukey's test was used to determine the effects of OA on LORR induced by propofol or etomidate. Unpaired t tests were used to assess the effects of OA on membrane expression

and phosphorylation levels of the GABA_A-R $\beta 3$ subunit. All statistical analyses were performed with R version 3.3.2 (R Core Team, 2016). $P < 0.05$ was considered statistically significant.

To estimate effect size as a benchmark for assessing the magnitude of differences between WT and *PRIP-1 KO* mice, Cohen's d value for unpaired t tests and Mann-Whitney U tests, and Cohen's f value for one-way ANOVA were computed using the test function in the R-package `compute.es` (Del Re, 2013). Cohen defined a small effect as $d = 0.20$ or $f = 0.10$, medium effect as $d = 0.50$ or $f = 0.25$, and a large effect as $d = 0.80$ or $f = 0.40$ (Cohen, 1992).

Results

Effect of PRIP-1 Deficiency on Sensitivity to Propofol, Etomidate, and Pentobarbital. First, we determined the ED₅₀ for the number of mice showing LORR (% LORR) with each anesthetic in WT and *PRIP-1 KO* mice. Table 1 summarizes LORR and TAS values at each dose for propofol, etomidate, and pentobarbital in *PRIP-1 KO* and WT mice. The dose-response curve of % LORR for each anesthetic is shown in Fig. 1. The ED₅₀ of propofol in *PRIP-1 KO* mice was greater than that in WT mice (Fig. 1A, left panel). Propofol at the maximum dose of 140 mg/kg could not evoke LORR in all *PRIP-1 KO* mice tested. TAS for propofol in *PRIP-1 KO* mice was lower than that in WT mice (Table 1). There were no differences between genotypes for etomidate or pentobarbital ED₅₀ values (Fig. 1, B and C, left panels).

Effect of PRIP-1 Deficiency on Induction and Maintenance of Hypnosis Induced by Propofol, Etomidate, and Pentobarbital. Because the highest dose of each anesthetic in this study was most effective at inducing a stable LORR occurrence and/or increased anesthetic scores for *PRIP-1 KO* mice (Table 1; temporal anesthetic score changes after each anesthetic treatment are shown in Supplemental Figs. 1–3), we estimated the hypnotic potency of these anesthetics at the highest doses by measuring the latency and duration of LORR. Latency to LORR in *PRIP-1 KO* mice after injection of propofol (140 mg/kg i.p.) was significantly longer than that

in WT mice (Fig. 1A, right panel). The duration of propofol-induced LORR in *PRIP-1 KO* mice was significantly shorter than that in WT mice. There were no significant differences in latency or duration of LORR between genotypes after etomidate (30 mg/kg i.p.; Fig. 1B, right panel) or pentobarbital (40 mg/kg i.p.; Fig. 1C, right panel) administration.

Effect of PRIP-1 Deficiency on Immobilization Produced by Propofol, Etomidate, and Pentobarbital.

LTWR was examined to evaluate the immobilizing potency of each anesthetic in WT and *PRIP-1 KO* mice. *PRIP-1 KO* mice showed attenuation of propofol (140 mg/kg i.p.)-evoked LTWR (% MPE) in comparison with WT mice (Fig. 2A). The magnitude of propofol-induced LTWR (AUC) in *PRIP-1 KO* mice was significantly smaller than that in WT mice. There were no significant differences between genotypes in % MPE and AUC of LTWR produced by etomidate (30 mg/kg i.p.; Fig. 2B) or pentobarbital (40 mg/kg i.p.; Fig. 2C).

Hypnotic Effect of Propofol in PRIP-1 KO Mice Was Rescued by OA Pretreatment.

PRIP-1 possesses a binding site for GABA_A-R subunits and PP1/2A, and functionally regulates not only trafficking but also phosphorylation of GABA_A-R. We have electrophysiologically demonstrated dysfunction of the extrasynaptic GABA_A-R response in *PRIP-1 KO* mice (Migita et al., 2011; Zhu et al., 2012). Therefore, we examined whether PP1/2A inhibitor rescued the perturbed anesthetic action of propofol. Pretreatment with PP1/2A inhibitor OA before propofol administration reduced LORR latency and prolonged LORR duration in *PRIP-1 KO* mice (Fig. 3A), but did not do the same with etomidate administration (Fig. 3B). The TAS of propofol (140 mg/kg i.p.), but not etomidate (30 mg/kg i.p.), increased after OA pretreatment (0.1 mg/kg i.p.) in *PRIP-1 KO* mice (Table 2). OA pretreatment did not alter these values in WT mice. These results indicated that recovery of the hypnotic effect of propofol in *PRIP-1 KO* mice was provided by phosphorylation of GABA_A-R.

