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SENSORY PHYSIOLOGY

Propofol-induced pain sensation involves multiple mechanisms in sensory neurons

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Abstract Propofol, a commonly used intravenous anesthetic agent, is known to at times cause pain sensation upon injection in humans. However, the molecular mechanisms underlying this effect are not fully understood. Although propofol was reported to activate human transient receptor potential ankyrin 1 (TRPA1) in this regard, its action on human TRP vanilloid 1 (TRPV1), another nociceptive receptor, is unknown. Furthermore, whether propofol activates TRPV1 in rodents is controversial. Here, we show that propofol activates human and mouse TRPA1. In contrast, we did not observe propofol-evoked human TRPV1 activation, while the ability of propofol to activate mouse TRPV1 was very small. We also found that propofol caused increases in intracellular Ca^{2+} concentrations in a considerable portion of dorsal root ganglion (DRG) cells from mice lacking both TRPV1 and TRPA1, indicating the existence of TRPV1- and TRPA1-independent mechanisms for propofol action. In addition, propofol produced action potential generation in a type A γ-amino butyric acid ($GABA_A$) receptor-dependent manner. Finally, we found that both T-type and L-type Ca^{2+} channels are activated downstream of $GABA_A$ receptor activation by propofol. Thus, we conclude that propofol may cause pain sensation through multiple mechanisms involving not only TRPV1 and TRPA1 but also voltage-gated channels downstream of $GABA_A$ receptor activation.

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Introduction

Propofol (2,6-diisopropylphenol) is one of the most common intravenous drugs in the clinical field used to induce a loss of consciousness [12]. It is known as a modulator and an activator of type A γ-amino butyric acid ($GABA_A$) receptors in the central nervous system [1, 14], but it is also reported to affect the function of glycine receptors in the spinal cord [44]. Many clinicians including anesthesiologists use it for sedation and induction or maintenance of general anesthesia in hospitals because of its rapid onset and short-acting duration [12, 18]. Although it is valuable for its safe use in clinical medicine, it has a serious side-effect, namely the production of intense pain upon injection that patients feel along the limbs where propofol flows after injection into the vein [22]. This distressing effect occurs in more than 20 % of adult patients and at a higher rate in children [40]. Although many clinical trials have been conducted in efforts to attenuate this side-effect, a solution to reduce such pain has not been established because the precise mechanism of propofol-evoked pain sensation has not been elucidated [18].

Recent work indicates that transient receptor potential (TRP) channels, especially TRP vanilloid 1 (TRPV1) and TRP ankyrin 1 (TRPA1), which are expressed in peripheral neurons detecting noxious stimuli such as thermal and irritant chemical stimuli [8, 39], are involved in propofol-evoked pain sensation [13, 28]. TRP channels are non-selective cation channels and play important roles in nociception under physiological and pathological situations [9]. TRPV1 is a polymodal receptor that responds to noxious heat (above 42 °C), capsaicin (Cap, a main ingredient of hot chili peppers), and low pH [41] at peripheral nerve endings. TRPV1 function

is important for the development of hyperalgesia since mice genetically lacking TRPV1 displayed a decrease in behavioral responses to inflammatory mediator-induced hypersensitivity, such as ATP-induced thermal hyperalgesia [30]. Studies have revealed that TRPV1 function is enhanced by certain kinases such as protein kinase C epsilon (PKCε) [26, 32] and phosphoinositide 3-kinase (PI3K) [38] causing a sensitization of TRPV1 activity. In contrast, the membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) negatively modulates TRPV1 activity [5], although basal PIP_2 activity was reported to be essential for TRPV1 activation [21]. Additionally, it is known that a Ca^{2+}-binding protein, calmodulin, is involved in the desensitization of TRPV1 in an extracellular Ca^{2+}-dependent manner [31, 34]. Overall, TRPV1 has become a viable target in the pain research field. In comparison, TRPA1 is a chemical-detecting receptor that responds to irritants and pungent chemicals such as allyl isothiocyanate (AITC) and cinnamaldehyde, which activate TRPA1 via covalent modification of cytosolic cysteine residues [17, 25]. TRPA1 is also activated by formalin, which is known to induce tissue damage and inflammation. Furthermore, bradykinin, one of the inflammatory mediators, also activates TRPA1 though activation of bradykinin 2 receptors linked to phospholipase C activation [9]. It is also reported that TRPA1 is directly activated or modulated by intracellular Ca^{2+} [43, 46]. Thus, TRPA1 has a polymodal nature to detect various stimuli similar to TRPV1. Consequently, these two TRP channels have become important targets in pain research especially for the development of innovative drugs.

Previous studies reported that TRPA1 could be activated by propofol in mice and humans, although the involvement of TRPV1 activation in propofol-evoked pain sensation is still controversial [13, 28]. While propofol was reported to activate $GABA_A$ receptors in mouse dorsal root ganglion (DRG) neurons to increase intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), the precise mechanism for $[Ca^{2+}]_i$ increases downstream of $GABA_A$ receptor activation remains unclear.

Our present study indicates that propofol activates both mouse TRPV1 and TRPA1 (albeit to a different extent), while $GABA_A$ receptor activation by propofol causes $[Ca^{2+}]_i$ increases through activation of voltage-gated calcium channels and action potential generation in mouse DRG cells. Finally, we found that human TRPV1 was not activated by propofol in vitro.

Materials and methods

All procedures involving the care and use of animals were approved by the Institutional Animal Care and Use Committee of National Institutes of Natural Sciences and carried out in accordance with the National Institutes of Health Guide for the care and use of laboratory animals.

