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Volatile anesthetic effects on isolated GABA synapses and extrasynaptic receptors

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ABSTRACT

The volatile anesthetics enhance GABAergic inhibitory transmission at synaptic and extrasynaptic sites at central neurons. In the present study, we investigated the effects of three volatile anesthetics (isoflurane, enflurane and sevoflurane) on synaptic and extrasynaptic $GABA_A$ receptor responses using mechanically dissociated rat hippocampal CA1 neurons in which functional native nerve endings (boutons) were retained. The extrasynaptic $GABA_A$ receptors were activated by exogenous GABA application while synaptic ones were assessed by miniature and evoked inhibitory postsynaptic currents (mIPSCs and eIPSCs, respectively). All volatile anesthetics concentration-dependently enhanced the exogenous GABA-induced postsynaptic responses. The structural isomers, isoflurane and enflurane, increased mIPSC frequency while sevoflurane had no effect. None of these anesthetics altered mIPSC amplitudes at their clinically relevant concentrations. Sevoflurane prolonged event kinetics by increasing decay time of mIPSCs and eIPSCs at clinically relevant concentration. On the other hand, both isoflurane and enflurane only prolonged the kinetics of these events at 1 mM of high concentration. For GABAergic eIPSCs, both isoflurane and enflurane decreased the evoked response amplitude and increased the failure rate (Rf), while sevoflurane decreased the amplitude without affecting Rf. These results suggest that isoflurane and enflurane at the clinically relevant concentrations predominantly act on GABAergic presynaptic nerve endings to decrease action potential dependent GABA release. It was concluded that these anesthetics have heterogeneous effects on mIPSCs and eIPSCs with different modulation of synaptic and extrasynaptic $GABA_A$ receptors.

1. Introduction

Volatile anesthetics inhibit neuronal activities throughout the central nervous system (CNS) with complex behavioral components such as sedation, analgesia, hypnosis, unconsciousness, and immobility. The previous studies have demonstrated that volatile anesthetics bind to γ-aminobutyric acid type A ($GABA_A$) receptors which consist of anesthetics-sensitive α1, α2, and β1 subunits (Mihic et al., 1997; Koltchine et al., 1999; Siegwart et al., 2003), and these often prominently prolong the current decay phase of $GABA_A$ inhibitory postsynaptic currents (IPSCs) and enhance neuronal inhibition in brain slice and primary cultured neuron preparations (Jones et al., 1992; Zimmerman et al., 1994; Nishikawa and MacIver, 2001; Nishikawa et al., 2005). Furthermore, recent studies of the molecular targets of volatile anesthetics identified a tonic conductance generated by activation of extrasynaptic $GABA_A$ receptors (Bai et al., 2001; Caraiscos et al., 2004b; Hemmings et al., 2005a; Bonin and Orser, 2008; Bieda et al., 2009). The extrasynaptic $GABA_A$ responses (one of postsynaptic targets) in cultured cells and dissociated neurons were also increased by volatile anesthetics (Wakamori et al., 1991; Wu et al., 1996; Sebel et al., 2006). Moreover, volatile anesthetics altered presynaptic targets that affect synaptic vesicle exocytosis as detected by laser-scanning fluorescence microscopy or by isotope method using isolated nerve terminal (Westphalen and Hemmings, 2003; Hemmings et al., 2005b). Examination of volatile anesthetic actions at single synapses provides more direct information by reducing interference by surrounding tissues such as other neurons and glial cells (Akaike et al., 2002; Akaike and Moorhouse, 2003). Here, we examined how three volatile anesthetics (isoflurane, enflurane and sevoflurane) modulate GABA release by measuring both the frequency of miniature IPSCs (mIPSCs) and the failure rate (Rf) of action potential-evoked IPSCs (eIPSCs). Comparisons of the amplitudes of mIPSCs and eIPSCs along with exogenous GABA-induced

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currents assessed volatile anesthetic effects on synaptic and extrasynaptic $GABA_A$ receptors.

2. Materials and methods

2.1. Mechanical dissociation of hippocampal CA1 neurons

All experiments were performed in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences of the Physiological Society of Japan. Wistar rats (11–17 days old) were decapitated under pentobarbital anesthesia (50 mg/kg, i.p.). The brain was quickly removed and immersed in ice-cold artificial cerebrospinal fluid. The hippocampus was removed and cut at a thickness of 400 μm with a microslicer (Leica VT1000S) in ice-cold incubation solution. The brain slices were kept in incubation solution saturated with 95% O_2 and 5% CO_2 at room temperature (22–25 °C) for at least 1 h. For mechanical dissociation, the slices were transferred to a 35 mm culture dish. Mechanical dissociation with a vibrating fire-polished glass pipette (40–50 Hz, S-I 10 cell Isolator, K.T. Labs, Japan) yielded isolated CA1 neurons with functional excitatory and inhibitory nerve terminals ('synaptic bouton' preparation) (Akaike et al., 2002; Jang et al., 2002; Akaike and Moorhouse, 2003). Before electrophysiological recording, neurons settled and attached to the bottom of the culture dish within 15 min.

