

Effects of Alcohols and Anesthetics on Recombinant Voltage-Gated Na⁺ Channels

Munehiro Shiraishi and R. Adron Harris

Waggoner Center for Alcohol and Addiction Research and the Institute for Cellular and Molecular Biology, University of Texas at Austin, Austin, Texas

Received December 18, 2003; accepted February 19, 2004

ABSTRACT

Voltage-gated Na⁺ channels (Na⁺ channels) mediate the rising phase of action potentials in neurons and excitable cells. Nine subtypes of the α subunit (Na_v1.1–Na_v1.9) have been shown to form functional Na⁺ channels to date. Recently, anesthetic concentrations of volatile anesthetics and ethanol were reported to inhibit Na⁺ channel functions, but it is not known whether all subtypes are inhibited by anesthetics. To investigate possible subtype-specific effects of anesthetics on Na⁺ channels, mRNA of Na_v1.2, Na_v1.4, Na_v1.6, and Na_v1.8 α subunit-encoded genes were injected individually or together with a β subunit mRNA into *Xenopus* oocytes. Na⁺ currents were recorded using the two-electrode voltage-clamp technique. Isoflurane, at clinically relevant concentrations, inhibited the currents produced by Na_v1.2, Na_v1.4, and Na_v1.6 by ~10% at the holding potential of –90 mV and by ~30% at –60 mV, but

it did not affect the Na_v1.8-mediated current. An anesthetic fluorocyclobutane (1-chloro-1,2,2-trifluorocyclobutane) also inhibited the Na_v1.2 channel, whereas the nonanesthetic fluorocyclobutane (1,2-dichlorohexafluorocyclobutane) had no effect. The perfluorinated heptanol [CF₃(CF₂)₅CH₂OH], which produces anesthesia, inhibited the Na_v1.2 channel like other alcohols tested (ethanol, heptanol, and CF₃CH₂OH), even though this compound does not affect GABA, glycine, α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, or kainate receptors. In contrast, most intravenous anesthetics did not have significant effects on the Na_v1.2 channel at clinically relevant concentrations although urethane inhibited. These results show that isoflurane inhibits the Na⁺ channel functions except Na_v1.8 in a voltage-dependent manner. These findings indicate that the Na⁺ channel is a neuronal target for anesthetic action.

Voltage-gated sodium channels (Na⁺ channels) are responsible for the rising phase of action potentials in neurons and electrically excitable cells. They consist of an α subunit (approximately 260 kDa) in association with auxiliary β subunits (33–36 kDa). To date, nine subtypes of the α subunit, designated Na_v1.1 to Na_v1.9, have been shown to form functional Na⁺ channels (Catterall, 2000; Goldin, 2001).

It has been thought that axonal conduction, and by inference voltage-gated Na⁺ channels, are not affected by reasonable concentrations of anesthetics (Franks and Lieb, 1994). However, more recent studies show that anesthetics at clinically relevant concentrations can suppress Na⁺ channel function in synaptosomes (Ratnakumari and Hemmings, 1998), neurohypophysial nerve terminals (Ouyang et al., 2003), and in cells transfected with a rat neuronal sodium

channel, Na_v1.2 channel (Rehberg et al., 1996). These studies raise the possibility that Na⁺ channel inhibition can contribute to anesthesia. In line with this, *in vivo* studies have shown that Na⁺ channel inhibitors, such as lidocaine, reduce minimum alveolar concentration (MAC) values of volatile anesthetics. This is at least consistent with inhibition of Na⁺ channels participating in anesthesia *in vivo* (Doherty and Frazier, 1998; Hodgson et al., 1999). It is also important to note that synaptosomal Na⁺ channels can be inhibited by several volatile anesthetics, including 1-chloro-1,2,2-trifluorocyclobutane (F3), but they are not affected by the nonimmobilizer 1,2-dichlorohexafluorocyclobutane (F6; Ratnakumari and Hemmings, 1998; Ratnakumari et al., 2000).

It is possible that the old ideas on axonal conduction can be reconciled with anesthetic inhibition of Na⁺ channel function, because there are nine different α subunits, which may vary in their anesthetic sensitivity. Moreover, the old ideas were mostly based on studies using molluscan axons. There have been several studies of volatile anesthetics on cloned Na⁺ channels with conflicting results. Human cardiac chan-

This study was supported by National Institutes of Health Grants GM47818 and AA06399 (to R.A.H.).

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.

DOI: 10.1124/jpet.103.064063.

ABBREVIATIONS: MAC, minimum alveolar concentration; F3, 1-chloro-1,2,2-trifluorocyclobutane; F6, 1,2-dichlorohexafluorocyclobutane; HEK, human embryonic kidney; PK, protein kinase; TTX, tetrodotoxin; DRG, dorsal root ganglion; FC2, CF₃CH₂OH; FC7, CF₃(CF₂)₅CH₂OH; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; NMDA, *N*-methyl-D-aspartate; DMSO, dimethyl sulfoxide; ANOVA, analysis of variance; I-V, current-voltage.

nels (Na_v1.5) expressed in the HEK293 cells were inhibited by isoflurane and halothane (Stadnicka et al., 1999), and rat brain channels (Na_v1.2) were also inhibited by halothane and enflurane (Rehberg et al., 1996). However, others showed that halothane inhibition of Na_v1.2 and Na_v1.4 required PKC coexpression, and Na_v1.5 was insensitive to halothane even with the PKC (Patel et al., 2000). To study the effects of various anesthetics on selected cloned Na⁺ channels in the same expression system, we chose the *Xenopus* oocyte expression system. Although the expression system has advantages in studying channel subtypes specifically and mutated channels, other studies are required to configure physiological relevance.

Genes encoding Na_v1.1, Na_v1.2, Na_v1.3, and Na_v1.7 are located on chromosome 2 in mouse, and these channels share similarities in DNA sequence, biophysical characteristics, predominant expression in the brain, and sensitivity to tetrodotoxin (TTX). Na_v1.5, Na_v1.8, and Na_v1.9 are located on mouse chromosome 9, and these are resistant to TTX. The other isoforms, Na_v1.4 and Na_v1.6, are on mouse chromosome 11 and 15, respectively. From these Na⁺ channels, we selected the Na_v1.2 subunit, which is predominantly expressed in brain; Na_v1.4, which is the skeletal muscle channel; Na_v1.6, which is widely expressed in the central nervous system and peripheral nervous system; and Na_v1.8, which is expressed in dorsal root ganglion (DRG).