TABLE 1
Effects of anesthetics on TAS and LORR in WT and *PRIP-1 KO* mice

Anesthetic	Dose	WT		<i>PRIP-1 KO</i>		Effect Size ^e
		n^a	TAS ^b	n^a	TAS ^b	
	<i>mg/kg</i>					
Propofol	40	0/6	0 (0–3)	0/6	0 (0–21)	0.40
	60	3/7	1 (0–25)	0/7	0 (0–3)	1.03
	80	4/6	36.5 (0–43)	1/6	16.5 (0–24) ^d	1.51
	100	6/7	52 (0–110)	6/14	7.5 (0–45) ^d	1.31
	120	7/7	97 (14–161)	7/13	21 (6–127) ^d	0.92
	140	7/7	71 (54–149)	10/14	29 (6–79) ^d	1.87
Etomidate	5	0/6	0 (0–1)	0/6	0 (0–3)	0.07
	8	2/10	2.5 (0–81)	2/11	0 (0–41)	0.67
	10	7/8	21 (0–78)	8/9	17 (0–86)	0.21
	20	6/6	47 (36–147)	6/6	48 (19–101)	0.05
	30	9/9	125 (82–180)	10/10	107.5 (58–180)	0.72
Pentobarbital	10	0/6	0 (0–0)	0/6	3 (0–15)	0.77
	15	2/6	11.5 (0–29)	2/5	12 (6–68)	0.64
	20	5/7	15 (5–22)	5/7	15 (3–43)	0.17
	30	6/6	38.5 (24–46)	9/9	30 (18–43)	0.82
	40	6/6	88 (41–136)	6/6	71 (42–164)	0.28

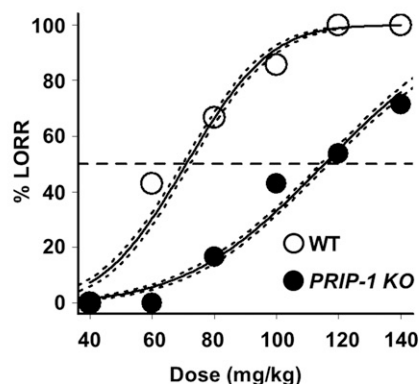
^aDenominators represent the number of animals tested in each group. Numerators represent animals that showed LORR.

^bTAS express the sum of anesthetic scores as median (range).

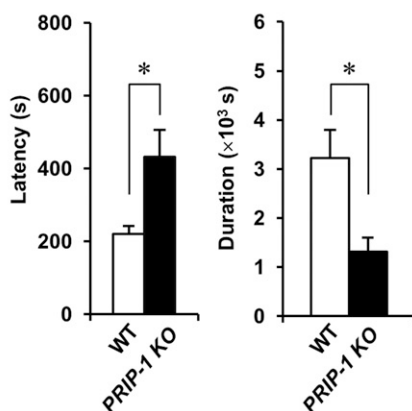
^cEffect size (Cohen's d) for TAS compared with WT mice with the same treatment.

^d $P < 0.05$ versus WT mice with the same treatment (Mann-Whitney U test).

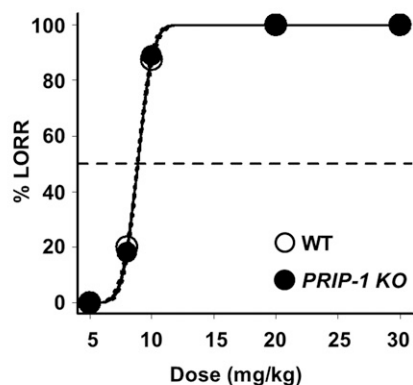
A Propofol



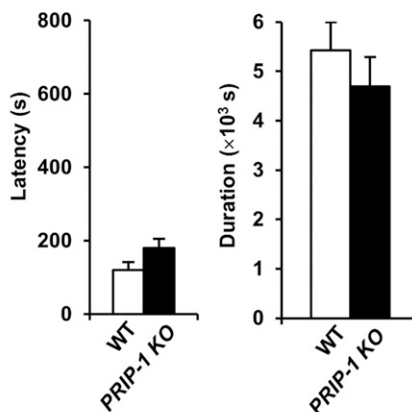
Propofol 140 mg/kg i.p.



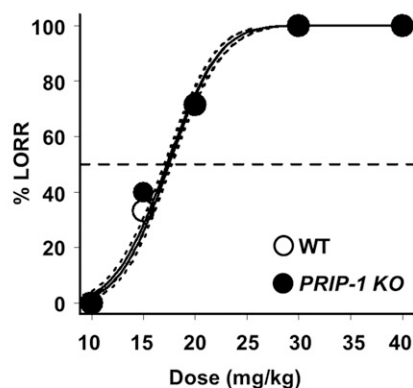
B Etomidate



Etomidate 30 mg/kg i.p.