2.2. Electrical measurements

$GABA_A$ receptor mediated currents were recorded in whole-cell patch recording mode (Molecular Devices, MultiClamp 700B, Foster city, CA, USA) at a holding potential (V_H) of 0 mV which is near the equilibrium potential of excitatory glutamatergic postsynaptic currents under our ionic conditions (Jang et al., 2001). The external standard solution was used during electrical recordings. GABAergic IPSCs were isolated pharmacologically by the continuous presence of glutamatergic blockers (20 μM CNQX, 50 μM D-AP5). Patch pipettes were made from borosilicate glass tubes (1.5 mm o.d., 0.9 mm i.d.; G-1.5, Narishige, Tokyo, Japan) in two stages on a vertical pipette puller (PC-10, Narishige) and had resistances of 3–6 MΩ. Neurons were visualized with phase-contrast on an inverted microscope (DMI3000B, Leica, Wetzar, Germany). Currents were monitored on an oscilloscope (Kenwood DCS-7040, Melrose, MA, USA) and a pen recorder (Linearcorder WR3701, Graphtec corp., Tokyo, Japan). All membrane currents were filtered at 1 kHz (3611 Decade Filter, NF Electronic Instruments, Tokyo, Japan), digitized at 3 kHz using pCLAMP 10.0 (Molecular Devices).

For focal electrical stimulation of single boutons (Akaike et al., 2002; Jang et al., 2002; Akaike and Moorhouse, 2003), a bipolar stimulating pipette (θ glass tube with inner diameter about 0.5 μm) was filled with external standard solution with the anode and cathode in separate compartments of the θ glass tube. The stimulating electrode was placed close to the postsynaptic soma membrane of a dissociated single neuron while recording whole-cell currents under voltage clamp. The stimulating pipette was then carefully moved along the surface membrane of the soma and dendrites while delivering periodic shocks until eIPSCs appeared. The individual boutons were stimulated by current pulses (100 μs duration, 0.1–0.2 mA current intensity) at a frequency of 0.1 Hz from an isolator (SS-202 J, Nihon Kohden, Tokyo, Japan) (Maeda et al., 2009). The eIPSCs appeared in all-or-nothing fashion indicating that the stimulating pipette was positioned just above a single GABAergic bouton (Akaike et al., 2002; Akaike and Moorhouse, 2003). Current strength of focal stimulation was adjusted to produce a 10–40% failure rate. This focal eIPSCs were blocked reversibly by 300 nM tetrodotoxin (Akaike et al., 2002). All experiments were performed at room temperature (22–25 °C).

2.3. Solutions

The ionic composition of external incubation solution for slice preparation was (mM): NaCl 124, KCl 5, KH_2PO_4 1.2, $MgSO_4$ 1.3, $CaCl_2$ 2.4, glucose 10 and $NaHCO_3$ 24, bubbled with 95% O_2 and 5% CO_2 to adjust to pH to 7.4. The external standard solution was (mM): NaCl 150, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10 and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid 10, pH adjusted to 7.4 with Tris[hydroxymethyl]amino methane-OH (Tris-base) at room temperature. The ionic composition of the internal (patch pipette) solution was (mM): Cs-methanesulfonate 80, CsF 65, TEA-Cl 5, ethylene glycol tetraacetic acid 2, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid 10 and adenosine 5′-triphosphate magnesium salt (ATP-Mg) 4, pH was adjusted to 7.2 with Tris-base. ATP-Mg was dissolved in the internal solution just before use.

2.4. Drugs

For selective recording of GABAergic currents, the external test solution contained 20 μM CNQX and 50 μM D-AP5 (Tocris) to block AMPA- and N-methyl-D-aspartate (NMDA)-type glutamatergic receptors. 300 nM tetrodotoxin was used to selectively block Na channels for recording mIPSCs. Bicuculline (10 μM) and gabazine (1 μM) were used as $GABA_A$ receptor antagonists (Sigma, St. Louis, MO). All drugs and ligands including the anesthetics were applied by the "Y-tube system" which enables solution exchange within 20 ms (Murase et al., 1990). The half-maximum concentration in the concentration–response relationship for GABA-induced postsynaptic current was about 50 μM. At concentrations more than 10 μM, GABA-induced current decayed rapidly to a steady-state level due to desensitization (Uneyama et al., 1993; Wu et al., 1996). To examine enhanced effects of volatile anesthetics on exogenous GABA-induced current, therefore, we used a concentration of 1 μM at which GABA elicits little desensitization of $GABA_A$ receptors (Fig. 1A).