In this study, we used isoflurane, halothane, F3, and F6 as volatiles, and we used two fluorinated alkanols, CF₃CH₂OH (FC2) and CF₃(CF₂)₅CH₂OH (FC7), to study effects on the Na⁺ channels. Although these fluorinated alkanols produce anesthesia in vivo, the mechanisms of the action are not understood. FC2 potentiated the function of GABA receptors like other anesthetics, whereas FC7 did not affect GABA, glycine, AMPA, or kainate receptors (Ueno et al., 1999). Moreover, these injectable anesthetics, ketamine, propofol, and urethane, were tested on the Na⁺ channel at their clinically relevant concentrations. Propofol (1 μM) potentiates the function of GABA_A receptors by more than 100% (Sanna et al., 1995), but it has only small effects on glycine and NMDA receptors (Mascia et al., 1996). On the other hand, ketamine strongly inhibits NMDA receptors, but it does not have significant effects on GABA_A, glycine, and AMPA receptors (Yamakura et al., 2001). Urethane has been widely used as an anesthetic in animal experiments, but its mechanisms of action are still unclear. Urethane potentiated GABA_A and glycine receptors and inhibited NMDA and AMPA receptors (Hara and Harris, 2002). But each of these effects was very small at an anesthetic concentration (10 mM) of urethane. For example, this concentration of urethane enhanced the GABA and glycine receptor functions only by 23 and 33%, respectively, although isoflurane and halothane potentiated both receptors more than 100% at their anesthetic EC₅₀ (Downie et al., 1996; Yamakura and Harris, 2000). Therefore, it was of interest to determine whether the Na⁺ channel is a target for any of these injectable anesthetics.

Materials and Methods

Xenopus laevis female frogs were purchased from Xenopus Express, Inc. (Homosassa, FL). Isoflurane was from Marsam Pharmaceutical Inc. (Cherry Hill, NJ), and cyclopropane was from Ohio Medical Product (Madison, WI). FC2 and FC7 were obtained from

TDC Research Inc. (Gainesville, FL). Halothane, lidocaine, propofol, ketamine, urethane, tetrodotoxin, and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). F3 and F6 were obtained from Lancaster Synthesis Inc. (Windham, NH).

mRNA Synthesis. The cDNA encoding rat Na_v1.2 α subunit (gift from Dr. W. A. Catterall, University of Washington, Seattle, WA), the cDNA of human Na_v1.4 α subunit (gift from Dr. A. L. George, Vanderbilt University, Nashville, TN), the cDNA of rat Na_v1.6 α subunit (gift from Dr. A. L. Goldin, University of California, Irvine, CA), the cDNA of rat Na_v1.8 α subunit (gift from Dr. A. N. Akopian, The University of Texas Health Science Center, San Antonio, TX), the cDNA of rat Na_v β₁ subunit (gift from Dr. A. L. Goldin, University of California, Irvine), and the cDNA of human Na_v β₁ subunit (gift from Dr. A. L. George, Vanderbilt University) were used for cRNA synthesis using either T7 or SP6 RNA polymerase. In vitro transcripts were prepared using the mMMESSAGE mMACHINE kit (Ambion, Austin, TX). The quality and quantity of these RNAs were determined by using the RNA 6000 Nano assay with Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA).

Oocyte Expression. The use of experimental animals was approved by the Animal Care and Use Committees of the University of Texas. Oocytes were isolated and placed in modified Barth's saline containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.82 mM MgSO₄, 0.91 mM CaCl₂, 0.33 mM Ca(NO₃)₂, and 10 mM HEPES adjusted to pH 7.5. Oocytes were injected with 40 nl of a 10:1 mixture of Na⁺ channel β subunit and Na⁺ channel α subunit mRNA or 40 nl of a Na⁺ channel α subunit alone, with the α subunit concentration approximately 0.5 ng/nl of injected solution. The injected oocytes were singly placed in Corning cell wells (Corning Glassworks, Corning, NY) containing incubation medium (sterile modified Barth's saline supplemented with 10 mg/l streptomycin, 100,000 units/l penicillin, 50 mg/l gentamycin, 90 mg/l theophylline, and 220 mg/l pyruvate) and incubated at 15 to 19°C. One to 5 days after injection, the oocytes were used in electrophysiological recording.

Two-Electrode Voltage-Clamp Recording from Oocytes. Oocytes injected with the cRNA were placed in a rectangular chamber (~100-μl volume) and continuously perfused (2 ml/min) with Frog Ringer solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, and 10 mM HEPES, pH 7.2) using a peristaltic pump (Cole-Parmer Instrument Co., Chicago, IL). Two-electrode voltage-clamp recordings were obtained from injected oocytes using a Warner Instruments model OC-725C (Hamden, CT). The amplitude of expressed Na⁺ currents was typically 2 to 15 μA. These currents were completely blocked by external applications of tetrodotoxin (200 nM) or replacement of Na⁺ (data not shown). Recording electrodes were made with a puller (P-97; Sutter Instruments Company, Novato, CA) from borosilicate capillary glass (Frederick Haer and Co., Bowdoinham, ME) and had a final resistance of <0.5 MΩ when filled with 3 M KCl. All electrophysiological recordings were carried out at a room temperature of around 23°C. Pulses were applied, and data were acquired using pClamp software (Axon Instruments, Foster City, CA). The transients and leak currents were subtracted using the P/N procedure (Bezanilla and Armstrong, 1975). The actual bath concentrations of the anesthetics (isoflurane, halothane, cyclopropane, and F3) and nonanesthetic (F6) were determined by gas chromatography. Isoflurane, halothane, ethanol, and FC2 were directly dissolved in the Frog Ringer, whereas heptanol and FC7 was first dissolved in dimethyl sulfoxide (DMSO) and then diluted in Frog Ringer to a final concentration of DMSO not exceeding 0.05%. Saturated solutions of F3 and F6 obtained by 100 μl of each compound in 20 ml of Frog Ringer were equilibrated in a sealed glass syringe protected from light for at least 24 h before use. A solution in a closed vial was bubbled with 100% cyclopropane for at least 10 min at room temperature to obtain saturated solution. The concentration of cyclopropane in the chamber was quantified by gas chromatography, and the value was 0.46 ± 0.03 atm (Hara et al., 2002). This concentration corresponds to 5 MAC of cyclopropane. Final solutions of those compounds were prepared from the saturated solutions immediately before ap-

plication. The loss of concentrations from prepared solution to bath was approximately 50% for all compounds, except for ethanol and FC2. All compounds were tested at a concentration corresponding to MAC (or the predicted MAC for F6) in the final bath concentration. MAC is the minimum alveolar concentration of anesthetic required eliminating movement in response to a noxious stimulus in 50% of subjects. Calculated 1 MAC for isoflurane, halothane, cyclopropane, F3, F6, ethanol, FC2, heptano, and FC7 were 0.32 mM, 0.25 mM, 0.18 atm, 0.8 mM, 17.8 μ M, 190 mM, 31 mM, 0.23 mM, and 26 μ M, respectively (Saidman et al., 1967; Mihic et al., 1994; Dildy-Mayfield et al., 1996; Eger et al., 1999; Ueno et al., 1999). Regarding injectable anesthetics, ketamine and urethane were first dissolved in water and propofol was dissolved in DMSO to make stock solutions. Calculated EC₅₀ of propofol, ketamine, and urethane considering protein binding in serum were 1 μ M, 2 μ M, and 10 mM, respectively.