Animals

C57BL/6NCr (wild-type, WT) mice (5–8 weeks old, SLC) were used as a control. TRPV1/TRPA1 double-knockout (V1A1DKO) mice were obtained from a mating between TRPV1-knockout (V1KO) and TRPA1-knockout (A1KO) mice (both were generously provided by Dr. David Julius, UCSF, San Francisco, CA, USA) [4, 7], which were backcrossed on a C57BL/6NCr background. Mice were housed in a controlled environment (12 h light/12 h dark cycle; room temperature 22–24 °C; 50–60 % relative humidity) with free access to food and water. The genotyping of V1A1DKO mice used for the experiments in Figs. 4 and 5 was performed by PCR.

Isolation of dorsal root ganglion (DRG) cells

Mouse DRG at thoracic and lumbar levels in each genotype was rapidly dissected and dissociated by incubation at 37 °C for 20 min in a solution of culture medium, which contained Earle's balanced salts solution (Sigma-Aldrich), fetal bovine serum (FBS) (10 %, BioWest or Gibco Life technology), penicillin-streptomycin (50 units/mL and 50 mg/mL, respectively, Gibco Life technology), GlutaMAX (2 mM, Gibco Life technology), and vitamin solution (1 %, Sigma-Aldrich), with 0.25 % collagenase type XI (Sigma-Aldrich). Cells were gently triturated using fire-polished Pasteur pipettes and centrifuged in a culture medium to separate cells from debris. Cells were resuspended and plated onto 12-mm cover slips coated with poly-D-lysine (Sigma-Aldrich). Ca^{2+}-imaging experiments were performed 12–20 h, and the patch-clamp recordings were performed 12–24 h after the incubation of isolated DRG cells as described below.

Electrophysiology

HEK293T cells and isolated mouse DRG cells were used for patch-clamp recordings. HEK293T cells were maintained in D-MEM (Wako) supplemented with 10 % FBS (BioWest), penicillin-streptomycin (50 units/mL and 50 mg/mL, respectively, Gibco Life technology), and GlutaMAX (2 mM, Gibco Life technology) and seeded at a density of 5×10^5 cells per 35-mm dish 24 h before transfection. For patch-clamp recordings of HEK293T cells, either 1 μg human TRPA1 (hTRPA1), human TRPV1 (hTRPV1), mouse TRPV1 (mTRPV1, a generous gift from Dr. Xu), or mouse TRPA1 (mTRPA1) channel expression vector and 0.1 μg pGreen-Lantern 1 vector were transfected into HEK293T cells using Lipofectamine and Plus reagents (Invitrogen). Patch-clamp recordings of HEK293T cells were performed 18–36 h after the transfection. Mouse DRG cells were prepared as described above. The extracellular solution contained 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose

at pH 7.4 adjusted with NaOH. The intracellular solution for the experiments with HEK293T cells contained 140 mM KCl, 5 mM ethylene glycol tetraacetic acid (EGTA), and 10 mM HEPES at pH 7.4 adjusted with KOH. For the recordings of mouse DRG cells, the intracellular solution contained 67 mM KCl, 65 mM K gluconate, 1.0055 mM $CaCl_2$, 1 mM $MgCl_2$, 4 mM Mg ATP, 1 mM 2Na-GTP, 5 mM EGTA, and 10 mM HEPES at pH 7.3 adjusted with KOH. The free Ca^{2+} concentration was 20 nM (calculated by CaBuf; www.kuleuven.be/fysio/trp/cabuf). A Ca^{2+}-free bath solution used in the Ca^{2+}-free experiments was made by removing 2 mM $CaCl_2$ and adding 5 mM EGTA to the standard bath solution. Data for analysis were sampled at 10 kHz and filtered at 5 kHz for whole-cell recordings and 2 kHz for single-channel recordings (Axopatch 200B amplifier with pClamp software, Molecular Devices). In the experiments with mouse DRG cells at a current-clamp mode, the cells in which the resting potential was under −40 mV were selected. All of the patch-clamp experiments were performed at room temperature. The cover slips were mounted in a chamber connected to a gravity flow system to deliver various stimuli. Chemical stimulation was applied by running a bath solution containing various chemical reagents.

Ca^{2+} imaging

Mouse DRG cells on cover slips were incubated at 37 °C for 30 min in a culture medium containing 5 μM Fura-2-acetoxymethyl ester (Molecular Probes). The cover slips were washed with a standard bath solution identical to the extracellular solution in the patch-clamp recordings and a Ca^{2+}-free bath solution identical to the extracellular solution used in the patch-clamp experiments. Fura-2 fluorescence was measured in a standard bath solution. Fura-2 was excited with 340- and 380-nm wavelength lights, and the emission was monitored at 510 nm with a CCD camera, CoolSnap ES (Roper Scientific/Photometrics) at room temperature. Chemical stimulations were applied as described above for the patch-clamp recordings. Data were acquired using IPlab software (Scanalytics) and analyzed with ImageJ and Excel software (Microsoft). Ionomycin (5 μM, Sigma-Aldrich) was applied to confirm cell viability, and values were normalized to those evoked by ionomycin for each experiment. Cells in which an increase in normalized intensity during propofol application was over 0.2 were considered activated.

Chemicals

Chemicals used in this study were purchased as described below. Propofol (2,6-diisopropylphenol), HC-030031, capsaicin, ionomycin, nifedipine, γ-amino butyric acid (GABA), and (+)-bicuculline were from Sigma-Aldrich. Allyl isothiocyanate (AITC) was from Kanto Chemical. Picrotoxin, verapamil hydrochloride, flunarizine dihydrochloride, and NNC 55-0396 dihydrochloride were from Tocris. Propofol, HC-030031, picrotoxin, flunarizine, nifedipine, and (+)-bicuculline were dissolved in dimethyl sulfoxide (DMSO) as stock solutions. Capsaicin and AITC were dissolved in ethanol and methanol, respectively. The others were dissolved in water. All of the dissolved chemicals were diluted (from 1:10,000 to 1:1000) into the solution for the patch-clamp and Ca^{2+}-imaging experiments. The concentration of DMSO did not exceed 0.15 %.

