Sevoflurane Induced Suppression of Inhibitory Synaptic Transmission Between Soma-Soma Paired Lymnaea Neurons

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In this study, we utilized a recently developed model system (Feng et al. 1997b), where a specific inhibitory synapse between individually identifiable pre- [right pedal dorsal 1 (RPeD1)] and postsynaptic [visceral dorsal 4 (VD4)] neurons from the mollusk Lymnaea stagnalis was reconstructed between the cell bodies. This soma-soma model has several advantages over conventionally used neurite-neurite synapses (Feng et al. 1997b). For example, because the synapses develop between the cell bodies (in the absence of neurites), they tend to perturb neuronal communication in the nervous system by either interfering with postsynaptic transmitter receptors and presynaptic transmitter release or alter various ion channel conductances (see Bazil and Minneman 1989; Dilger et al. 1994; Franks and Lieb 1988; Hirota and Roth 1997; Pocock and Richards 1991; Puil and El-Beheiry 1990; Richards 1983; Spencer et al. 1995, 1996; Weight et al. 1992). However, the precise cellular and synaptic mechanisms underlying anesthetic action have not been fully defined because synaptic transmission between defined sets of pre- and postsynaptic neurons in vertebrates is often difficult to study directly.

Due to their relatively simpler nervous systems, various molluscan species have been used extensively to determine how anesthetics affect cell-cell communication in the nervous system. For instance, identified neurons from Lymnaea (Franks and Lieb 1988, 1991a; Girdlestone et al. 1989a,b; Spencer et al. 1995, 1996), Helix (Judge and Norman 1982), and Aplysia (Arimura and Ikemoto 1986) have been used to determine how halothane, isoflurane, and enflurane affect synaptic transmission in the nervous system. Girdlestone et al. (1989a,b) found that clinically relevant concentrations of halothane (1–2%) induced complete “anesthesia” (suppression of whole body reflexes) in Lymnaea. Using isolated ganglionic preparations; they subsequently showed that both halothane and isoflurane suppressed chemical, but not electrical, synaptic transmission between identified Lymnaea neurons (Girdlestone et al. 1989a,b). Halothane (1–2%) was also shown to suppress the peptidergic synaptic transmission between cultured Lymnaea neurons (Spencer et al. 1995, 1996). Similarly, cholinergic synaptic transmission between identified Aplysia neurons was found to be blocked by clinically relevant concentrations of enflurane (Arimura and Ikemoto 1986). Studies from the laboratories of Franks and Lieb (1988, 1991b) have since identified and characterized various ion channels that are altered by halothane in cultured Lymnaea neurons. Together, the above studies have established the usefulness of several simpler invertebrate preparations for research on the cellular and synaptic mechanisms by which anesthetics affect cell-cell interactions in the nervous system. However, even in the above simple model systems, sevoflurane is yet to be tested for its actions on synaptic transmission.

In this study, we utilized a recently developed model system (Feng et al. 1997b), where a specific inhibitory synapse between individually identifiable pre- [right pedal dorsal 1 (RPeD1)] and postsynaptic [visceral dorsal 4 (VD4)] neurons from the mollusk Lymnaea stagnalis was reconstructed between the cell bodies. This soma-soma model has several advantages over conventionally used neurite-neurite synapses (Feng et al. 1997b). For example, because the synapses develop between the cell bodies (in the absence of neurites), they

INTRODUCTION

Inhalation anesthetics such as halothane, isoflurane, and sevoflurane induce unconsciousness (general anesthesia) in animals and are therefore extensively used during surgical procedures (Krnjevic 1991). Anesthetics are generally thought to perturb neuronal communication in the nervous system by either interfering with postsynaptic transmitter receptors and presynaptic transmitter release or alter various ion channel conductances (see Bazil and Minneman 1989; Dilger et al.
are suitable for direct electrophysiological analysis. In addition, both the somata and their synapses can be exposed simultaneously and directly to various anesthetic agents.