Data Analysis. Data were obtained from 4 to 20 oocytes taken from at least three different frogs for each experiment. Data are shown as means \pm S.E.M. Statistical analysis was conducted by one-way analysis of variance (ANOVA) for multiple comparisons and *t* test for comparisons between two groups, using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Differences were considered as statistically significant at *P* value less than 0.05.

Current-Voltage Relation and Conductance-Voltage Relation (Steady-State Activation) of the Na⁺ Channels. The currents were elicited by 50-ms (200 ms for Na_v1.8) depolarizing pulses from -80 to 50 mV in 10-mV increments from a holding potential of -90 mV or from -60 to 50 mV in 10-mV increments from a -60 mV holding potential. To calculate the conductance, following equation was used:

$$G = I_p / (V_R - V), \quad (1)$$

where *G* is conductance, *I_p* is the sodium current, *V_R* is the measured reversal potential, and *V* is the test pulse voltage. Normalized conductance was fit with a single Boltzmann relationship of the following form:

$$G(V) = 1 / \{1 + \exp(V_{1/2} - V) / k\}, \quad (2)$$

where *V_{1/2}* is the half-maximal activation voltage and *k* is a slope factor.

Steady-State Inactivation of the Channels. The currents were elicited by test pulse to the potential that induces maximal currents obtained from current-voltage (*I-V*) relation after 200-ms prepulses (500 ms for Na_v1.8) to potentials varying from -140 to 20 mV. The

peak test pulse current was plotted as a function of prepulse potential, normalized, and fit with a Boltzmann function.

$$I/I_{\max} = 1 / \{1 + \exp(V - V_{1/2}) / k\}, \quad (3)$$

where *I/I_{max}* is normalized peak current, *V_{1/2}* is the voltage at which one-half of the channels are available for opening, and *k* is a slope factor.

Anesthetic Effects on Na⁺ Channels. All anesthetics were tested at two different holding potentials, *V_{max}* and *V_{1/2}*, to see holding potential-dependent effects on Na⁺ channels. *V_{max}* indicates a holding potential at which most of the channels are available for activation, *V_{1/2}* means a prepulse potential that induces half-maximal current. *V_{max}* and *V_{1/2}* were obtained from steady-state inactivation curves.

Currents were elicited by a 50-ms depolarizing pulse to a potential that causes maximal conductance obtained from the conductance-voltage relation curve from a holding potential (*V_{max}* or *V_{1/2}*) every 10 s. During the recording, anesthetics were applied for 2 min. Effects of anesthetics on steady-state activation and inactivation of the channels were also studied.

Results

Characteristics of Na_v1.2, Na_v1.4, Na_v1.6, and Na_v1.8 Currents. No measurable Na⁺ currents were detected in noninjected oocytes under our experimental conditions. Na⁺ currents were normally activated at a threshold around -50 mV in oocytes expressing Na_v1.2, Na_v1.4, and Na_v1.6. Peak

TABLE 1

Effects of isoflurane on Na⁺ channel gating

All channels are coexpressed with β_1 subunits. Values are shown as mean \pm S.E.M.

	Activation		Inactivation	
	Control <i>V_{1/2}</i>	Isoflurane <i>V_{1/2}</i>	Control <i>V_{1/2}</i>	Isoflurane <i>V_{1/2}</i>
	<i>mV</i>			
Na _v 1.2	-32.7 \pm 5.4	-32.4 \pm 6.5	-58.7 \pm 3.8	-67.6 \pm 3.0*
Na _v 1.4	-37.4 \pm 3.0	-38.0 \pm 2.3	-56.3 \pm 3.3	-64.3 \pm 4.7*
Na _v 1.6	-33.8 \pm 3.9	-32.2 \pm 4.0	-60.1 \pm 2.5	-68.8 \pm 2.8*
Na _v 1.8	-3.2 \pm 3.0	-3.9 \pm 1.9	-35.3 \pm 4.8	-36.1 \pm 3.9

* *P* < 0.05, compared with control using paired *t* test.

