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Propofol anesthesia is reduced in phospholipase C-related inactive protein type-1 knockout mice

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Abbreviations

Phospholipase C-related inactive protein type-1 (PRIP-1); loss of righting reflex

(LORR): loss of tail-pinch withdrawal response (LTWR); okadaic acid (OA); protein phosphatase (PP); total anesthesia score (TAS).

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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 okadaic acid (OA), a protein phosphatase 1/2A inhibitor, pretreatment 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 β3 subunit in *PRIP-1 KO* mice. These results suggest that PRIP-1-mediated $GABA_A$-R β3 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-δ1 but catalytically inactive (Kanematsu et al, 1992; Kanematsu et al, 1996; Matsuda et al, 1998). PRIP-1 has various binding partners including GABA type A receptor ($GABA_A$-R) β1–3 subunits (Terunuma et al. 2004), $GABA_A$-R-associated protein (Kanematsu et al, 2002), protein phosphatases 1 (PP1) and 2A (PP2A; Yoshimura et al, 2001; Kanematsu et al, 2006; Yanagihori et al, 2006), and Akt protein kinase (Fujii et al, 2010). PRIP-1 acts as a bridge between the γ2 $GABA_A$-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; Yanagihori et al, 2006; Kanematsu et al, 2007). 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 (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 et al, 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).

Here, 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 the anesthetic action of these anesthetics.

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 ± 2°C under a 12-h 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 Physiological 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 µL/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 µL/g body weight i.p.) 30 min 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 µL/g body weight i.p.) with OA (0.01 mg/mL) or saline. Other animals received OA (1 pg/5 µL) or vehicle (saline, 5 µL) intracerebroventricularly (i.c.v.) under 1–3% isoflurane (Escain; Pfizer, Tokyo, Japan) for general anesthesia and lidocaine (Xylocaine jelly 2%; AstraZeneca, Osaka, Japan) for local surface anesthesia, as described previously (Maeda et al, 2005). Thirty min after i.p. injection, or 25 min after i.c.v. injection, mice were euthanized with pentobarbital (150 mg/kg i.p.) and 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 × 28 × 15 cm) with an electric heat pad, and the righting reflex was assessed every 2 min for a maximum of 2 h. Anesthesia was evaluated using the following scoring system (Irifune et al, 2003): 0, normal righting reflex; 1, righting within 2 s; 2, righting latency 2–10 s; 3, no righting within 10 s. Anesthetic scores were determined every 2 min 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, 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 s (cutoff time) at 15 min after anesthetic administration. The nociceptive response was measured by the time (test latency) when the mouse showed any response to tail-pinch.

Baseline nociceptive response (baseline latency) was measured 30 min before drug injection. To estimate LTWR, the percentage of maximal possible effect (% MPE) was evaluated using the formula: % MPE = 100 × (test latency − baseline latency) / (cutoff time − baseline latency). The magnitude of LTWR was assessed by the area under the % MPE *vs.* time 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 (PBS) 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 previously described (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 1000 × *g* for 10 min, and then supernatant was further centrifuged at 10 000 × *g* for 20 min 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, Hercules, CA). Membranes were incubated with the following primary antibodies: anti-$GABA_A$-R β2,3 subunit antibody (Millipore, Temecula, CA); anti-$GABA_A$-R β3 subunit antibody (Millipore); anti-$GABA_A$-R β3 phospho-Ser408/409 antibody (PhosphoSolutions, Aurora, CO); anti-β-actin antibody (Abcam, Cambridge, MA); or anti-calnexin antibody (Synaptic Systems, Göttingen, Germany). Membranes were washed with Tween 20–PBS 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 carried out on the LORR dose–response data for each anesthetic using 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 % MPE of LTWR are

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expressed as mean ± SEM. To compare the effects of anesthetics on LORR and LTWR between genotypes, unpaired *t*-tests were performed. One-way 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 β3 subunit. All statistical analyses were performed with R version 3.3.2 (R Core Team, 2016). A value of $P < 0.05$ was considered 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).

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Results

Effect of PRIP-1 deficiency on sensitivity to propofol, etomidate, and pentobarbital

First, we determined the ED_{50} 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 Figure 1. The ED_{50} of propofol in *PRIP-1 KO* mice was greater than that in WT mice (Figure 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 ED_{50} values (Figure 1B 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 Figures S1–S3), we estimated the hypnotic potency of

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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 (Figure 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.; Figure 1B, right panel) or pentobarbital (40 mg/kg i.p.; Figure 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 to WT mice (Figure 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.; Figure 2B) or pentobarbital (40 mg/kg i.p.; Figure 2C).