RPedD1 and VD4 are respiratory central pattern generating (CPG) neurons, and when stimulated electrically (either in vivo or in vitro), RPedD1 produces 1:1 inhibitory postsynaptic potentials (IPSPs) in VD4. This synaptic transmission is dopaminergic and similar to that seen in the intact brain (Syed and Winlow 1991a). The soma-soma synapse between RPedD1 and VD4 provided us with an excellent opportunity to test both pre- and postsynaptic mechanisms by which clinically relevant concentrations of sevoflurane (1–4%) affected inhibitory synaptic transmission in the nervous system.

METHODS

Animals

Laboratory-raised stocks of the fresh water snail *Lymnaea stagnalis* were maintained at room temperature (18–20°C) in well-aerated artificial pond water and fed lettuce (Ridgway et al. 1991). Snails with a shell length of 20–25 mm (approximate age 4–6 mo) were used in all experiments.

Cell culture

Animals were dissected under sterile cell culture conditions as described earlier (Syed et al. 1990). The isolated central ring ganglia were washed several times (6–7 washes of 10 min each) with antibiotic saline (Gentamycin, 50 μg/ml). These were subsequently enzyme treated (Trypsin, Sigma type III) for 20–40 min and pinned to the bottom of a dissection dish (Syed et al. 1990). Fine forceps were used to remove the connective sheath surrounding the somata. A fire-polished glass pipette attached to a microsyringe (Gilmont) was used to extract individual neurons from the intact ganglion. The isolated cells were plated on polyl-lysine-coated tissue culture dishes (Falcon 3001), containing 3 ml of either defined medium (DM) or brain conditioned medium (CM) (see Ridgway et al. 1991). Soma-soma synapses were prepared by juxtaposing freshly isolated somata of identified neurons (see Feng et al. 1997 for details). The paired neurons were left undisturbed overnight at room temperature.

Electrophysiology

SHARP ELECTRODE RECORDINGS. Conventional intracellular recording techniques were used (Syed and Winlow 1991b). Specifically, glass microelectrodes (1.5 mm ID, WPI) were pulled on a vertical electrode puller (Kopf, 700C) and filled with a saturated solution (3 M) of K₂SO₄ (resistance 20–40 MΩ). Neurons were viewed under a Zeiss (Axiovert 135 or Telaval 31) inverted microscope and impaled using Narishige micromanipulators (model MO-103). The intracellular signals were amplified via a preamplifier (NeuroData, IR-283), displayed on a storage oscilloscope (Tektronix R5103N), and regularly signals were amplified via a preamplifier (NeuroData, IR-283), using Narishige micromanipulators (model MO-103). The intracellular signals were amplified via a preamplifier (NeuroData, IR-283), displayed on a storage oscilloscope (Tektronix R5103N), and recorded on a Gould chart recorder (Recorder 2200 s). All experiments were performed at room temperature (18–22°C).

WHOLE CELL PATCH-CLAMP RECORDINGS. Whole cell voltage-clamp (membrane rupture) or current-clamp recordings were made using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA). Patch electrodes (1–2 MΩ) were pulled from PG150T-7.5 glass tubing (Warner Instrument Hamden, CT), on a vertical pipette puller (Kopf 750, Tujunga, CA). To study K⁺ currents, pipettes were filled with filtered pipette solution containing (pH 7.4; in mM): 29 KCl, 1/11 Ca/BAPTA buffer, and 10 HEPES, supplemented with 2 ATP-Mg and 0.1 GTP-Tris. To study Ca²⁺ currents, pipettes were filled with filtered (0.22 μm filter) cesium pipette solution consisting of (in mM) 29 CsCl, 1/11 Ca/BAPTA buffer, 1 tetraethylammonium (TEA Cl), 10 HEPES, 2 ATP-Mg, and 0.1 GTP-Tris, and the pH was adjusted to 7.4 (with CsOH). Potassium currents and membrane potentials were recorded in standard *Lymnaea* saline consisting of (in mM) 51.3 NaCl, 1.7 KCl, 1.5 MgCl₂, and 4.1 CaCl₂. To record Ca²⁺ currents, the bath solution consisted of (in mM) 52 TEA Cl, 1 MgCl₂, 4 CaCl₂, 1 4-aminopyridine (4-AP), and 5 HEPES (pH 7.4). After obtaining a gigahm-seal, the series resistance was compensated (<0.8 MΩ) using the membrane test function (pClamp-7, Axon Instruments). The Ca²⁺ current measured in this study was filtered at 1 kHz using a 4-pole Bessel filter and digitized at a sampling frequency of 2 kHz. The voltage command generation and data acquisition were carried out using a Dell (XPS R350) computer equipped with a Digidata 1200 interface (Axon Instruments) in conjunction with pClamp-7 software (Axon Instruments).