C Pentobarbital



Pentobarbital 40 mg/kg i.p.

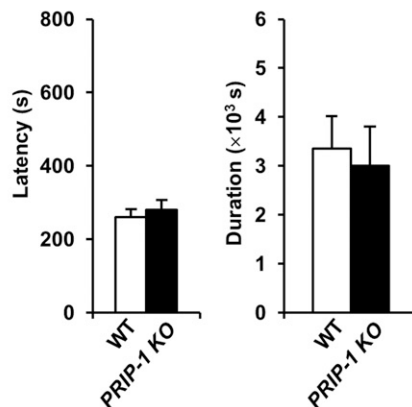


Fig. 1. Effect of PRIP-1 deficit on hypnosis produced by propofol, etomidate, and pentobarbital. (A) Dose–response curves for LORR induced by propofol in WT and *PRIP-1 KO* mice. Probit analysis was used to fit curves of ED₅₀ values (solid lines) and 95% confidence intervals (dashed curves). ED₅₀ values were 71.0 mg/kg (95% confidence interval, 69.8–72.2 mg/kg) in WT mice and 115 mg/kg (114–117 mg/kg) in *PRIP-1 KO* mice. Significant prolongation of latency to LORR was observed with administration of propofol (140 mg/kg i.p.) in *PRIP-1 KO* mice (unpaired *t* test: $t_{15} = 2.95$, $P < 0.05$, Cohen's $d = 1.30$). *PRIP-1 KO* mice emerged from propofol-induced LORR significantly earlier than WT mice ($t_{15} = 3.16$, $P < 0.05$, $d = 1.80$). (B) ED₅₀ values of etomidate were 8.85 mg/kg (8.78–8.91 mg/kg) in WT mice and 8.85 mg/kg (8.79–8.92 mg/kg) in *PRIP-1 KO* mice, and (C) those of pentobarbital were 17.5 mg/kg (17.2–17.8 mg/kg) in WT mice and 17.2 mg/kg (16.9–17.5 mg/kg) in *PRIP-1 KO* mice. There were no significant differences in response to etomidate (30 mg/kg i.p.) or pentobarbital (40 mg/kg i.p.) between WT and *PRIP-1 KO* mice (etomidate: latency, $t_{17} = 1.76$, $P > 0.05$, $d = 0.81$; duration, $t_{17} = 0.94$, $P > 0.05$, $d = 0.43$; pentobarbital: latency, $t_{10} = 0.68$, $P > 0.05$, $d = 0.36$; duration, $t_{10} = 0.41$, $P > 0.05$, $d = 0.22$). Group $n \geq 5$ mice. * $P < 0.05$ versus WT mice. Data are mean \pm S.E.M.

Effect of OA on Protein Expression of GABA_A-R β 2,3 Subunits and the Phosphorylated β 3 Subunit in *PRIP-1 KO* Mice. The GABA_A-R β 3 subunit is thought to be a common target of propofol and etomidate. Therefore, we performed Western blotting to evaluate the effect of OA pretreatment on phosphorylation states for the cortical GABA_A-R β 3 subunit. There was less phosphorylation of the GABA_A-R β 3 subunit in the frontal cortex of *PRIP-1 KO* mice than in WT mice (Fig. 4A). Both i.p. and i.c.v.

administration of OA enhanced GABA_A-R β 3 subunit phosphorylation without affecting overall levels of the β 2,3 subunit in *PRIP-1 KO* mice. In addition, these OA treatments induced enhancement of β 3 subunit membrane expression and phosphorylation levels (Fig. 4B). The β 3 subunit membrane expression levels in *PRIP-1 KO* mice were lower than those in WT mice after saline treatment (Fig. 4C). After i.p. or i.c.v. OA treatments, β 3 subunit membrane expression increased in both genotypes. Phosphorylation levels of the