Hypnotic effect of propofol in* PRIP-1 KO *mice was rescued by OA pretreatment

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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 (Figure 3A), but did not do the same with etomidate administration (Figure 3B). 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.

Effect of OA on protein expression of $GABA_A$-R β2,3 subunits and the phosphorylated β3 subunit in* PRIP-1 KO *mice

The $GABA_A$-R β3 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 β3 subunit. There was less phosphorylation of the $GABA_A$-R β3 subunit in the frontal cortex of *PRIP-1 KO* mice

than in WT mice (Figure 4A). Both i.p. and i.c.v. administration of OA enhanced $GABA_A$-R β3 subunit phosphorylation without affecting overall levels of the β2,3 subunit in *PRIP-1 KO* mice. In addition, these OA treatments induced enhancement of β3 subunit membrane expression and phosphorylation levels (Figure 4B). β3 subunit membrane expression levels in *PRIP-1 KO* mice were lower than those in WT mice after saline treatment (Figure 4C). Following i.p. or i.c.v. OA treatments, β3 subunit membrane expression increased in both genotypes. Phosphorylation levels of the membrane $GABA_A$-R β3 subunit in i.p. and i.c.v. saline-administered *PRIP-1 KO* mice tended to be lower than in WT mice (Figure 4D). After i.p. or i.c.v. OA injections, phosphorylated β3 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 while OA did not alter the effect of propofol in WT mice. Furthermore, we confirmed that the dephosphorylated state of the $GABA_A$-R β3 subunit in *PRIP-1 KO* mice was recovered by i.p. OA administration, similar to i.c.v. administration.

Propofol mainly exerts its anesthetic action via the $GABA_A$-R β3 subunit (Jurd et al, 2003; Belelli et al, 2005; Feng and Macdonald, 2004; Drexler et al, 2009). Etomidate potentiates $GABA_A$-R containing β3 or β2 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 β3 subunit membrane expression and induces its dephosphorylation (Figure 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 β2/3 subunits in *PRIP-1 KO* mice did not seem to differ from that of WT mice, suggesting that the reduction in β3 subunit membrane expression might be compensated by the β2 subunit. Propofol affinity for the open state of α1β2γ2L $GABA_A$-R is approximately 3–7-fold weaker than etomidate (Rüsch et al, 2004; Ruesh

et al, 2013). In addition to the β3 subunit, α1/4 and β2 $GABA_A$-R subunits may be responsible for pentobarbital anesthesia (Zeller et al, 2007; Mercado and Czajkowski, 2008). This evidence suggests that non-significant but moderate effective changes of etomidate- and pentobarbital-anesthesia in *PRIP-1 KO* mice would be because of membrane expression of β2-containing $GABA_A$-R. There is a possibility that downregulation of β3-containing $GABA_A$-R in cell surface attenuate 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 et al, 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 α4β3 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 β3 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 β3 subunit in membrane fraction (Figure 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 β3 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 β3 subunit.

Contrary to our finding that PRIP-1 deficiency induced dephosphorylation of the $GABA_A$-R β3 subunit in the frontal cortex, another study has reported that phosphorylation levels of the β3 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 *vs*. 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 β3 subunit (Terunuma et al, 2004), which support 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 β3 subunit. Inhibition of PP2A function by OA injection may

also contribute to phosphorylation of the β3 subunit, because OA has a higher affinity to PP2A than PP1 (Chen et al, 1994). PP2A regulates basal levels of β3 subunit phosphorylation (Jovanovic et al, 2004). As mentioned above, preservation of β3 subunit phosphorylation increases cell surface expression levels of β3-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 β3 subunit phosphorylation by blocking PP1/2A activation increases surface expression of β3-containing $GABA_A$-R in *PRIP-1 KO* mice. In WT mice, OA pretreatment induced slight, but not 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 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 the 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 α1–6, β1/2, γ1–3, 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. While 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 phosphorylation and membrane trafficking may emerge as novel candidates for agent-specific regulatory factors in the control of anesthetic action.

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Authorship contributions

Study design: Yoshikazu, Tetsuya, Kazuyoshi, and Shinya.