Statistical analysis

Data are presented as mean±standard error of mean (SEM). The abbreviation *n* indicates the number of data points. The Mann-Whitney *U* test, unpaired *t* test, chi-square test, and non-parametric multiple comparison were applied for statistical analyses. *P* values less than 0.05 were considered significant. Data from the propofol-evoked hTRPA1 current recordings were fitted with a Hill's equation to generate a dose-response curve, and EC_{50} values were calculated. Data related to the inhibitory effect of HC-030031 on propofol-evoked humanTRPA1 currents were fitted by a logistic curve to calculate the IC_{50} value. All statistical analyses were performed using Origin software (OriginLab).

Results

Patch-clamp studies of the propofol-evoked TRP channel currents

First, we utilized a patch-clamp method to examine propofol actions in HEK293T cells expressing either hTRPA1, mTRPA1, hTRPV1, or mTRPV1. As shown in Fig. 1A, 100 μM of propofol activated both hTRPA1 and mTRPA1 with an outwardly rectifying current-voltage relationship, although an increase in currents upon propofol washout was observed in the case of mTRPA1, probably due to the bimodal effects of the compound on mTRPA1. Once large current responses were observed upon propofol application, the following AITC responses were small as previously reported [24]. Propofol-evoked current activation was observed in relation to mTRPV1, but not hTRPV1, although both hTRPV1 and mTRPV1 responded to capsaicin (Cap, 1 μM) similarly with clear outward rectification in the same cells, indicating that propofol actions on TRPV1 differ depending on species.

Next, we tried to determine the dose dependency for the propofol effects on the four TRP channels at −60 mV (Fig. 1B). When we analyzed propofol-evoked currents during the propofol application, we found that propofol was most

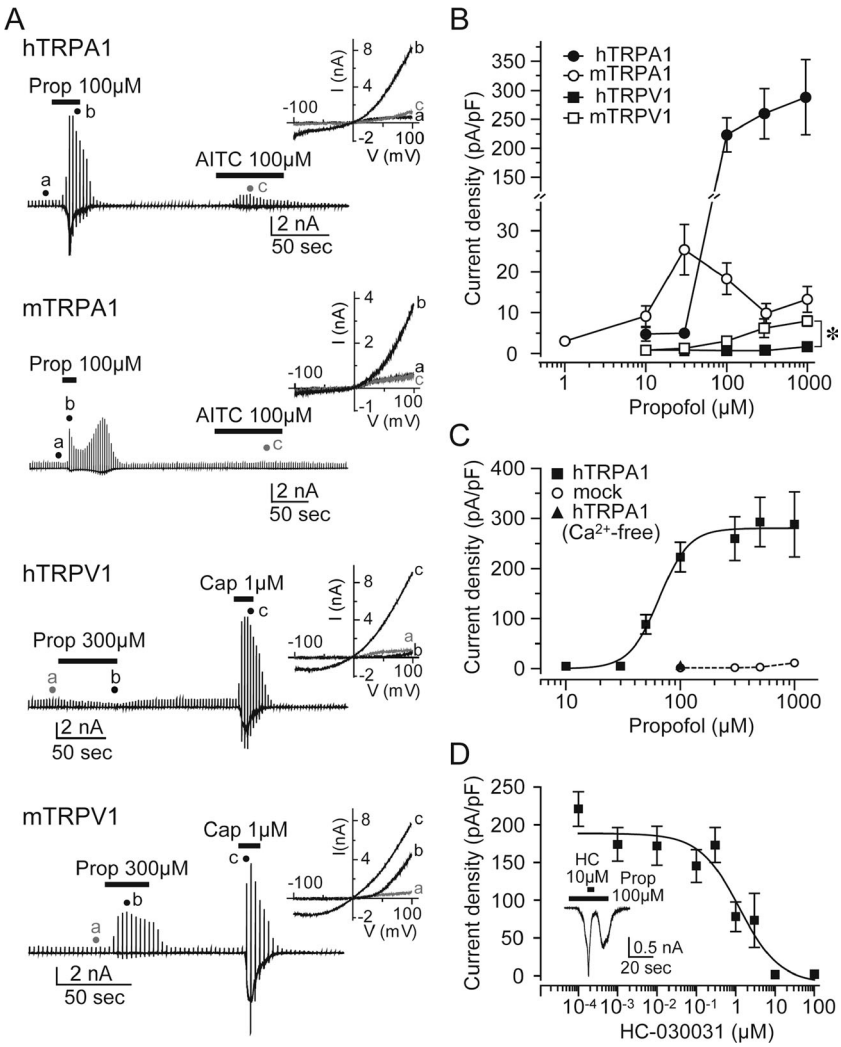

Fig. 1 Effects of propofol on human and mouse TRPA1 and TRPV1. **A** Representative whole-cell current traces in HEK293T cells expressing human TRPA1 (hTRPA1), mouse TRPA1 (mTRPA1), human TRPV1 (hTRPV1), or mouse TRPV1 (mTRPV1) in response to propofol (Prop, 100 or 300 μM) application. AITC (100 μM) or capsaicin (Cap, 1 μM) was applied after propofol. Ramp pulses from −100 to +100 mV in 300 ms were applied every 3 s, and current-voltage curves at the time points of *a*, *b*, and *c* are shown in *insets*. **B** Dose dependencies of the effects of propofol on hTRPA1-, mTRPA1-, hTRPV1-, and mTRPV1-mediated currents at −60 mV (n=5–6). *p<0.01, analyzed with Mann-Whitney U test. **C** Dose dependencies of the effects of propofol on hTRPA1-mediated currents at −60 mV in the presence and absence of extracellular Ca^{2+} (n=4–18). **D** Dose-dependent inhibition of propofol-activated hTRPA1 currents by HC-030031 at −60 mV (n=2–8)