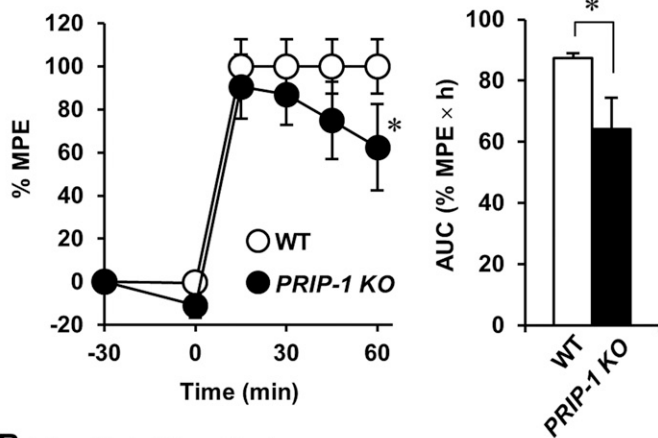
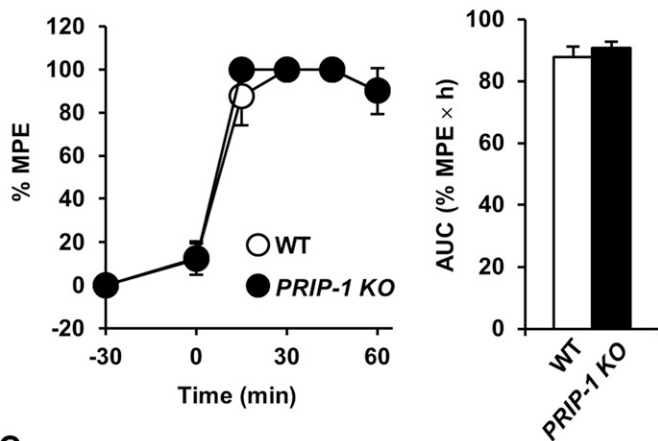
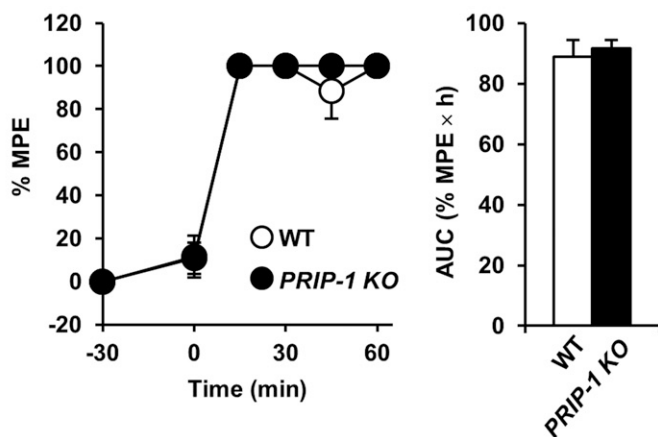
A Propofol 140 mg/kg i.p.**B Etomidate 30 mg/kg i.p.****C Pentobarbital 40 mg/kg i.p.**

Fig. 2. Effect of PRIP-1 deficit on immobilization induced by propofol, etomidate, and pentobarbital. (A) Time course and magnitude of propofol-induced LTWR. Significant reduction of percentage of MPE and AUC were observed in *PRIP-1 KO* mice (unpaired *t* test: % MPE at 60 minutes, $t_{15} = 2.30$, $P < 0.05$, Cohen's $d = 1.12$; AUC, $t_{15} = 2.25$, $P < 0.05$, $d = 1.10$). (B, C) There were no significant differences in LTWR produced by etomidate or pentobarbital between WT and *PRIP-1 KO* mice (etomidate: AUC, $t_{15} = 0.82$, $P > 0.05$, $d = 0.40$; pentobarbital: AUC, $t_9 = 0.49$, $P > 0.05$, $d = 0.27$). Group $n \geq 6$ mice. * $P < 0.05$ versus WT mice. Data are mean \pm S.E.M.

membrane GABA_A-R $\beta 3$ subunit in i.p. and i.c.v. saline-administered *PRIP-1 KO* mice tended to be lower than in WT mice (Fig. 4D). After i.p. or i.c.v. OA injections,

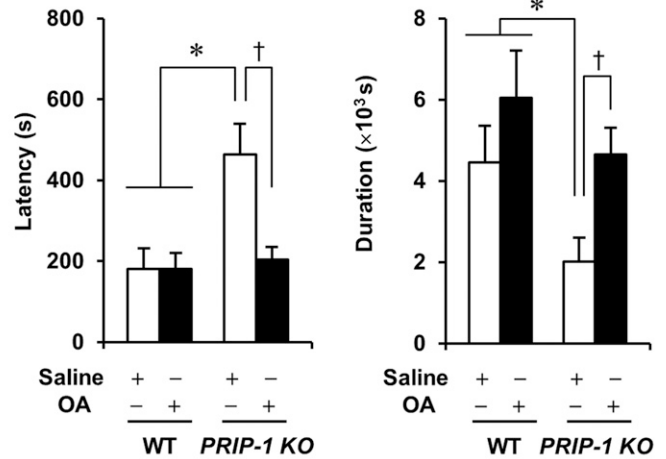
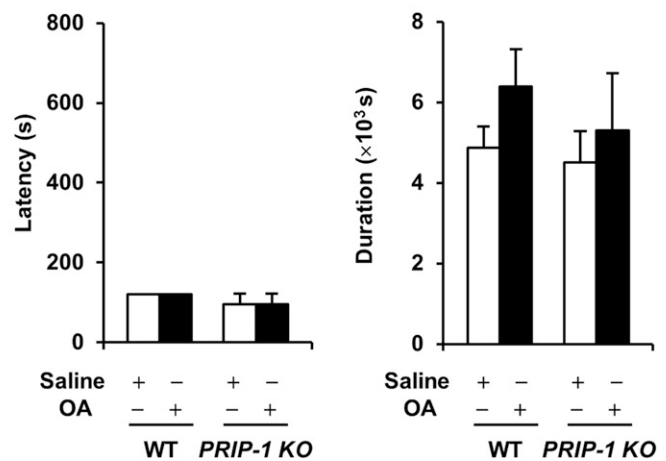
A OA 0.1 mg/kg i.p. + Propofol 140 mg/kg i.p.**B OA 0.1 mg/kg i.p. + Etomidate 30 mg/kg i.p.**