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Footnotes

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Figure legends

Figure 1 Effect of PRIP-1 deficit on hypnosis produced by propofol, etomidate, and pentobarbital

(A) Dose–response curves for loss of righting reflex (LORR) induced by propofol in wild-type (WT) and *PRIP-1 KO* mice. Probit analysis was used to fit curves of ED_{50} values (solid lines) and 95% confidence intervals (dashed curves). ED_{50} 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)** ED_{50} 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. *$P < 0.05$ *vs.* WT mice. Data are mean ± SEM.

Figure 2 Effect of PRIP-1 deficit on immobilization induced by propofol, etomidate, and pentobarbital

(A) Time course and magnitude of propofol-induced loss of tail-pinch withdrawal response (LTWR). Significant reduction of percentage of maximal possible effect (% MPE) and AUC were observed in *PRIP-1 KO* mice (unpaired *t*-test: % MPE at 60 min, $t_{15} = 2.30$, $P < 0.05$, Cohen's $d = 1.12$; AUC, $t_{15} = 2.25$, $P < 0.05$, $d = 1.10$). **(B)** and **(C)** There were no significant differences in LTWR produced by etomidate or pentobarbital between wild-type (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. *$P < 0.05$ *vs.* WT mice. Data are mean ± SEM.

Figure 3 Effect of okadaic acid (OA) pretreatment on hypnosis induced by propofol and etomidate in *PRIP-1 KO* and wild-type (WT) mice

(A) Latency and duration of propofol-induced loss of righting reflex (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 pre-injection 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 $d > 1.50$, *vs.* WT mice; $^{\dagger}P < 0.05$, $d > 1.60$, *vs. PRIP-1 KO* mice pretreated with saline. Data are mean ± SEM.

Figure 4 Western blot analysis of whole cell and membrane fraction of $GABA_A$-R β3 and phosphorylated β3 subunits in i.p. or i.c.v. okadaic acid (OA)-treated wild-type (WT) and *PRIP-1 KO* mice

(A) In WT mice, significant differences were not observed in $GABA_A$-R β2,3 and phosphorylated β3 subunits (P-β3; lanes 1–4). In *PRIP-1 KO* mice, there were no changes in total protein levels of $GABA_A$-R β2,3 subunits (lanes 5–8). The amount of P-β3 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. β-actin was used as an internal control. **(B)** In WT mice, surface β3 and P-β3 expression increased after i.p. or i.c.v. administration of OA. Surface β3 and P-β3 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. The band intensity was normalized to Gephyrin. **(C)** and **(D)** The results of western blotting of the membrane fraction are represented in a graph. **(C)**

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Membrane expression of the β3 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 β3 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 β3 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-β3 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-β3 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-β3 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 $d = 2.68$, *vs.* mice treated with saline; $^{\dagger}P < 0.05$, $d \geq 2.27$, *vs.* WT mice with the same treatments. Data are mean ± SEM.

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

Anesthetic	Dose (mg/kg)	WT		*PRIP-1 KO*		Effect size[c]
		n[a]	TAS[b]	n[a]	TAS[b]	
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)*	1.51
	100	6/7	52 (0–110)	6/14	7.5 (0–45)*	1.31
	120	7/7	97 (14–161)	7/13	21 (6–127)*	0.92
	140	7/7	71 (54–149)	10/14	29 (6–79)*	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

[a] Denominators represent the number of animals tested in each group. Numerators represent animals that showed LORR. [b] TAS express the sum of anesthetic scores as median (range). [c] Effect size (Cohen's *d*) for TAS compared with WT mice with the same treatment. *$P < 0.05$, *vs*. WT mice with the same treatment (Mann–Whitney *U*-test).

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

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

[a] Denominators represent the number of animals tested in each group. Numerators represent animals that showed LORR. [b] TAS express the sum of anesthetic scores as median (range). [c] Effect size (Cohen's *d*) for TAS compared with WT mice with the same

treatment. [d] Cohen's *d* for TAS compared with *PRIP-1 KO* mice pretreated with OA (0 mg/kg). *$P < 0.05$, *vs.* WT mice with the same treatments; †$P < 0.05$, *vs. PRIP-1 KO* mice pretreated with OA (0 mg/kg) (Mann–Whitney *U*-test).

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Figure 1.