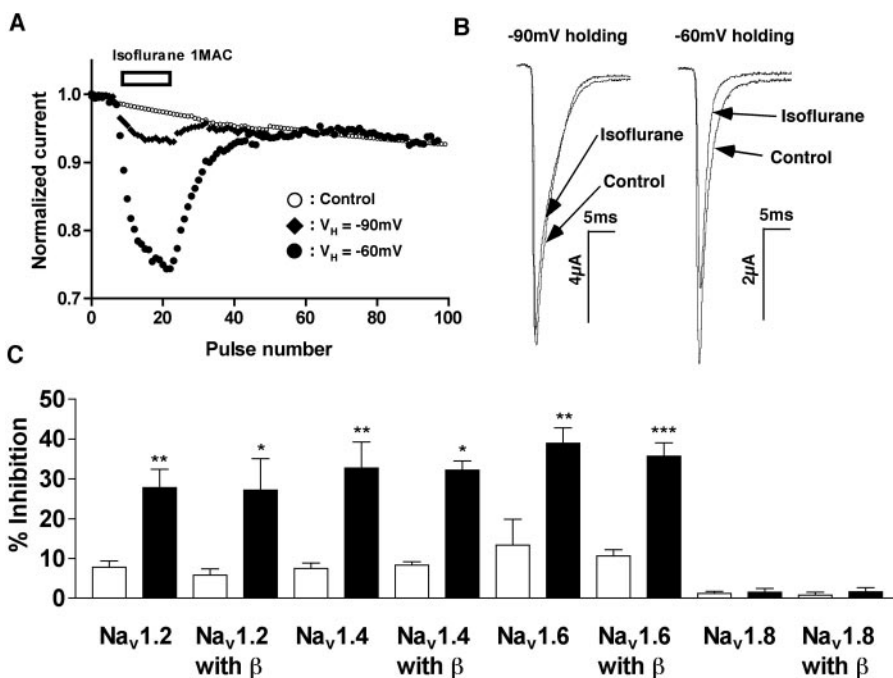


Fig. 1. A, time course of isoflurane-induced suppression on Na_v1.2 + β_1 -mediated currents. The currents were elicited by a 50-ms depolarizing pulse to -20 mV from a holding potential of either -60 or -90 mV every 10 s. Peak currents were normalized to the initial control current. The open bar indicates duration of 1 MAC isoflurane treatment. B, representative tracings of the peak Na⁺ currents before (control) and after application of isoflurane (1 MAC) elicited from two different holding potentials. In this case, a -60-mV holding potential caused half-maximal current (*V_{1/2}*). C, summary of isoflurane inhibition at *V_{max}* (open column) and *V_{1/2}* (closed column) on Na_v1.2, Na_v1.4, Na_v1.6, and Na_v1.8 channels with or without β_1 subunits. Open columns indicate percentage of inhibition by 1 MAC isoflurane at the holding potential of -90 mV. Closed columns indicate percentage of inhibition by 1 MAC isoflurane at holding potentials, which induce half-maximal currents in single oocytes. Data were obtained from 4 to 20 oocytes on each experiment and are represented as means \pm S.E.M. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001, compared with control using one-way ANOVA.

inward currents were normally elicited by depolarizing pulses to -20 mV from a holding potential of -90 mV for $\text{Na}_v1.2$, $\text{Na}_v1.4$, and $\text{Na}_v1.6$. These currents were completely blocked by 200 nM tetrodotoxin (data not shown). $\text{Na}_v1.8$ currents were activated at a threshold about -10 mV and peak inward currents were elicited by a depolarizing pulse to 20 mV. The currents were eliminated by replacement of external Na^+ (data not shown). Steady-state activation curves were obtained (see *Materials and Methods*) and the $V_{1/2}$ of $\text{Na}_v1.2$, $\text{Na}_v1.4$, and $\text{Na}_v1.6$ were approximately -30 mV. Steady-state inactivation curves were also obtained and half-maximal currents were elicited by a step pulse from holding potentials of approximately -60 mV on $\text{Na}_v1.2$, $\text{Na}_v1.4$, and $\text{Na}_v1.6$. The current profile of $\text{Na}_v1.8$ was distinct from other channels tested (Table 1).

Effects of Isoflurane on Amplitudes of the Na^+ Channel-Induced Inward Currents. Effects of 1 MAC of isoflurane on peak amplitude currents induced by $\text{Na}_v1.2$, $\text{Na}_v1.4$, $\text{Na}_v1.6$, or $\text{Na}_v1.8$ expressed in oocytes with or without β_1 subunits were tested. $\text{Na}_v1.2$ -mediated currents were elicited by a 50-ms depolarizing pulse to -20 mV from either -90 mV (V_{max}) or -60 mV ($V_{1/2}$) every 10 s. Isoflurane at 1 MAC (2-min treatment) inhibited the currents by 8 ± 2 and $28 \pm$

5% at the V_{max} and $V_{1/2}$, respectively (Fig. 1, A and B). Isoflurane also inhibited the currents induced by $\text{Na}_v1.2$ with β_1 subunits by 6 ± 2 and $27 \pm 8\%$ at the V_{max} and $V_{1/2}$; thus, Na^+ channel β_1 subunits did not affect the inhibition by isoflurane. Likewise, $\text{Na}_v1.4$ - and $\text{Na}_v1.6$ -mediated currents were inhibited by isoflurane, and these actions also did not depend on expression of β_1 subunits (Fig. 1C). Isoflurane reduced the currents caused by $\text{Na}_v1.4$ and $\text{Na}_v1.6$ with β_1 subunits by 32 ± 2 and $36 \pm 3\%$ at the $V_{1/2}$, respectively; however, $\text{Na}_v1.8$ -mediated currents were not inhibited by isoflurane with or without β_1 subunits even at the $V_{1/2}$ (Fig. 1C). As a positive control, lidocaine (100 μM) inhibited the currents induced by $\text{Na}_v1.8$ with β_1 by 26 ± 4 and $49 \pm 17\%$ at the V_{max} and $V_{1/2}$, respectively. Thus, isoflurane suppressed the function of the voltage-gated Na^+ channels, which are expressed in brain ($\text{Na}_v1.2$ and $\text{Na}_v1.6$), skeletal muscle ($\text{Na}_v1.4$), and peripheral nerves ($\text{Na}_v1.6$) but not the channel expressed in dorsal root ganglion ($\text{Na}_v1.8$).

Effects of Isoflurane on the Steady-State Activation and Inactivation of the Na^+ Channels. These experiments were carried out with coexpression of β_1 subunits, and the steady-state activation curve of $\text{Na}_v1.2$ was calculated by a family of currents elicited by 50-ms depolarizing pulses