effective at hTRPA1, in which propofol effects were almost saturated at 100 μM. The effects of propofol on mTRPA1 exhibited a bell-shaped curve, possibly due to its bimodal action. High concentrations of propofol caused small but significant mTRPV1 activation at −60 mV, although the current activation looked negligible at a negative potential in Fig. 1A. When the curve of the dose-dependent activation of hTRPA1 by propofol was fitted with a Hill equation, Hill co-efficient and EC_{50} value were 3.3 and 65.4 μM, respectively (Fig. 1C), representing attainable concentrations in the clinical setting [11]. Surprisingly, 100 μM propofol did not cause measurable hTRPA1 activation at −60 mV in the absence of extracellular Ca^{2+}, suggesting that propofol-induced hTRPA1 activation requires extracellular Ca^{2+}, similar to the extracellular Ca^{2+}-dependent activation of green anole lizard TRPA1 by heat [23] and to the very small propofol-induced inward currents in HEK293 cells expressing rat TRPA1 in the absence of extracellular Ca^{2+} [28]. Propofol-evoked currents were inhibited reversibly by HC-030031, a specific TRPA1 antagonist, with an IC_{50} value of 1.2 μM, further supporting the notion that propofol activates hTRPA1 (Fig. 1D).

In order to examine whether hTRPA1 is directly activated by propofol in a membrane-delimited manner, we performed single-channel recordings in an inside-out mode of a membrane excised from a HEK293T cell expressing hTRPA1. Clear single-channel openings at a membrane potential of +60 mV were observed upon application of propofol (30 μM), and robust hTRPA1 channel activation by 30 μM AITC was induced in the same patch membrane (Fig. 2A), confirming the hTRPA1 activation by propofol. We next analyzed unitary amplitudes of the single-channel currents activated by propofol by fitting the amplitude histogram with a Gaussian equation, which provided 4.3±0.4 and 2.8±0.1 pA at +60 and −60 mV, respectively (Fig. 2B) without measurable currents at 0 mV (data not shown), leading to conductances of 71.7 and 46.7 pS at positive and negative potentials, respectively. These results indicate that propofol can activate hTRPA1 directly and that the intracellular component is not necessary for the mechanism of propofol-induced hTRPA1 activation.

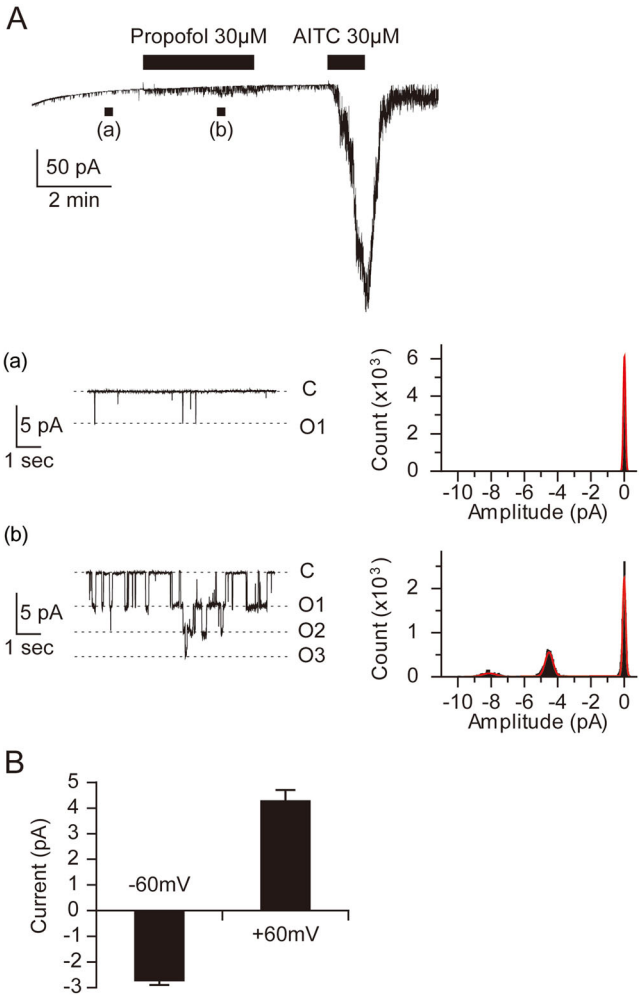

Fig. 2 Propofol-evoked hTRPA1 activation at a single-channel level. **A** A representative single-channel current trace at +60 mV in response to propofol (30 μM) and AITC (30 μM). Currents at *a* and *b* are expanded and shown with open channel histograms fitted with a Gaussian equation (*red line*). *C* closed level, *O1–3* open levels. **B** Mean unitary amplitudes of the propofol-activated single channels at −60 and +60 mV (n=3)