Gas chromatography

Sevoflurane (Maruishi Pharmaceutical, Japan) was vaporized in 100% O₂, using a sevoflurane Type-s MK III–VII (Acoma) vaporizer and bubbled for at least 15 min into the reservoirs containing *Lymnaea* saline (see Girdlestone et al. 1989a). To minimize gas loss over time, all anesthetic solutions were prepared fresh in sealed glass reservoirs. To determine the final anesthetic concentration, 1 ml samples (from the outlet of the experimental dish) were collected, and standards were prepared in 2–ml sealed vials, to which a 10–μl internal standard (enflurane) was added. Sevoflurane samples and standards were analyzed using a HP 5890A Gas Chromatograph, equipped with flame-ionizing detector (FID). The column used was a DB-1 (30 m × 0.53 mm × 5.0 μm film J&W Scientific), and the carrier gas (helium) flow was adjusted to 6.0 ml/min. The oven program were set to run at 40°C for 5.2 min, then switched to 125°C at a rate of 30°C/min. Sample injections of 1.0 μl were split 1:3. The chromatograph acquisition and analysis was performed with a HP 3396A Integrator. The above program produced retention times of 2.4 min for sevoflurane and 3.4 min for the internal standard enflurane. Analysis of perfusate, collected from the outlet of perfusion system, allowed us to establish molar concentrations of anesthetic in the viscosity of neurons. For example, the lowest concentration (1%) used in this study corresponded to 0.3 mM; whereas the highest sevoflurane concentration (7%) was estimated to be 2.5 mM. Therefore 1% sevoflurane used in this study corresponded to an aqueous equivalent of mammalian minimal alveolar concentration (MAC) (see Franks and Lieb 1997).

Anesthetic delivery

To minimize gas loss, Teflon tubing was used throughout the perfusion system, and sevoflurane solution was delivered directly to the somata using a computer-controlled pressurized perfusion system (Multi-Flo RVA-6 Norscan Instruments Winnipeg, Canada). This perfusion system was connected to the micromanifold, with dead volume <2 μl and allowed us to switch between two solutions in <200 ms.

Statistics

All parametric data were presented as means ± SD. Differences between mean values from each experimental group were tested using either a Student’s t-test (for 2 groups) or repeated measures ANOVA (for multiple comparisons). Differences among groups were considered significant if P < 0.05 (P < 0.05).
RESULTS

Sevoflurane suppressed inhibitory synaptic transmission between RPeD1 and VD4 in a concentration-dependent manner

To test sevoflurane (1–4%) for its action on inhibitory synaptic transmission, synapses between the somata of RPeD1 and VD4 were reconstructed in vitro (Fig. 1A). Following 12–18 h of soma-soma pairing, simultaneous intracellular recordings were made from both cells, and a chemical synapse was demonstrated electrophysiologically (Fig. 1B). Specifically, current induced action potentials in RPeD1 produced 1:1 inhibitory postsynaptic potentials (IPSPs) in VD4 (n = 37, Fig. 1B). These synapses were similar to those seen in vivo (Syed and Winlow 1991a). To determine whether sevoflurane affected the amplitude of RPeD1-induced IPSPs in VD4, synapses were tested either in the absence or presence of the anesthetic (Fig. 1C). Single action potentials were induced in RPeD1 via current injection, and the average IPSPs amplitude was analyzed in VD4, either in the absence or presence of sevoflurane (Fig. 1C). Sevoflurane in a dose-dependent (Fig. 1D) and reversible manner (Fig. 1C), blocked RPeD1-induced IPSPs in VD4 (n = 6). It is important to mention that because both neurons were hyperpolarized by sevoflurane below the
threshold for their spiking activity, greater currents were often required to elicit action potentials in the presence of this anesthetic.