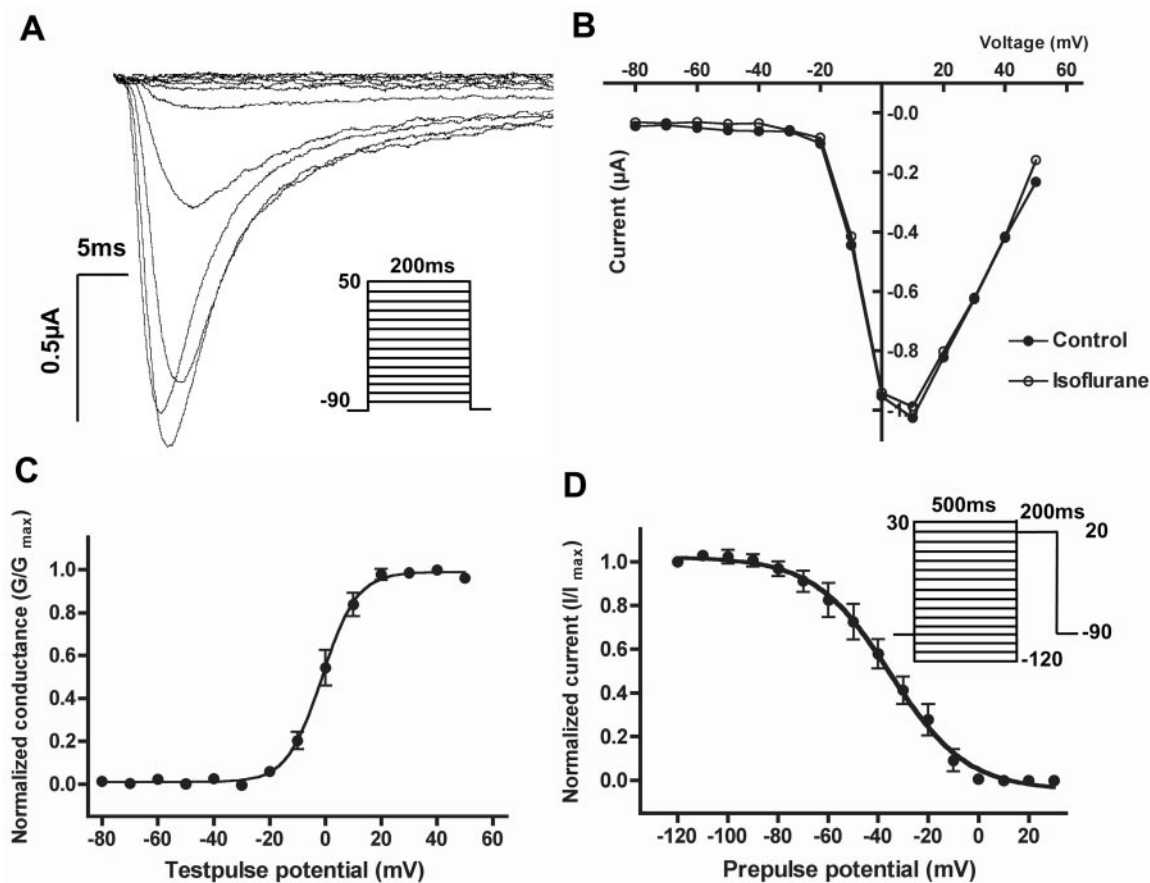


Fig. 2. A, representative I_{Na} traces of $\text{Na}_v1.2$ with β_1 subunits expressed in *Xenopus* oocytes, using the two-electrode voltage-clamp. The currents were elicited by 50-ms depolarizing pulses from -80 to 50 mV in 10 -mV increments from a holding potential of -90 mV with or without 2 MAC isoflurane exposure (2 min). Leak currents were subtracted by P/5 procedure. B, I-V relations of the channel in these three conditions. Currents were elicited by 50-ms depolarizing pulses from -60 to 50 mV in 10 -mV increments from a holding potential of -60 mV to observe the isoflurane effects at the more depolarized holding potential. C, activation curves for control (closed circle) and 2 MAC of isoflurane (open circle). Normalized conductance was calculated from B and the curve was fit with a single Boltzmann relationship. D, control and isoflurane modulated steady-state inactivation of the channel. Currents were elicited by 50-ms test pulses to -20 mV after 200-ms prepulses to potentials varying from -140 to 0 mV. Normalized peak amplitudes of the currents were plotted as a function of prepulse potential to obtain steady-state inactivation curves in control (closed circle) and 2 MAC isoflurane (open circle). The curves were fit with the Boltzmann relationship.

from -60 to 50 mV in 10-mV increments from a -60 mV holding potential (Fig. 2, A-C). Isoflurane (2 MAC) inhibited the current amplitudes (Fig. 2B) but did not alter the steady-state activation curve of the channel (Fig. 2C). In contrast to activation, the steady-state inactivation curve of Na_v1.2 was shifted to left by ~9 mV by isoflurane (Fig. 2D). Isoflurane had similar effects on Na_v1.4 and Na_v1.6 channels (Table 1). However, isoflurane did not have significant effects on the steady-state activation or inactivation of the Na_v1.8 channel (Fig. 3 and Table 1).

Effects of Inhaled Anesthetics on Na_v1.2. Isoflurane, halothane, and cyclopropane (2 MAC) were tested to the Na_v1.2 plus β₁ subunits (Fig. 4). Isoflurane inhibited the channel by 6 ± 1 and 32 ± 3% at the V_{max} and V_{1/2}, respectively. Inhibition by halothane was smaller than by isoflurane but statistically significant. The inhibition was 2.4 ± 1 and 11 ± 2% at the V_{max} and V_{1/2}, respectively. The nonhalogenated alkane cyclopropane also inhibited the channel by 6.6 ± 1.5 and 20 ± 3% at the V_{max} and V_{1/2}, respectively. It was of interest to determine whether the Na⁺ channel could distinguish between the anesthetic (F3) and nonimmobilizer (F6) cyclobutanes. F3 at 2 MAC inhibited the channels by 7 ± 2 and 34 ± 5% at the V_{max} and V_{1/2}, respectively, whereas F6 had no effects.

Effects of *n*- and Perfluorinated Alcohols on Na_v1.2. The fluorinated alkanols FC2 and FC7 produce anesthetic effects in vivo. Ethanol (C2) and heptanol (C7) were also tested to compare the effects on Na_v1.2 (with coexpression of β₁ subunits). In this experiment, FC2 and FC7 inhibited the Na_v1.2 induced currents in a concentration-dependent manner (Fig. 5). All alcohols and fluorinated alkanols had similar effects, ~30% inhibition, on the channel at 1 MAC.

Effects of Injectable Anesthetics on Na_v1.2. Propofol, ketamine, and urethane were tested to the Na_v1.2 plus β₁ subunits. Propofol and ketamine weakly inhibited the Na_v1.2 channel, but these small effects were not statistically significant. The inhibition of the channel by propofol was 3 ± 2 and 7 ± 1% at 1 and 2 μM, respectively, and that by ketamine was 3 ± 2 and 9 ± 2% at 2 and 4 μM, respectively. Propofol at higher concentration (10 μM) suppressed the Na⁺ channel function by 25 ± 4% with an IC₅₀ of 30 μM. In contrast, urethane at the anesthetic concentration significantly suppressed the Na⁺ channel function (Fig. 6).