The effects of propofol on mouse DRG cells

Thus far, propofol was clarified to directly activate hTRPA1 at an attainable concentration in a clinical setting. To further confirm the ability of propofol to activate TRPA1, we performed Ca^{2+}-imaging experiments using mouse DRG cells. A previous report showed that propofol-induced $[Ca^{2+}]_i$ increases were not observed in TRPA1-deficient DRG cells [28], while another report showed that propofol-induced $[Ca^{2+}]_i$ increases were still observed in DRG cells from V1A1DKO mice [13]. Some DRG cells from WT mice responded to propofol (50 μM), and a greater number of cells responded to AITC (100 μM) and/or Cap (1 μM) (Fig. 3a). Such propofol-induced $[Ca^{2+}]_i$ increases were abolished completely in the absence of extracellular Ca^{2+} (Fig. 3b), indicating that propofol-induced $[Ca^{2+}]_i$ increases through Ca^{2+} influx from outside of the cells. Next, in order to confirm the involvement of TRPV1 and/or TRPA1 in the propofol-induced $[Ca^{2+}]_i$ increases, we compared the propofol-induced $[Ca^{2+}]_i$ increases in DRG cells from WT, A1KO, V1KO, and V1A1DKO mice. Our patch-clamp studies using HEK293T cells (Fig. 1) suggested that propofol could induce $[Ca^{2+}]_i$ increases through both TRPV1 and TRPA1 in mouse DRG cells although the TRPA1 contribution looked greater. The propofol-induced $[Ca^{2+}]_i$ increases were observed in A1KO, V1KO, and V1A1DKO DRG cells (Fig. 3c), although the percentage of the propofol-responsive DRG cells was significantly smaller in V1A1DKO compared with WT, A1KO, and V1KO DRG cells (Fig. 3d). This result suggests that propofol actions on mouse DRG cells are almost similar even if either TRPA1 or TRPV1 is genetically abolished and that both TRPA1 and TRPV1 might be involved in propofol-induced $[Ca^{2+}]_i$ increases. In this regard, we might underestimate the TRPA1 and TRPV1 activation by 50 μM propofol in mouse DRG cells because our results in Fig. 1 indicate that 50 μM of propofol activates mTRPA1 and mTRPV1 to a much lesser degree than 100 μM AITC and 1 μM Cap, respectively.

To know what kind of differences exist in propofol-induced $[Ca^{2+}]_i$ increases across each genotype, we compared the maximal intensities of propofol-induced $[Ca^{2+}]_i$ increases. When the propofol-induced $[Ca^{2+}]_i$ increases were normalized to the values induced by ionomycin, the values were significantly smaller in A1KO and V1A1DKO DRG cells compared with V1KO DRG cells. This result indicated that TRPA1 is more profoundly involved in the propofol-induced $[Ca^{2+}]_i$ increases compared with TRPV1 (Fig. 3e), consistent with data obtained in the patch-clamp experiments using HEK293T cells expressing either mTRPV1 or mTRPA1 (Fig. 1). The fact that the propofol-induced $[Ca^{2+}]_i$ increases were still observed in the V1A1DKO DRG cells indicates the existence of another mechanism causing $[Ca^{2+}]_i$ increases by propofol. In order to examine what kinds of DRG cells respond to propofol, we compared cell sizes between propofol-responsive WT and V1A1DKO DRG cells, the former of which should contain TRPA1- and/or TRPV1-expressing cells. Mean sizes of the propofol-responsive V1A1DKO DRG cells were 24.9±0.5 μm, which were significantly larger than those of the propofol-responsive WT DRG cells (21.4± 0.4 μm). This result suggested that propofol-responsive DRG cells not expressing TRPA1 or TRPV1 are a little larger and could contain Aδ fiber neurons.

Involvement of $GABA_A$ receptors and voltage-gated Ca^{2+} channels in the propofol-induced $[Ca^{2+}]_i$ increases in V1A1DKO DRG cells

Because propofol is known to act on $GABA_A$ receptors [33], $GABA_A$ receptor activation by propofol in DRG