Sevoflurane blocked action potentials in both RPeD1 and VD4

To deduce the possible sites of sevoflurane actions, we began our analyses by examining effects on the intrinsic membrane properties of both RPeD1 and VD4. Specifically, individually isolated neurons RPeD1 and VD4 were maintained in vitro for 12–24 h. Current-clamp recordings were made from individual somata, and sevoflurane (4%) was applied directly to the cell body via a fast perfusion system. As shown in Fig. 2, sevoflurane completely blocked an induced (previously quiescent cells were depolarized just above the threshold for firing) train of action potentials in both RPeD1 and VD4. Throughout the course of anesthetic delivery, both cells remained hyperpolarized below their threshold for the activation of action potentials. Normal spiking activity, however, resumed immediately after wash out with normal saline.

To further examine the potential synaptic sites for anesthetic actions, we sought to determine whether sevoflurane modified the postsynaptic dopamine response in VD4.

Dopamine-induced effects on postsynaptic K⁺ conductance were mimicked by sevoflurane

RPeD1 is known to contain and release dopamine. Its synaptic transmission with VD4 is dopaminergic (Magoski et al. 1995). Moreover, RPeD1-induced effects on VD4 activity are mimicked by exogenous dopamine and involve changes in K⁺ channel activity (Barnes et al. 1994). To test the hypothesis that sevoflurane affected the dopamine-activated, postsynaptic K⁺ current in VD4, we made current-clamp and voltage-clamp recordings from individually cultured cells. Exogenous dopamine (10 μm) application hyperpolarized VD4 from its resting membrane potential of −53 to −72 mV (69.8 ± 1.65; mean ± SD, n = 4). These effects were mimicked by sevoflurane (4%; n = 9, Fig. 3A). When applied in the presence of sevoflurane (4%), dopamine produced a further hyperpolarization in VD4. The net response was, however, similar to that produced by dopamine alone (n = 5, Fig. 3A). The dopamine-induced hyperpolarizing response in VD4 was, however, fully matched by 7% (higher than clinical range) sevoflurane. Together, these data suggest that both dopamine and sevoflurane-induced hyperpolarizing responses in VD4 are highly reproducible and reversible and do not desensitize rapidly. Moreover, sevoflurane and dopamine-induced hyperpolarizing responses in VD4 were nonadditive.

We next sought to determine whether both dopamine and sevoflurane-induced hyperpolarizing effects in VD4 involved the same K⁺ channel. To test this possibility, voltage-clamp recordings were made from VD4 cells, and K⁺ conductance was analyzed (Fig. 3B). When held near its resting membrane potential (−50 mV), dopamine (10 μm) application alone initiated a 92-pA outward current in VD4. Similarly, sevoflurane at a concentration of 7% (higher than clinical range) and 4% activated a 40- and 30-pA outward conductance, respectively. Dopamine application in the presence of sevoflurane enhanced this outward current; however, the sum of total outward conductance activated by both compounds was equivalent to that induced by dopamine alone (n = 5, Fig. 3B). These data suggest that both dopamine and sevoflurane may activate the same K⁺ channel. Figure 3C shows ramp I-V relationship between sevoflurane-sensitive current conductances, demonstrating that the voltage-insensitive outward current is concentration dependent and has a reversal potential of −69.6 ± 3.1 mV (n = 8). These data are consistent with those presented in Fig. 3A and suggest that the current is most likely carried by K⁺ ions [according to the Nernst equation, the K⁺ equilibrium potential for Lymnaea neurons is −71 mV (under
our experimental conditions with 29 mM K inside and 1.7 mM outside the cell).