Discussion

The aim of this study was to identify subtype differences of Na⁺ channels to isoflurane and to investigate effects of other

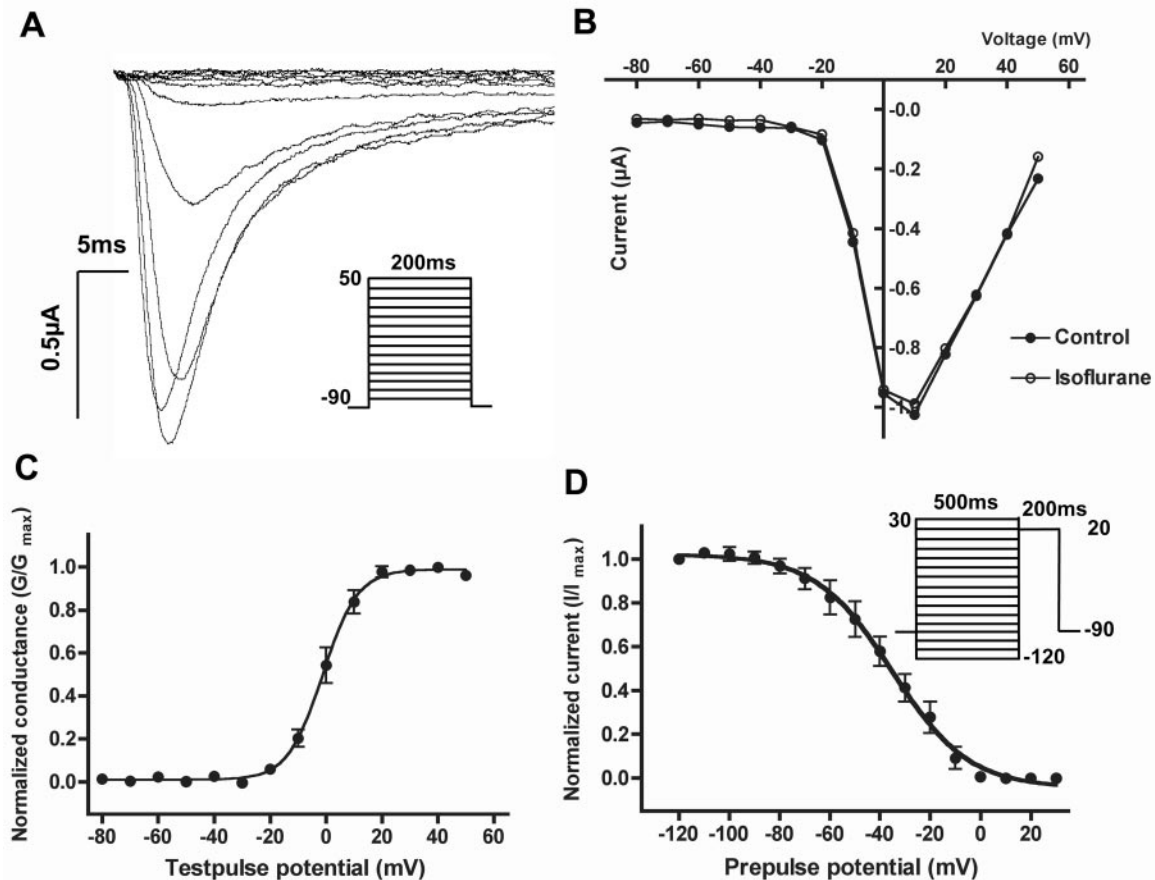


Fig. 3. A, representative I_{Na} traces of Na_v1.8 with β₁ subunits expressed in *Xenopus* oocytes, using the two-electrode voltage-clamp. The currents were elicited by 200-ms depolarizing pulses from -80 to 50 mV in 10-mV increments from a holding of -90 mV. Leak currents were subtracted by P/5 procedure. B, I-V relations of the channel in these two conditions. Currents were elicited by 200-ms depolarizing pulses from -80 to 50 mV in 10-mV increments from a holding potential of -90 mV. C, steady-state activation of the channel. Normalized conductance was obtained ($n = 7$), and the curve was fit with a single Boltzmann relationship. D, steady-state inactivation of the channel. Currents were elicited by 200-ms test pulses to 20 mV after 500-ms prepulses to potentials varying from -120 to 30 mV. Normalized peak amplitudes of the currents were plotted as a function of prepulse potential to obtain steady-state inactivation curves ($n = 7$). The curves were fit with the Boltzmann relationship.

anesthetics and alcohols on these channels. We evaluated the effects of isoflurane on several types of voltage-gated Na^+ channels in the same expression system (*Xenopus* oocytes). The channel-gating properties were very similar among all of the channels that we have tested (except $\text{Na}_v1.8$), and isoflurane at 1 MAC inhibited the peak amplitude of the currents mediated by $\text{Na}_v1.2$, $\text{Na}_v1.4$, and $\text{Na}_v1.6$ to around 30%. Others report that isoflurane suppresses firing frequencies of rat hippocampal neurons at clinically relevant concentrations (Yoshimura et al., 1985; Fujiwara et al., 1988). Moreover, recent studies showed that 30 μM lidocaine reduced firing frequency to 21 Hz from a control frequency of 35 Hz in DRG neurons (Scholz et al., 1998b; Scholz and Vogel, 2000). Importantly, 10% inhibition of the Na^+ currents by 30 μM lidocaine reduced the number of action potentials to 10 from a control response of 21 in 750 ms. Therefore, 10% inhibition at V_{max} holding potential and 30% inhibition at $V_{1/2}$ holding potential of the Na^+ channel function by anesthetics in our study would be expected to affect action potentials. This action of isoflurane was not affected by coexpression of β_1 subunits, and the effect of isoflurane on $\text{Na}_v1.2$ was dose-dependent. The inhibition of the Na^+ channels by isoflurane varied with holding potential; at the more depolarized holding potential, the effect of isoflurane was greater. In other words, the isoflurane effect was much greater when the fraction of the channels in the inactivated state was increased. Most of the channels were in the resting state that are ready for activation at the holding potential of -90 mV, whereas one-half of the channels were in the inactivated state at around -60 -mV holding potential. These results are mostly consistent with other publications. For example, Stadnicka et al. (1999) showed that isoflurane, as well as halothane, suppressed heart sodium channels expressed in HEK293 cells by acceleration of the transition from the open to the inactivated state and stabilization of the inactivated states (Stadnicka et al., 1999). Moreover, isoflurane inhibited voltage-gated Na^+ currents in a concentration- and voltage-dependent manner in isolated rat neurohypophysial nerve terminals, and the inhibition by isoflurane had no significant effects on $V_{1/2}$ of the activation curve, but the $V_{1/2}$ of the inactivation curve was consistently shifted in a negative direction (Ouyang et al., 2003).