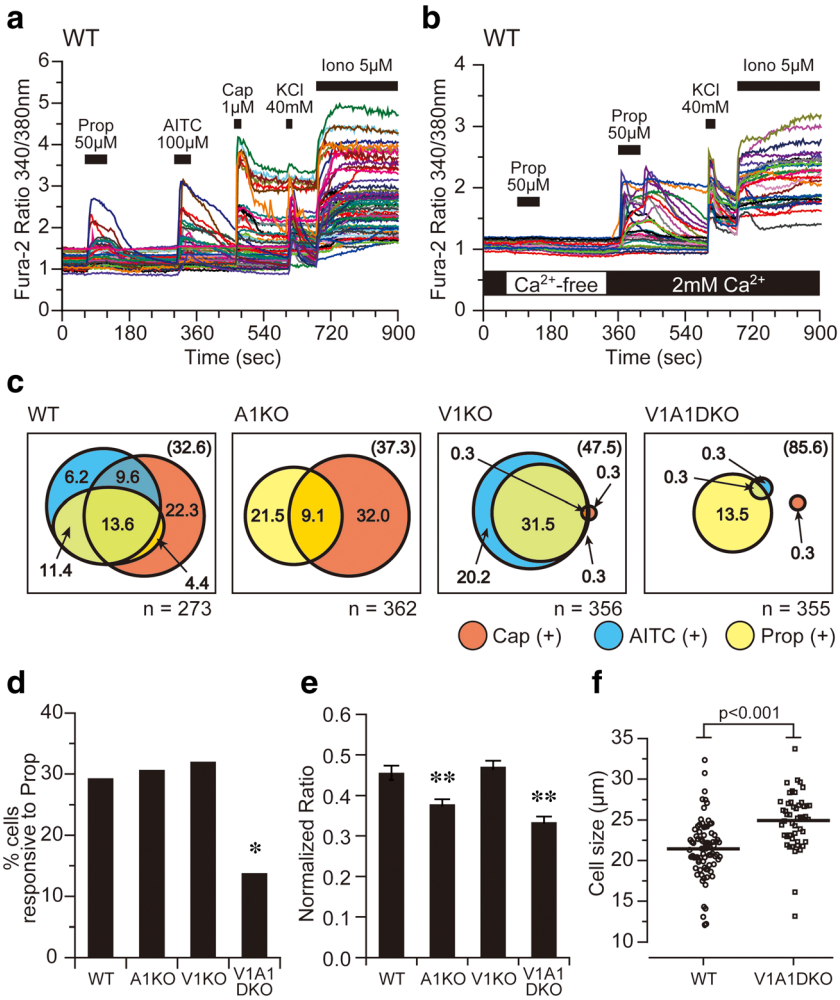

Fig. 3 Effects of propofol on intracellular Ca^{2+} concentrations in isolated DRG cells from wild-type (WT), TRPA1KO (A1KO), TRPV1KO (V1KO), and TRPV1/TRPA1 double KO (V1A1DKO) mice. **a**, **b** Effects of propofol on intracellular Ca^{2+} concentrations in WT DRG cells in the presence (**a**) or absence (**b**) of extracellular Ca^{2+}. **c** Venn diagrams of the effects of propofol, AITC, and capsaicin in WT, A1KO, V1KO, and V1A1DKO DRG cells. The *number in each box* indicates the percentage of cells responding to each agonist. The *number in parentheses in each box* indicates the percentage of cells responding to only KCl. **d**, **e** Percentages of the propofol-responsive cells (**d**) and normalized Fura-2 ratios in the propofol-responsive cells (**e**) in WT, A1KO, V1KO, and V1A1DKO DRG cells. *$p<0.05$; **$p<0.01$, analyzed with chi-square test (**d**) and non-parametric multiple comparison followed by Steel-Dwass test (**e**). **f** Comparison of the propofol-responsive cell sizes between WT and V1A1DKO DRG. *P* value was calculated by performing an unpaired *t* test

cells could cause depolarization. It is well known that intracellular chloride concentrations in DRG cells are quite high due to the lack of KCC2 expression [27]. Therefore, we hypothesized that depolarization involving $GABA_A$ receptor activation would activate voltage-gated Ca^{2+} channels to cause $[Ca^{2+}]_i$ increases upon propofol application, although such membrane depolarization should also activate voltage-gated Na^+ channels. In order to confirm the hypothesis, we performed Ca^{2+}-imaging experiments using DRG cells from V1A1DKO mice, which excludes the involvement of TRPA1 or TRPV1 in propofol-induced $[Ca^{2+}]_i$ increases. Propofol-induced $[Ca^{2+}]_i$ increases were drastically and reversibly inhibited by picrotoxin (Pic, 100 μM), a $GABA_A$ receptor antagonist (Fig. 4a, d). Another $GABA_A$ receptor antagonist, (+)-bicuculline (Bic, 30 μM), also inhibited propofol-induced $[Ca^{2+}]_i$ increases almost completely, indicating that a majority of the TRPV1/TRPA1-independent component of the propofol-induced $[Ca^{2+}]_i$ increases is caused by $GABA_A$ receptor activation (Fig. 4a, d). Among the voltage-gated Ca^{2+} channels that could be activated by depolarization downstream of $GABA_A$ receptor activation, we first tested two kinds of the L-type voltage-gated Ca^{2+} channel inhibitors, verapamil (Ver) and nifedipine (Nif). Both Ver (50 μM) and Nif (10 μM) significantly inhibited propofol-induced $[Ca^{2+}]_i$ increases while the inhibition by Ver was significantly greater than that by Nif and to the level obtained by Pic (Fig. 4b, d). These data indicated that L-type voltage-gated Ca^{2+} channels are activated downstream of $GABA_A$ receptor activation, although Ver is also known to inhibit T-type voltage-gated Ca^{2+} channels. Therefore, we next examined the effects of T-type voltage-gated Ca^{2+} channel inhibitors flunarizine (Flu) and NNC 55-0396 (NNC) on propofol-induced $[Ca^{2+}]_i$ increases. Both Flu (50 μM) and NNC (10 μM) reduced propofol-induced $[Ca^{2+}]_i$ increases, albeit to a lesser extent compared with Ver. Moreover, co-application of Nif and NNC further inhibited propofol-induced $[Ca^{2+}]_i$ increases compared with Nif or NNC treatment alone (Fig. 4c, d), suggesting that both L-type and T-type Ca^{2+} channels are activated by depolarization upon $GABA_A$ receptor activation.