Sevoflurane reduced presynaptic Ca\(^{2+}\) currents in RPeD1

Synaptic transmission between RPeD1 and VD4 requires Ca\(^{2+}\) influx through high-voltage–activated (HVA), and L-type–like Ca\(^{2+}\) channels (Feng et al. 1997a). To test whether sevoflurane-induced suppression of synaptic transmission between RPeD1 and VD4 involved presynaptic Ca\(^{2+}\) channels, Ca\(^{2+}\) currents were recorded from RPeD1 in a whole cell voltage-clamp configuration. Specifically, individual somata were isolated in culture, and macroscopic Ca\(^{2+}\) currents were

FIG. 3. Sevoflurane altered dopamine-sensitive K\(^+\) currents in VD4. A: isolated VD4 somata were current clamped, and dopamine was applied exogenously, either in the presence or absence of sevoflurane (4 and 7%). Dopamine (10 \(\mu\)M) hyperpolarized VD4 from a resting membrane potential of \(-53\) to \(-72\) mV. A similar hyperpolarizing response was induced by sevoflurane. When applied in the continuous presence of sevoflurane, dopamine did induce further hyperpolarizing response in VD4; however, the net response was similar to that produced by dopamine alone. B: VD4 (same somata as was used in A) was voltage clamped at a holding potential of \(-50\) mV, and dopamine-activated, outward current was recorded either in the absence or presence of sevoflurane. Pressure applications of dopamine (10 \(\mu\)M) alone evoked an outward current. In the presence of 4\% sevoflurane, further dopamine applications induced a smaller current that was completely matched by 7\% sevoflurane. The total outward current induced by both dopamine and sevoflurane was similar to that evoked by dopamine alone. C: ramp current-voltage (I-V) relationship of the sevoflurane-sensitive K current in a VD4 cell. The reversal potential of this voltage-insensitive current (in this particular cell) was \(-65\) mV. Inset: the activation of sevoflurane-induced outward current (at a holding potential of \(-50\) mV) was concentration dependent. These data were obtained from the same VD4 cell.
recorded either in the absence or presence of sevoflurane. Ca\(^{2+}\) current was evoked by 450-ms depolarizing pulses applied from a holding potential of \(-80\) to \(+50\) mV, in increments of 5 mV. As shown in Fig. 4A, inward Ca\(^{2+}\) current in RPeD1 neurons was reduced proportionally by increasing concentrations of sevoflurane. At depolarizing pulses, applied from a holding potential of \(-80\) to \(+10\) mV, significant reductions (27% ± 12) in the peak Ca\(^{2+}\) current \((n = 10, P < 0.05)\) were seen only at the higher (6% or equivalent to 6 \(\times\) MAC) concentration of anesthetic. At lower concentrations, however, changes in peak current amplitude were insignificant. The Ca\(^{2+}\) current inactivation rate was more sensitive to sevoflurane, and a marked increase was seen at concentrations as low as 2% (equivalent to 2 \(\times\) MAC; Fig. 4). Sevoflurane-induced effects on the HVA Ca\(^{2+}\) currents were reversible, and the current amplitude returned to its control level after the anesthetic was washed out with normal saline.

**DISCUSSION**

In the present study, we demonstrated that sevoflurane blocked the inhibitory synaptic transmission between neurons RPeD1 and VD4. Because sevoflurane also blocked action potentials in both pre- and postsynaptic neurons, we postulate that these effects on the intrinsic membrane properties of the cells can account for the suppression of synaptic transmission between the cells. Halothane was reported earlier to suppress action potentials in other, as yet unidentified *Lymnaea* neurons (Franks and Lieb 1997) and together with our data, these studies suggest that the anesthetic-induced effects on action potential parameters may be common to various different neuronal cell types. As compared with halothane, however (Franks and Lieb 1997), we found that most *Lymnaea* neurons (in addition to RPeD1 and VD4, data not shown), were sensitive to lower concentrations of sevoflurane (2–4%). These differences in neuronal responsiveness recorded in the above two studies can be attributed either to the nature of different anesthetic used or the type of cells examined. Furthermore, because sevoflurane hyperpolarized VD4 toward its reversal potential for K ions, we suggest that the anesthetic-induced suppression of inhibitory synaptic transmission may also involve voltage-insensitive K conductances.