In our experiments, we found that $\text{Na}_v1.8$ was insensitive to isoflurane. One group reported that halothane (Scholz et al., 1998a) blocked TTX-resistant Na^+ channels in DRG neurons, but the IC_{50} of halothane used was around 6 mM, which corresponds to about 24 MAC. We tested only 1 and 2 MAC of

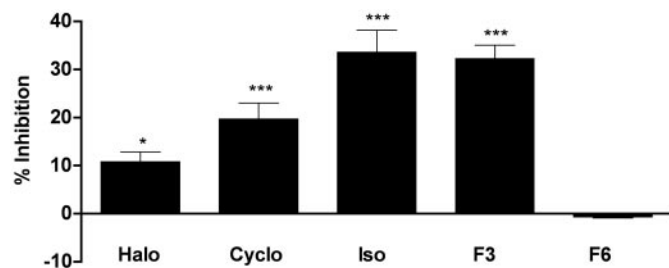


Fig. 4. Summary of inhibition produced by 2 MAC of halothane, cyclopropane, isoflurane, F3, and F6 on $\text{Na}_v1.2 + \beta_1$ channels at $V_{1/2}$ holding potentials. Data are represented as means \pm S.E.M. from four to 10 oocytes on each experiment. *, $P < 0.05$; ***, $P < 0.001$, compared with control using one-way ANOVA.

isoflurane on the $\text{Na}_v1.8$ channel and did not observe significant inhibition at pharmacologically relevant concentrations, but it is possible that isoflurane blocks the $\text{Na}_v1.8$ channel at much higher concentrations. There are other reports that volatile anesthetics suppress Na^+ channel functions in DRG neurons (Duch et al., 1998; Ratnakumari et al., 2000), but it is unclear whether the inhibitions are mediated by $\text{Na}_v1.8$ channels because DRG neurons express several types of Na^+ channels, including TTX-sensitive and -insensitive channels (Caffrey et al., 1992; Novakovic et al., 2001). The local anesthetic lidocaine is known to inhibit Na^+ channels in DRG neurons in a voltage- and use-dependent manner. The IC_{50} of lidocaine on TTX-resistant Na^+ currents is almost 200 μM (Roy and Narahashi, 1992; Scholz et al., 1998b). These results are consistent with our finding of 25% inhibition at 100 μM . One possible explanation for the insensitivity of $\text{Na}_v1.8$ channels to anesthetics is that they do not bind to the $\text{Na}_v1.8$ channel (but bind to other Na^+ channels). Another possibility is the differences in channel gating between $\text{Na}_v1.8$ and the others. The $\text{Na}_v1.8$ channel activates and inactivates slowly and does not inactivate completely. Because the isoflurane inhibition requires at least a fraction of the channels to be in the inactivated state and the reduced inactivation seen with the $\text{Na}_v1.8$ channel may reduce action

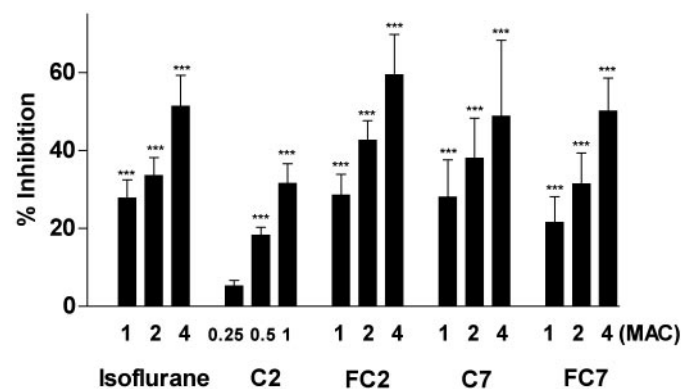


Fig. 5. Effects alcohols on $\text{Na}_v1.2 + \beta_1$ channels at $V_{1/2}$ holding potentials in comparison with the isoflurane effects. FC2, heptanol (C7), and FC7 were tested at concentrations of 1, 2, and 4 MAC. Ethanol (C2) was tested at 50, 100, and 200 mM, which correspond to 0.25, 0.5, and 1 MAC. Data are represented as means \pm S.E.M. from four to nine oocytes on each experiment. ***, $P < 0.001$, compared with control using one-way ANOVA.

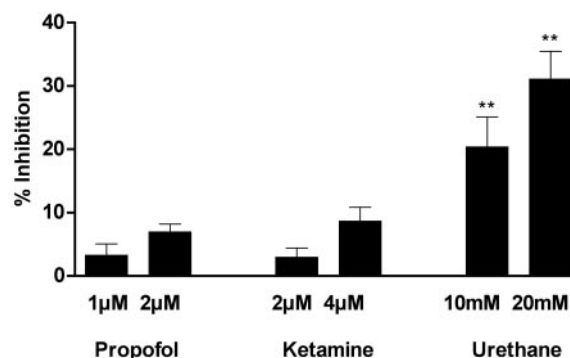


Fig. 6. Effects of injectable anesthetics on $\text{Na}_v1.2 + \beta_1$ channels at $V_{1/2}$ holding potentials. Percentage of inhibition by propofol, ketamine, and urethane at concentrations corresponding to 1 and 2 MAC are shown. Data are represented as means \pm S.E.M. from four to six oocytes on each experiment. **, $P < 0.01$, compared with control using one-way ANOVA.

of isoflurane. In other words, isoflurane may affect preferentially the inactivated state.

Halothane, cyclopropane, and F3 inhibited Na_v1.2 channel functions at clinically relevant concentrations, although the inhibitions by halothane and cyclopropane were smaller than that of isoflurane. Many channels, such as GABA_A, glycine, and NMDA receptors have been reported to be targets of the action of volatile anesthetics to date (Mihic et al., 1994; Harris et al., 1995; Downie et al., 1996; Hollmann et al., 2001). But not all of anesthetics can affect these receptors, for example, cyclopropane has no effect on GABA_A receptors and has only 39% potentiation at 1 MAC on glycine receptors (Hara et al., 2002). Importantly, all volatile anesthetics we tested inhibited the Na⁺ channel function. Furthermore, the immobilizer F3, which produces anesthesia in vivo, and non-immobilizer F6, are both predicted by their lipid solubilities to be anesthetic. They are structurally similar, but have distinct effects on the channel. This result suggests that the Na⁺ channel could be a target relevant to the immobilizing action of inhaled anesthetics. This is consistent with studies of synaptosomal Na⁺ channels (Ratnakumari et al., 2000).

Moreover, *n*-alcohols and perfluorinated alkanols were tested, because these alcohols can produce anesthesia in vivo (Eger et al., 1999). We used ethanol (C2) and heptanol (C7) as *n*-alcohols and FC2 and FC7 as fluorinated alkanols, and all of these compounds showed similar inhibition pattern at 1, 2, and 4 MAC. FC7 does not enhance GABA_A or glycine receptors, even though FC2 and the *n*-alcohols potentiate those channels (Ueno et al., 1999).