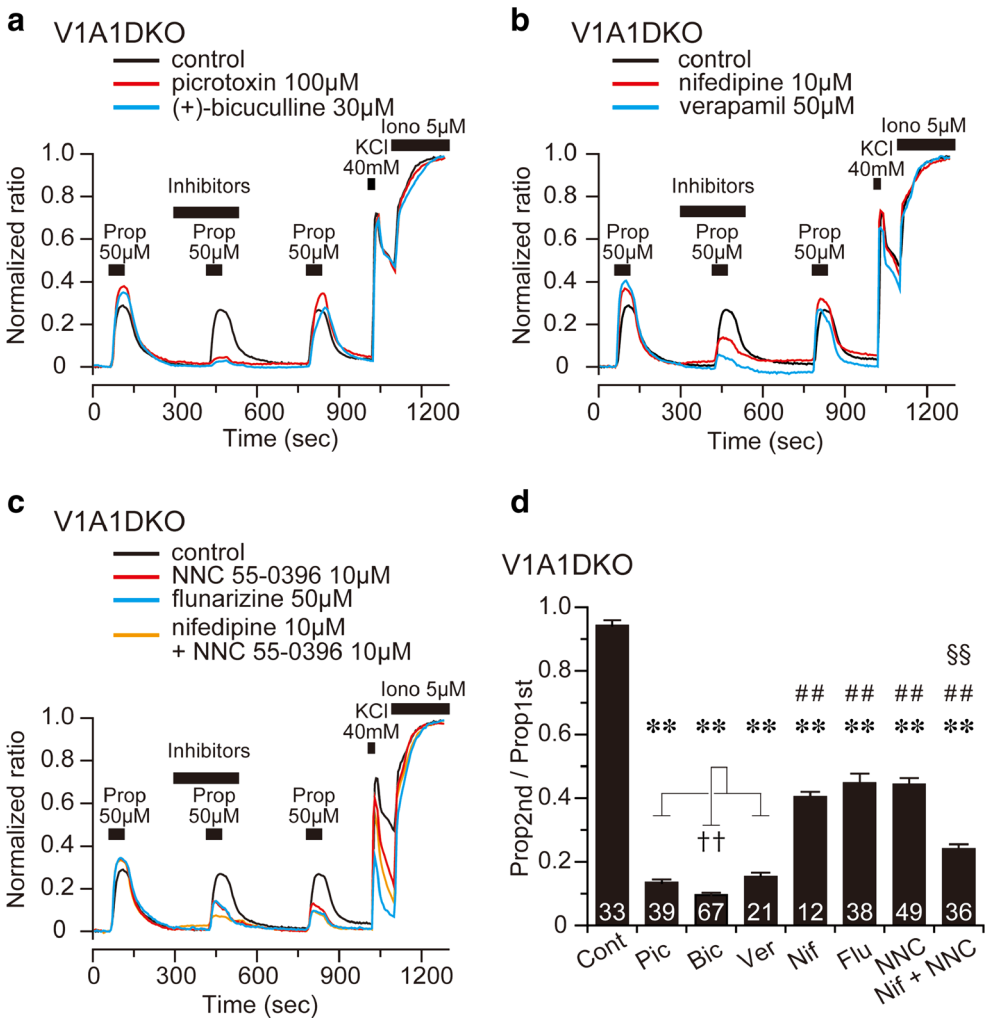

Fig. 4 Effects of antagonists at $GABA_A$ receptors and voltage-gated Ca^{2+} channels on the propofol-evoked changes in intracellular Ca^{2+} concentrations in V1A1DKO DRG cells. **a**–**c** Averaged changes in intracellular Ca^{2+} concentrations (indicated by ratios normalized to that caused by ionomycin) upon propofol application in the presence (*colored traces*) and absence (*black trace*) of the indicated compounds in V1A1DKO DRG cells. **d** Ratios of the second propofol ($Prop_{2nd}$)-evoked responses (with the indicated compounds) to the first propofol ($Prop_{1st}$)-evoked responses (without the compounds). $^{**}p<0.01$ vs. Cont; $^{\#\#}p<0.01$ vs. Pic, Bic, and Ver; $^{\S\S}p<0.01$ vs. Nif, Flu, and NNC; $^{\dagger\dagger}p<0.01$ vs. Pic and Ver, analyzed with non-parametric multiple comparison followed by Steel-Dwass test

Propofol-induced depolarization of mouse DRG cells through $GABA_A$ receptor activation

Given that propofol exhibited an ability to cause intracellular $[Ca^{2+}]_i$ increases in DRG cells through $GABA_A$ receptor activation (as shown in Fig. 4), we performed patch-clamp recordings of GABA-responsive V1A1DKO DRG cells to confirm whether $GABA_A$ receptor activation by propofol causes depolarization of DRG cells. As shown in Fig. 5A, propofol (50 μM) induced inward currents repeatedly, which could cause depolarization, in a GABA-responsive cell at −60 mV. The propofol-induced inward current was completely inhibited by picrotoxin (100 μM, n=4). Furthermore, not only GABA but also propofol depolarized isolated V1A1DKO DRG cells (Fig. 5B upper panel and C, n=4) followed by action potential generation, while such propofol-induced action potential generation observed in V1A1DKO DRG cells was inhibited by picrotoxin (Fig. 5B lower panel, n=4). These results obtained in our preparations indicate that $GABA_A$ receptor activation by propofol causes action potential generation in mouse DRG cells, suggesting that TRPA1 and TRPV1 are not sole targets for propofol actions as expected from the results presented in Fig. 3.

Discussion

In the present study, we found that the effects of propofol on TRPV1 varied in a species-dependent manner and that the ability of propofol to activate hTRPV1 is negligible (Fig. 1). These in vitro data indicate that hTRPV1 is not involved in propofol-induced pain sensation in humans. Regarding propofol-evoked TRPA1 activation, the ability of propofol to activate hTRPA1 was much greater than mTRPA1, consistent with many reports of propofol-induced pain sensation in humans [18] while few reports regarding propofol-induced pain-related behaviors in mice injected with propofol in their hindpaws. We also for the first time observed activation of hTRPA1 by propofol at a single-channel level (Fig. 2), indicating the activation of hTRPA1 in a membrane-delimited manner.