Fig. 3. Effect of OA pretreatment on hypnosis induced by propofol and etomidate in *PRIP-1 KO* and WT mice. (A) Latency and duration of propofol-induced LORR. Pretreatment with OA shortened the latency and extended the duration of LORR in *PRIP-1 KO* mice (latency: one-way ANOVA, $F_{3, 26} = 9.13$, $P < 0.001$, Cohen's $f = 1.03$; duration: $F_{3, 26} = 7.14$, $P < 0.01$, $f = 0.91$). (B) Latency and duration of etomidate-induced LORR. There were no significant differences in etomidate-induced LORR in either genotype after preinjection with OA (latency: $F_{3, 11} = 2.86$, $P > 0.05$, $f = 0.33$; duration: $F_{3, 11} = 0.39$, $P > 0.05$, $f = 0.40$). Group $n \geq 4$ mice. Tukey's post hoc comparison: * $P < 0.05$, Cohen's $d > 1.50$, versus WT mice; † $P < 0.05$, $d > 1.60$, versus *PRIP-1 KO* mice pretreated with saline. Data are mean \pm S.E.M.

phosphorylated $\beta 3$ levels in *PRIP-1 KO* mice were higher (~2-fold) than those in saline-injected *PRIP-1 KO* mice.

Discussion

In this study, we found that a lack of PRIP-1 attenuated the hypnotic and immobilizing effects of propofol but not etomidate or pentobarbital. Intraperitoneal pretreatment with OA rescued hypnotic effect of propofol in *PRIP-1 KO* mice whereas OA did not alter the effect of propofol in WT mice. Furthermore, we confirmed that the dephosphorylated state of the GABA_A-R $\beta 3$ subunit in *PRIP-1 KO* mice was recovered by i.p. OA administration, similar to i.c.v. administration.

TABLE 2
Effects of OA on anesthetic-induced TAS and LORR in WT and *PRIP-1 KO* mice

Anesthetic	Dose	OA Dose	WT		<i>PRIP-1 KO</i>		Effect Size
			<i>n</i> ^a	TAS ^b	<i>n</i> ^a	TAS ^b	
		<i>mg/kg</i>					
Propofol	140	0	6/6	121 (68–151)	7/8	38.5 (3–99) ^c	1.59 ^d
	140	0.1	6/6	169.5 (106–179)	11/12	148 (30–175) ^e	0.87, ^d 1.33 ^f
Etomidate	30	0	4/4	112 (110–149)	5/5	110 (89–177)	0.92 ^d
	30	0.1	4/4	179 (99–181)	5/5	166 (16–180)	0.70, ^d 0.79 ^f

^aDenominators represent the number of animals tested in each group. Numerators represent animals that showed LORR.

^bTAS express the sum of anesthetic scores as median (range).

^c*P* < 0.05 versus WT mice with the same treatments.

^dEffect size (Cohen's *d*) for TAS compared with WT mice with the same treatment.

^e*P* < 0.05 versus *PRIP-1 KO* mice pretreated with OA (0 mg/kg) (Mann-Whitney *U* test).

^fCohen's *d* for TAS compared with *PRIP-1 KO* mice pretreated with OA (0 mg/kg).

Propofol mainly exerts its anesthetic action via the GABA_A-R $\beta 3$ subunit (Jurd et al., 2003; Feng and Macdonald, 2004; Belelli et al., 2005; Drexler et al., 2009). Etomidate potentiates GABA_A-R containing $\beta 3$ or $\beta 2$ subunits (Reynolds et al., 2003; Belelli et al., 2005; Drexler et al., 2009). Our current and previous studies have shown that *PRIP-1* deficiency reduces $\beta 3$ subunit membrane expression and induces its dephosphorylation (Fig. 4; Zhu et al., 2012). This is because a lack of *PRIP-1* induces PP1/2A activation and AP2-clathrin-mediated internalization (Terunuma et al., 2004; Kittler et al., 2005; Kanematsu et al., 2007). However, in this study, the total expression level of $\beta 2/3$ subunits in *PRIP-1 KO* mice did not seem to differ from that of WT mice, suggesting that the reduction in $\beta 3$ subunit membrane expression might be compensated by the $\beta 2$ subunit.