The anesthetic-induced suppression of postsynaptic response has also been shown to involve ligand-gated ion channels (Weight et al. 1992). For instance, Arimura and Ikemota (1986) showed that enflurane-induced blockade of cholinergic synaptic transmission between *Aplysia* neurons involved a suppression of nicotinic Cl\(^-\) currents. A similar suppression of acetylcholine-induced, nicotinic Cl\(^-\) currents was observed in identified *Lymnaea* neurons (Franks and Lieb 1991a). In contrast, \(\gamma\)-aminobutyric acid (GABA)–activated Cl\(^-\) currents in rat dorsal root ganglia (DRG) neurons were significantly enhanced by halothane, isoflurane, and enflurane (Nakahiro et al. 1989). A similar halothane-induced enhancement of GABA-gated inward current was seen in cultured hippocampal neurons (Jones et al. 1992). In the present study, we demonstrated that a dopamine-sensitive, voltage-independent potassium conductance was enhanced by sevoflurane. Because this enhancement brought the resting membrane potential closer to the K\(^+\) equilibrium potential, we propose that the anesthetic-induced modulation of intrinsic membrane properties may be sufficient to account for the suppression of RPeD1-induced IPSPs in VD4. Consistent with this idea are our unpublished observations that RPeD1-induced IPSPs in VD4 persisted in the presence of anesthetic, when the postsynaptic membrane potential was adjusted closer to its spike threshold (data not shown).
by Franks and Lieb (1991b). These workers were the first to show that $I_{K, An}$ was cell type specific and was activated by both general anesthetics and synaptic inputs (Lopes et al. 1998). Specifically, they found that both halothane and dopamine activated the same K$^+$ current in the intact ganglia. In cell culture, however, the anesthetic-sensitive neurons did not respond to exogenous dopamine (Franks and Lieb 1997). Based on these results, they concluded that dopaminergic responses seen in the intact ganglia were most likely mediated indirectly via other neurons. Because in our cultures, VD4 exhibited robust postsynaptic response to dopamine, in a manner seen in vivo (Magoski et al. 1995), we postulate that dopamine and sevoflurane-induced effects most likely involved the same K$^+$ channel, although the underlying mechanisms remain to be determined.

Presynaptic transmitter release requires Ca$^{2+}$ influx through voltage-gated, N-Type Ca$^{2+}$ channels (Reuter 1996). Because most anesthetics are believed to suppress transmitter release, it therefore seemed logical that the anesthetic-induced synaptic depression might also involve these Ca$^{2+}$ channels. Because synaptic transmission between RPeD1 and VD4 requires Ca$^{2+}$ influx through voltage-gated Ca$^{2+}$ currents (Feng et al. 1997a), we asked whether anesthetic-induced synaptic depression between these cells involved Ca$^{2+}$ currents. Our data (at room temperature) demonstrated that the sevoflurane-induced modulation of Ca$^{2+}$ current amplitude was insignificant. These data are consistent with other earlier studies, in which the voltage-gated Ca$^{2+}$ currents were found to be relatively insensitive to clinically relevant concentration of volatile anesthetics (see Franks and Lieb 1993; Kress and Tas 1993). In the present study, sevoflurane exerted more pronounced effects on the rate of Ca$^{2+}$ current inactivation. Together, these data suggest that, although sevoflurane did not exert significant effects on whole cell total Ca current, these may nevertheless be sufficient to suppress transmitter release from RPeD1.

In summary, the data presented in this study demonstrate that clinically relevant concentrations of sevoflurane block inhibitory synaptic transmission between soma-soma paired Lymnaea neurons. The anesthetic-induced synaptic depression is both reversible and concentration dependent. We propose that sevoflurane may either block transmitter release by suppressing presynaptic Ca currents, or activate the dopamine-sensitive K conductance in the postsynaptic cell. The latter will hyperpolarize the membrane potential toward the reversal for K conductance and thus block the RPeD1-induced IPSPs in VD4. Although sevoflurane did not significantly suppress Ca$^{2+}$ currents in RPeD1, these data do not, however, rule out the possibility that the resulting changes may indeed be sufficient to perturb Ca$^{2+}$-dependent exocytosis.

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