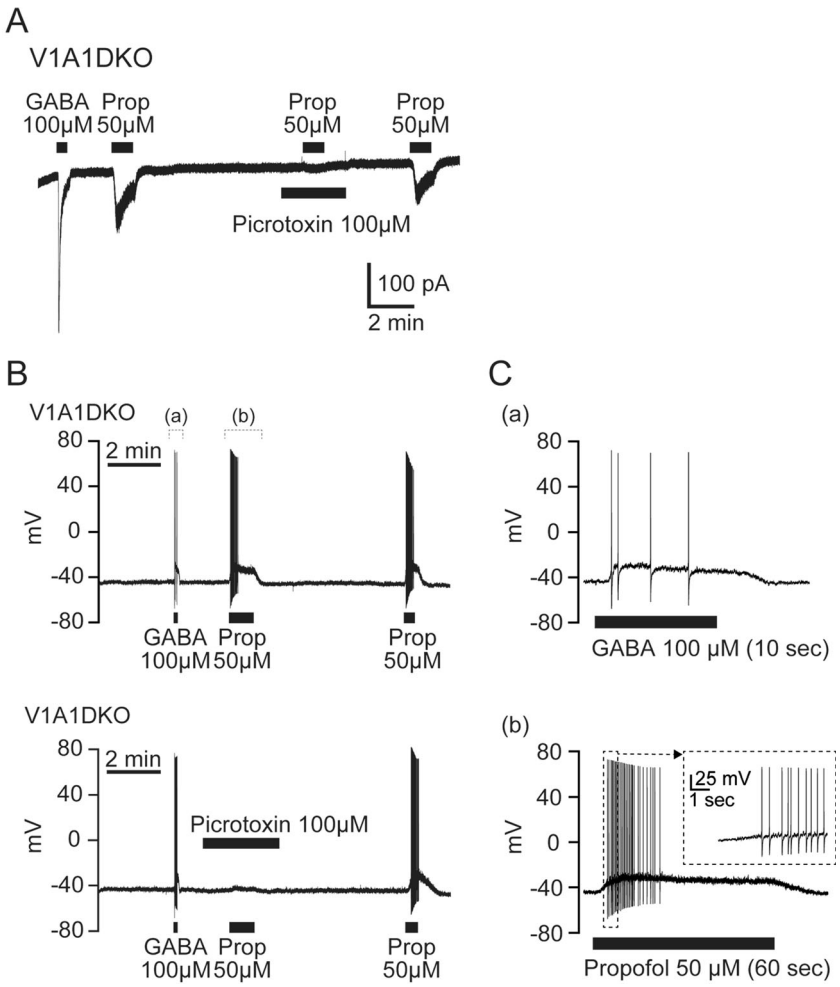

Fig. 5 GABA and propofol induce depolarization and action potential generation in V1A1DKO DRG cells. **A** A representative trace of GABA- and propofol-induced inward currents in V1A1DKO DRG (n=4, Cm=25.9±3.8 pF). Picrotoxin inhibited the propofol-induced inward currents. Vm=−60 mV. **B** GABA- and propofol-induced depolarization and action potentials in V1A1DKO DRG cells (n=6, Cm=19.5±1.3 pF). Picrotoxin inhibited the propofol-induced action potentials (n=4, Cm=17.0±0.6 pF). **C** Traces indicated as (*a*) and (*b*) in **B** are expanded as (*a*) and (*b*), respectively

Propofol-induced pain sensation in patients arises immediately after injection [18, 40]. We hypothesized that such an acute response could be mediated by activation of ion channels expressed in peripheral neurons. Previous reports showed that TRP channels, especially TRPV1 and TRPA1, might be involved in the propofol-induced $[Ca^{2+}]_i$ increases in Ca^{2+}-imaging studies using DRG neurons [13, 28]. However, we found alternative components that responded to propofol in Ca^{2+}-imaging studies using V1A1DKO DRG cells, suggesting that other targets might be involved in the mechanism of the propofol-induced $[Ca^{2+}]_i$ increases as well. Regarding the involved molecules mediating propofol-induced $[Ca^{2+}]_i$ increases, we determined that both T-type and L-type Ca^{2+} channels are activated upon depolarization caused by propofol-induced $GABA_A$ receptor activation. T-type Ca^{2+} channels are known to be expressed in small- and medium-sized DRG neurons [2, 36], corresponding to the size of propofol-responsive V1A1DKO DRG cells as shown in Fig. 3f. After depolarization by $GABA_A$ receptor activation, T-type Ca^{2+} channels are expected to be more activated than L-type Ca^{2+} channels, and propofol may thereby induce considerable amounts of depolarization. The effects of verapamil (which inhibits both L-type and T-type Ca^{2+} channels) were similar to effects caused by picrotoxin, indicating that L-type and T-type Ca^{2+} channels are the main targets downstream of depolarization by $GABA_A$ receptor activation, although we cannot rule out the possibility that other proteins are inhibited by verapamil.

It is widely believed that many general anesthetics activate $GABA_A$ receptors to produce their anesthetic effects in the brain [1, 14, 16, 35]. In spinal cord neurons, chloride concentrations can be changed dynamically depending on tissue conditions such as injury or inflammation to reduce the expression of Cl^- transporters, including KCC2 [10]. Intracellular Cl^- concentrations in neurons are also known to be high in early developmental stages when GABA may excite neurons [20]. In the case of peripheral sensory neurons such as DRG neurons, intracellular Cl^- concentrations are high owing to the low expression of KCC2 [27], indicating that Cl^- channel openings cause depolarization with Cl^- efflux. Whether $GABA_A$ receptor activation could cause excitatory responses of peripheral neurons in rodents depends on the intracellular Cl^- concentrations [3, 15, 37]. In this regard, it is possible that DRG neurons could be depolarized, followed by action

potential generation, through $GABA_A$ receptor activation due to high intracellular Cl^- concentrations. Indeed, we clearly observed action potential generation in some V1A1DKO DRG cells during application of propofol, which was drastically reduced by antagonizing $GABA_A$ receptor activity as shown in Fig. 5. Additionally, our in vitro study shows that propofol activates hTRPA1 with an EC_{50} value of 65.4 μM, which is close to the previously reported values of 48 μM in the oocyte system [42] and 23 μM in HEK293 cells [29] using human $GABA_A$ receptor containing β2 subunits, suggesting that propofol activates not only TRPA1 but also $GABA_A$ receptors within the same concentration ranges if it can reach peripheral nerve endings across blood vessels. Propofol-induced anesthetic effects are mediated by activation of $GABA_A$ receptors containing β2 or β3 subunits, and such effects vary depending on the subunit components of $GABA_A$ receptors [16, 19, 45]. It was reported that a subset of $GABA_A$ receptors containing β2/β3 subunits were expressed in unmyelinated sensory fibers [6] and that bilateral effects of $GABA_A$ receptor activation were observed in formalin-induced pain behaviors, meaning that low concentrations of muscimol attenuated while higher concentrations enhanced pain behaviors [6, 15]. Although we did not evaluate the effects of $GABA_A$ receptor activation by propofol on central terminals of DRG neurons or spinal cord neurons, it is possible that propofol might have a considerable influence on peripheral nociception by activating peripheral $GABA_A$ receptors and voltage-gated Ca^{2+} channels in addition to voltage-gated Na^+ channels.

In conclusion, the pain-producing effects of propofol may relate to its actions on both TRPA1 and $GABA_A$ receptors as shown in our study. Importantly, compounds acting on $GABA_A$ receptors in the periphery may be expected to exhibit similar abilities to induce pain sensation through activation of voltage-gated channels.

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Conflict of interest The authors declare no conflict of interest.

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