Propofol affinity for the open state of $\alpha 1\beta 2\gamma 2L$ GABA_A-R is approximately 3- to 7-fold weaker than etomidate (Rüsch et al., 2004; Ruesch et al., 2012). In addition to the $\beta 3$ subunit, $\alpha 1/4$ and $\beta 2$ GABA_A-R subunits may be responsible for pentobarbital anesthesia (Zeller et al., 2007; Mercado and Czajkowski, 2008). This evidence suggests that nonsignificant but moderate effective changes of etomidate- and pentobarbital-anesthesia in *PRIP-1 KO* mice would be because of membrane expression of $\beta 2$ -containing GABA_A-R. There is a possibility that down-regulation of $\beta 3$ -containing GABA_A-R in cell surface attenuates propofol anesthesia.

Tonic inhibition mediated by extrasynaptic GABA_A-R activity is more likely to be involved in anesthetic action than phasic inhibition mediated by synaptic activity (Akk et al., 2004; Bieda and MacIver, 2004; Belelli et al., 2009; Bieda et al., 2009; Kubo et al., 2009; Herd et al., 2014). Extrasynaptic GABA_A-R activity partly depends on receptor phosphorylation by various kinases such as protein kinase A and protein kinase C (Comenencia-Ortiz et al., 2014). For example, protein kinase C-induced phosphorylation of $\alpha 4\beta 3$ subunits, which mediate tonic inhibition, stabilizes GABA_A-R in the cell surface. Consequently, phosphorylated GABA_A-R causes enhancement of GABA-mediated currents (Abramian et al., 2010). In contrast, dephosphorylation of the $\beta 3$ subunit by PP1/2A triggers AP2-clathrin-dependent internalization (Terunuma et al., 2004; Kittler et al., 2005; Kanematsu et al., 2007), probably resulting in the reduction of GABA-mediated currents.

Our current and previous studies revealed that *PRIP-1* deficiency reduces expression levels of both total and phosphorylated

GABA_A-R $\beta 3$ subunit in membrane fraction (Fig. 4; Terunuma et al., 2004; Zhu et al., 2012). Functionally, *PRIP-1* deficiency reduces basal tonic currents but not phasic currents in hippocampal and cortical neurons (Zhu et al., 2012). Therefore, *PRIP-1* could modulate extrasynaptic GABA_A-R activity by preserving the $\beta 3$ subunit phosphorylation state and/or by regulating PP1/2A phosphatase activity. *PRIP-1* might be involved in the induction and maintenance of propofol-induced anesthesia through the trafficking of GABA_A-R and phosphorylation state of GABA_A-R $\beta 3$ subunit.

Contrary to our finding that *PRIP-1* deficiency induced dephosphorylation of the GABA_A-R $\beta 3$ subunit in the frontal cortex, another study has reported that phosphorylation levels of the $\beta 3$ subunit in the hippocampus in *PRIP-1 KO* mice are similar to those of WT mice (Terunuma et al., 2004). This discrepancy may be because of differences in the brain region of interest or in the experimental methods used (crude versus immunoprecipitated protein samples). However, the same study also reported that, in *PRIP-1 KO* mice, PP1 rather than PP2A phosphatase activity is enhanced and PP1/2A inhibitors partially regain phosphorylation and function of the $\beta 3$ subunit (Terunuma et al., 2004), which supports our i.p. and i.c.v. observations.

Decreased PP1 activity in OA-treated *PRIP-1 KO* mice is thought to lead to phosphorylation of the $\beta 3$ subunit. Inhibition of PP2A function by OA injection may also contribute to phosphorylation of the $\beta 3$ subunit because OA has a higher affinity to PP2A than PP1 (Chen et al., 1994). PP2A regulates basal levels of $\beta 3$ subunit phosphorylation (Jovanovic et al., 2004). As mentioned earlier, preservation of $\beta 3$ subunit phosphorylation increases cell surface expression levels of $\beta 3$ -containing GABA_A-R and GABAergic inhibitory transmissions (Abramian et al., 2010; Comenencia-Ortiz et al., 2014). OA pretreatment in *PRIP-1 KO* mice enhanced the induction and maintenance of hypnosis induced by propofol more effectively than etomidate, suggesting that enhancement of $\beta 3$ subunit phosphorylation by blocking PP1/2A activation increases surface expression of $\beta 3$ -containing GABA_A-R in *PRIP-1 KO* mice.