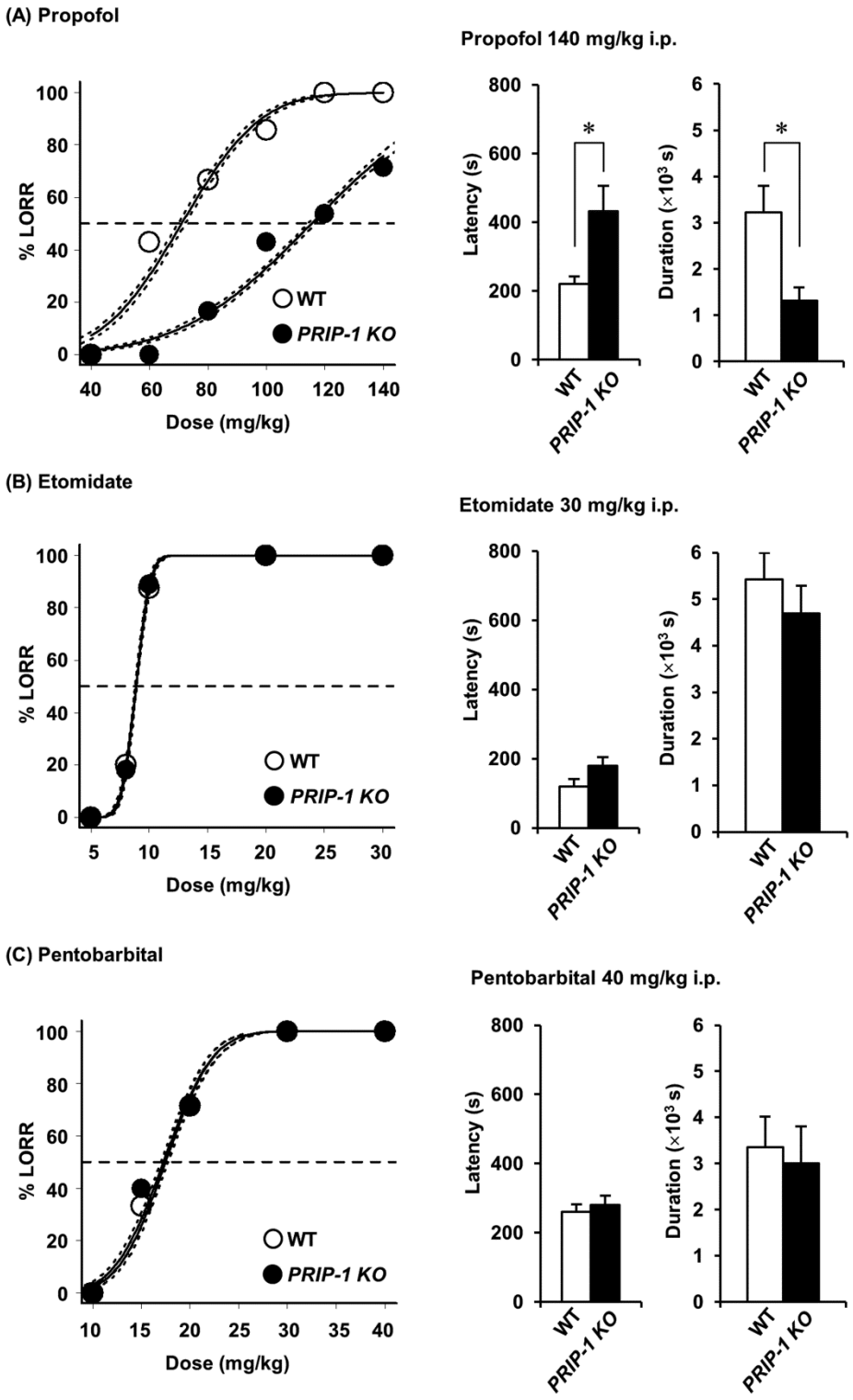

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Figure 2.