2.5. Preparation of anesthetics in aqueous solution

In this study, volatile anesthetics were completely and rapidly dissolved in 100% dimethyl sulfoxide at 1 M as a stock solution (O'Leary et al., 2000) just before use. The necessary amount of the stock solution was mixed into 10 ml external solution in a tightly capped glass test tube. During the electrical recording, the external test solution containing anesthetics was continuously applied to the isolated neuron by Y-tube system through silicon tube from capped glass test tube sealed closely by parafilm to avoid the evaporation. Another method which prepared test solution with volatile anesthetics was also used. For example, volatile anesthetics was injected into 100 ml of external solution in a tightly capped glass flask, and the test solution was sonicated for a few minutes until the droplet of volatile anesthetics had completely dissolved (Wu et al., 1996; Yamashita et al., 2001).

The concentrations of three anesthetics used were selected to approximate clinically relevant concentrations: 300 μM isoflurane, 600 μM enflurane and 300 μM sevoflurane (Krasowski and Harrison, 1999; Cheng and Kendig, 2002).

2.6. Data analysis

For analysis of exogenous GABA-induced postsynaptic responses, peak amplitudes were normalized to the peak of the respective control currents in response to 1 μM GABA. Concentration–response curves for GABA were fitted by a sigmoidal dose–response equation with Origin Pro 7.5 software (OriginLab Corporation, Northampton, MA, USA). The relative peak amplitudes of the exogenous GABA-induced postsynaptic responses were obtained from curve fitting by use of following equation:

$$Y = A_1 + (A_2 - A_1)/\left(1 + 10^{(\log X_0 - X)p}\right)$$

where Y is the measured response of the relative amplitudes of Cl^- current (I_{Cl}) A_2 and A_1 are the maximum and the minimum responses respectively of the relative amplitudes of I_{Cl} obtained with a control, p is the slope parameter of the dose–response curve, X is the applied dose, and $\log X_0$ is the center of the curve that is the concentration for half the relative amplitudes of I_{Cl}.

mIPSC events were counted and analyzed in pre-set epochs before, during, and after each test condition using MiniAnalysis Program (Synaptosoft Inc, Decatur, GA, USA). Briefly, the mIPSCs events were initially screened automatically using an amplitude threshold of 5 pA and then visually accepted or rejected based upon their 10–90% rise and 90–37% decay times. The mIPSC decay times were calculated from the averaged trace by 4–7 neurons at 13–190 events each. The eIPSC decay times were also analyzed from 3–6 neurons at 4–21 events each using MiniAnalysis Program, but the events were visually accepted. The amplitude and failure rate (Rf) of eIPSCs were analyzed with pCLAMP 10.0. The data were analyzed at a steady-state level during the drug application over a period of 2–3 min beginning 30 s after adding sevoflurane, and 2–5 min after adding isoflurane (shown in Fig. 2B) or enflurane. The average values of synaptic events during control period (before and after drugs application period for 2–3 min each) were calculated, and effects of drugs were quantified as relative changes in the amplitude, frequency, and decay time of mIPSCs, and in the amplitude, Rf, and decay time of eIPSCs compared with the respective controls. Values were reported as mean ± standard error (SEM) of the mean of values normalized to the individual control levels.

The significance of enhanced effects of anesthetics on the amplitude of GABA-induced outward current (Fig. 1), and the differences in the current amplitude, decay time, frequency, and Rf distribution (Figs. 2–7) were tested by Student's *t*-test using the relative values. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Effects of volatile anesthetics on extrasynaptic $GABA_A$ receptors

At a holding potential (V_H) of 0 mV, exogenous 1 μM GABA postsynaptically rapidly and reversibly induced an outward current in mechanically dissociated hippocampal CA1 neurons (Fig. 1Aa). The GABA-induced current was increased when coapplied with 300 μM isoflurane regardless of whether dissolved in DMSO or sonicated (Fig. 1Aa, b). Since the GABA responses were equally enhanced by both formulations of isoflurane (Fig. 1A), isoflurane loss during drug preparation and perfusion was unlikely to be a factor.