In WT mice, OA pretreatment induced slight, but not statistically significant, increases in anesthetic duration provided by propofol or etomidate. Inhibition of dephosphorylation by OA might induce opposite effect of propofol and etomidate in WT mice. Relatively increased phosphorylation of *PRIP-1* by OA may liberate the active form of PP1, leading

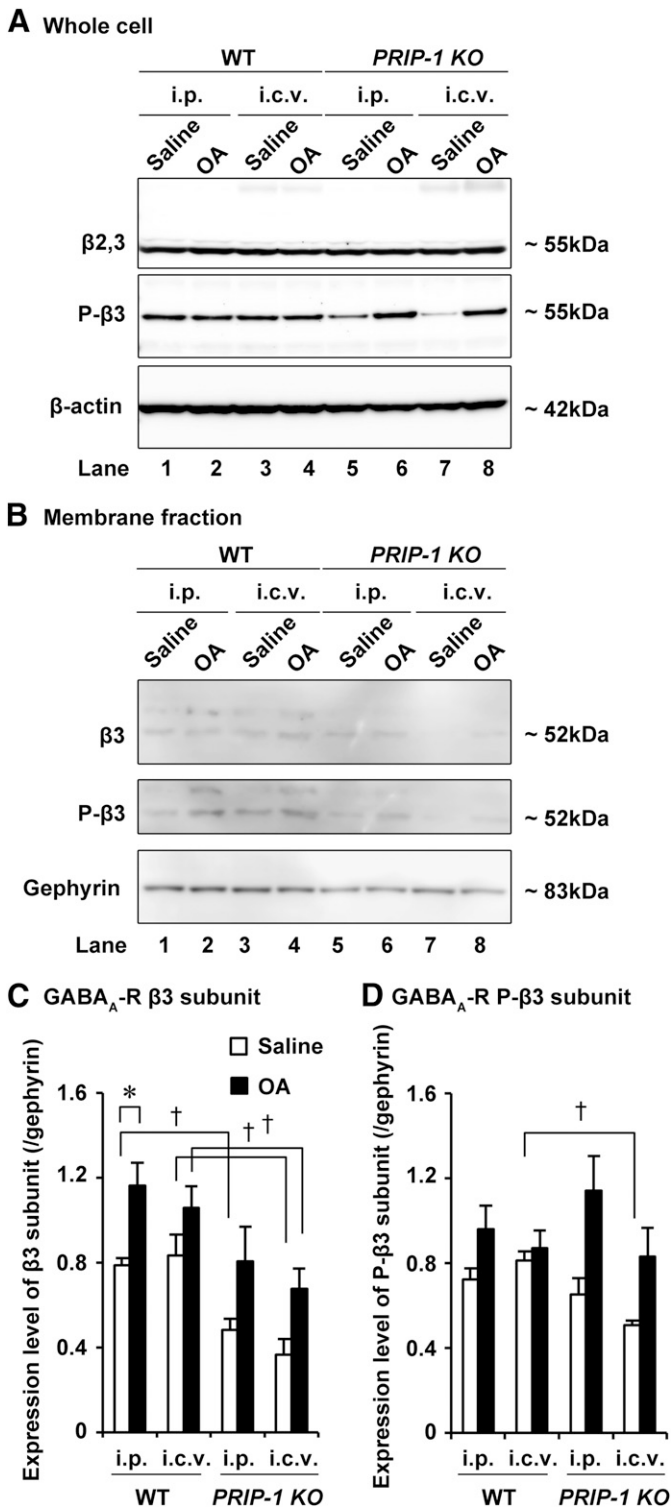


Fig. 4. Western blot analysis of whole cell and membrane fraction of GABA_A-R β_{3} and phosphorylated β_{3} subunits in i.p. or i.c.v. OA-treated wild-type (WT) and *PRIP-1 KO* mice. (A) In WT mice, significant differences were not observed in GABA_A-R $\beta_{2,3}$ and phosphorylated β_{3} subunits (P- β_{3} ; lanes 1–4). In *PRIP-1 KO* mice, there were no changes in total protein levels of GABA_A-R $\beta_{2,3}$ subunits (lanes 5–8). The amount of P- β_{3} increased after i.p. or i.c.v. administration of OA (lane 6, 8) compared with i.p. or i.c.v. saline (lane 5, 7) administration. We used β -actin as an internal control. (B) In WT mice, surface β_{3} and P- β_{3} expression increased after i.p. or i.c.v. administration of OA. Surface β_{3} and P- β_{3} expression decreased in *PRIP-1 KO* mice and recovered by i.p. or i.c.v. treatment with OA. Gephyrin was used as an internal control of the membrane fraction.

to activation of PP2A (Sugiyama et al., 2012). It is possible to inhibit the hypnotic effect of propofol and etomidate. In contrast, blockade of PP1/2A phosphatase activity by OA may provide potentiation of propofol and etomidate action in WT mice. Taken together, in our present study, administration of OA in WT mice provide slight potentiation of propofol and etomidate action.