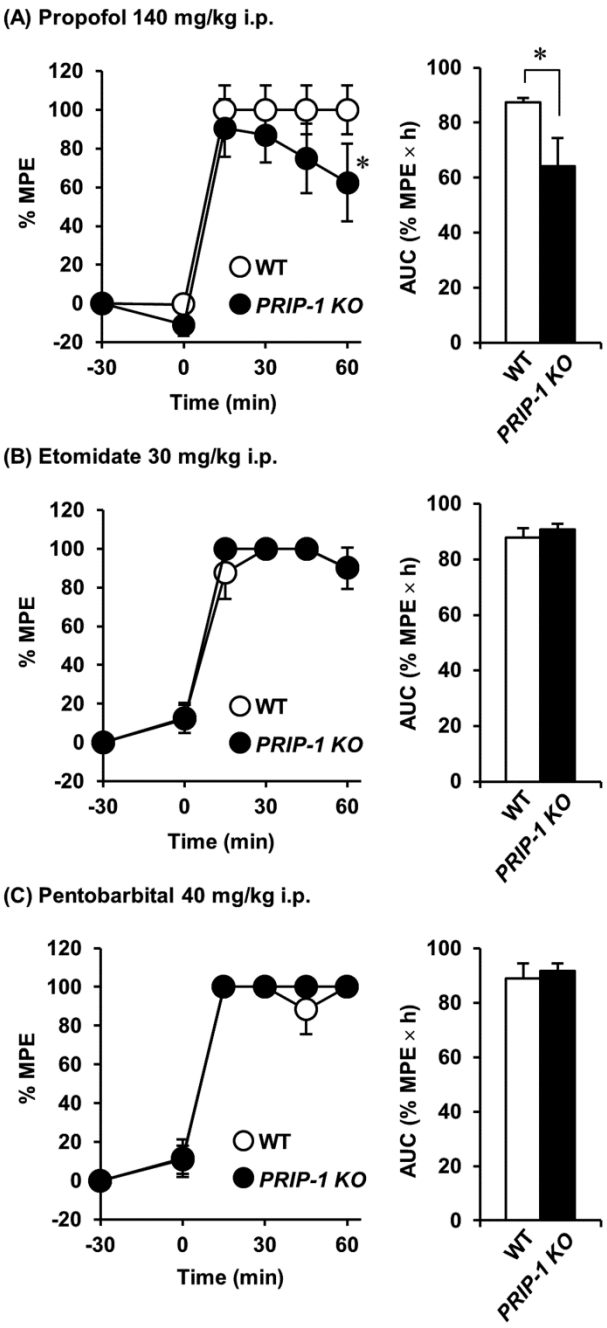

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Figure 3.

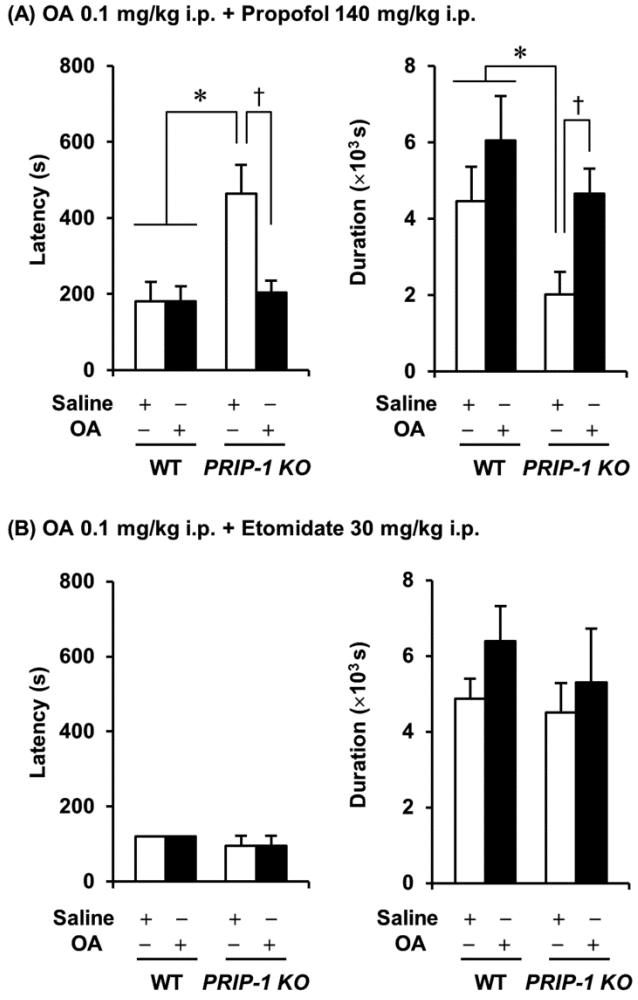

Figure 4.