Study Limitations. The anesthetics administered i.p. in this study are usually given via intravenous bolus injection or continuous infusion in humans and large animals, so the current results from i.p. administration in the mouse should be interpreted with caution. We could not analyze the effects of *PRIP-1* deficiency on membrane expression and phosphorylation of GABA_A-R $\alpha 1$ –6, $\beta 1/2$, $\gamma 1$ –3, and δ subunits. These subunit complexes play important roles in receptor phosphorylation, phasic/tonic inhibition, and endocytosis (Belelli et al., 2009; Comenencia-Ortiz et al., 2014). The effects of phosphorylation on GABA_A-R are diverse and appear to be highly dependent on the subunit composition (Terunuma et al., 2004; Kittler et al., 2005; Yanagihori et al., 2006; Abramian et al., 2010; Comenencia-Ortiz et al., 2014). Therefore, our current data would be but a part of the interaction among anesthetics, GABA_A-R phosphorylation, and its regulatory protein *PRIP-1*. Further studies are required to provide comprehensive mechanisms behind the effects of *PRIP-1* deficiency on anesthetic action.

Conclusions

We found that the *PRIP-1*-mediated GABA_A-R phosphorylation state is important for the induction of and emergence from propofol-induced anesthesia. Enhancement of GABA_A-R phosphorylation could potentiate propofol anesthesia. Although there might be various technical difficulties, our data imply the anesthetic and/or therapeutic possibility of GABA_A-R phospho-regulation. The preoperative estimation of the GABA_A-R phosphorylation state and its regulatory protein activity, such as *PRIP-1*, PP1/2A, or protein kinase C, in a patient would suggest a better propofol and/or other anesthetic administration plan to prevent adverse events such as awakening during and delayed emergence from anesthesia. *PRIP-1* and other proteins involved in GABA_A-R

The band intensity was normalized to gephyrin. (C, D) The results of Western blotting of the membrane fraction are represented in a graph. (C) Membrane expression of the β_{3} subunit in *PRIP-1 KO* mice significantly decreased compared with WT mice (i.p. treatment: unpaired *t* test, $t_4 = 4.87$, $P < 0.05$, Cohen's $d = 3.98$; i.c.v. treatment: $t_4 = 3.81$, $P < 0.05$, $d = 3.11$). OA treatment induced increases in surface β_{3} subunit expression levels in both genotypes (WT: i.p.: $t_4 = 3.28$, $P < 0.05$, $d = 2.68$; i.c.v.: $t_4 = 1.60$, $P = 0.18$, $d = 1.31$; *PRIP-1 KO*: i.p.: $t_4 = 1.90$, $P = 0.13$, $d = 1.55$; i.c.v.: $t_4 = 2.57$, $P = 0.06$, $d = 2.10$). Slight differences were detected in β_{3} membrane expression levels between WT and *PRIP-1 KO* mice administered OA (i.p.: $t_4 = 1.82$, $P = 0.14$, $d = 1.49$; i.c.v.: $t_4 = 2.78$, $P < 0.05$, $d = 2.27$). (D) Surface P- β_{3} expression in *PRIP-1 KO* mice tended to be lower than that in WT mice (i.p.: $t_4 = 0.75$, $P = 0.49$, $d = 0.61$; i.c.v.: $t_4 = 6.45$, $P < 0.05$, $d = 2.27$). OA administration enhanced membrane P- β_{3} subunit levels in WT and *PRIP-1 KO* mice (WT: i.p.: $t_4 = 1.89$, $P = 0.13$, $d = 1.53$; i.c.v.: $t_4 = 0.64$, $P = 0.56$, $d = 0.52$; *PRIP-1 KO*: i.p.: $t_4 = 2.72$, $P = 0.05$, $d = 2.22$; i.c.v.: $t_4 = 2.40$, $P = 0.07$, $d = 1.96$). Membrane P- β_{3} subunit levels were comparable between genotypes treated with OA (i.p.: $t_4 = 0.93$, $P = 0.41$, $d = 0.76$; i.c.v.: $t_4 = 0.25$, $P = 0.82$, $d = 0.20$). * $P < 0.05$, Cohen's $d = 2.68$, versus mice treated with saline; † $P < 0.05$, $d \geq 2.27$ versus WT mice with the same treatments. Data are mean \pm S.E.M.

phosphorylation and membrane trafficking may emerge as novel candidates for agent-specific regulatory factors in the control of anesthetic action.

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Wrote or contributed to the writing of the manuscript: Yoshikazu, Tomonori, Shuji, Junko, Keisuke, Kohei, Tetsuya, Kazuyoshi, Takashi, Masato, Shinya.

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