Propofol was reported to inhibit Na⁺ channels in Chinese hamster ovary cells expressing Na_v1.2 channels with an IC₅₀ of about 10 μM in the range of the threshold potential of action potential firing (Rehberg and Duch, 1999). Ouyang et al. (2003) reported the IC₅₀ value for propofol inhibition as 4.1 μM at a holding potential of -70 mV (more depolarized holding potential in their experiments and the effects are stronger), and 10 μM propofol inhibited Na⁺ channels by approximately 70%. Their results agree with ours, although our effects are weaker. One possible reason for this difference is that we used a heterologous expression system, whereas they used neurohypophysial nerve terminals. Isoflurane sensitivity that we obtained from our study was also lower than that reported in their article. Na⁺ channels can be modulated by PKA- and PKC-mediated phosphorylation (Cantrell and Catterall, 2001). Moreover, direct interaction of Na⁺ channels with G proteins might also modulate the function of Na⁺ channels (Ma et al., 1994, 1997). Although propofol significantly inhibited the Na⁺ channels at the higher concentration, an important point is that propofol, at clinically relevant concentrations, did not show significant inhibition of Na⁺ channels expressed in *Xenopus* oocytes. The free serum concentration of propofol during anesthesia is about 1 μM (Smith et al., 1994). This is similar to the ketamine results, where we did not find significant effects of ketamine at concentrations of 2 or 4 μM. There are reports of inhibition by ketamine on Na⁺ channels. For example, that *S*-(+)-ketamine and *R*-(-)-ketamine suppressed Na_v1.2 and Na_v1.4 channels expressed in HEK293 cells with IC₅₀ values of 59 to 333 μM (Haeseler et al., 2003). Another study showed that ketamine inhibited both TTX-sensitive and TTX-resistant Na⁺ channels with IC₅₀ values of 150 to 850 μM in rat DRG neurons (Zhou and Zhao, 2000). We estimated the anesthetic

EC₅₀ of ketamine as 2 μM based on the free serum concentration of ketamine measured in blood on awaking (Idvall et al., 1979; Dayton et al., 1983), and we did not observe statistically significant inhibition by ketamine at clinically relevant concentrations. For urethane, we observed significant inhibition by urethane at both 1 and 2 MAC. Urethane affects many kinds of neurotransmitter-gated ion channels, such as GABA_A, glycine, NMDA, and AMPA receptors, whereas the effects of urethane on these channels were relatively small compared with volatile anesthetics (Hara and Harris, 2002). In our experiments, 1 MAC (10 mM) of urethane suppressed the Na⁺ channel function at a similar degree to isoflurane. Thus, the Na⁺ channel might be a major player in anesthetic action produced by urethane.

Volatile anesthetics have been suggested to produce a part of anesthesia immobility by inhibiting Na⁺ channels, because systemic injection of local anesthetics, which predominantly reduce Na⁺ channel functions, decreases MAC values in animals (DiFazio et al., 1976; Doherty and Frazier, 1998). More recently, Hodgson et al. (1999) showed that lidocaine epidural anesthesia reduced the MAC value of sevoflurane from 1.18 to 0.52% (Hodgson et al., 1999). Thus, Na⁺ channels may be a target for the action of many anesthetics (Sonner et al., 2003). Although further experiments are necessary to understand the mechanism, the resistance of Na_v1.8 channels to anesthetics produces an opportunity to comprehend the molecular action of anesthetics on these channels.

Acknowledgments

We appreciate Dr. Edmond I. Eger II and Dr. Michael J. Laster (University of California, San Francisco) for helpful discussion and for determining concentrations of volatile anesthetics. We also thank Drs. S. John Mihic and Hitoshi Morikawa (Waggoner Center for Alcohol and Addiction Research, University of Texas at Austin) for excellent advice and discussions. We are grateful for those who kindly provided Na⁺ channel clones as listed under *Materials and Methods*: Drs. Armen N. Akopian, William A. Catterall, Alfred L. George, and Alan L. Goldin.

References

- Bezanilla F and Armstrong CM (1975) Kinetic properties and inactivation of the gating currents of sodium channels in squid axon. *Phil Trans R Soc Lond B Biol Sci* **270**:449–458.
- Caffrey JM, Eng DL, Black JA, Waxman SG, and Kocsis JD (1992) Three types of sodium channels in adult rat dorsal root ganglion neurons. *Brain Res* **592**:283–297.
- Cantrell AR and Catterall WA (2001) Neuromodulation of Na⁺ channels: an unexpected form of cellular plasticity. *Nat Rev Neurosci* **2**:397–407.
- Catterall WA (2000) From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels. *Neuron* **26**:13–25.
- Dayton PG, Stiller RL, Cook DR, and Perel JM (1983) The binding of ketamine to plasma proteins: emphasis on human plasma. *Eur J Clin Pharmacol* **24**:825–831.
- DiFazio CA, Neiderlehner JR, and Burney RG (1976) The anesthetic potency of lidocaine in the rat. *Anesth Analg* **55**:818–821.
- Diddy-Mayfield JE, Eger EI 2nd, and Harris RA (1996) Anesthetics produce subunit-selective actions on glutamate receptors. *J Pharmacol Exp Ther* **276**:1058–1065.
- Doherty TJ and Frazier DL (1998) Effect of intravenous lidocaine on halothane minimum alveolar concentration in ponies. *Equine Vet J* **30**:300–303.
- Downie DL, Hall AC, Lieb WR, and Franks NP (1996) Effects of inhalational general anaesthetics on native glycine receptors in rat medullary neurones and recombinant glycine receptors in *Xenopus* oocytes. *Br J Pharmacol* **118**:493–502.
- Duch DS, Rehberg B, and Vysotskaya TN (1998) Volatile anesthetics significantly suppress central and peripheral mammalian sodium channels. *Toxicol Lett* **100–101**:255–263.
- Eger EI 2nd, Ionescu P, Laster MJ, Gong D, Hudlicky T, Kendig JJ, Harris RA, Trudell JR, and Pohorille A (1999) Minimum alveolar anesthetic concentration of fluorinated alkanols in rats: relevance to theories of narcosis. *Anesth Analg* **88**:867–876.
- Franks NP and Lieb WR (1994) Molecular and cellular mechanisms of general anaesthesia. *Nature (Lond)* **367**:607–614.
- Fujiwara N, Higashi H, Nishi S, Shimoi K, Sugita S, and Yoshimura M (1988)

- Changes in spontaneous firing patterns of rat hippocampal neurones induced by volatile anaesthetics. *J Physiol (Lond)* **402**:155–175.
- Goldin AL (2001) Resurgence of sodium channel research. *Annu Rev Physiol* **63**:871–894.
- Haeseler G, Tetzlaff D, Bufler J, Dengler R, Munte S, Hecker H, and Leuwer M (2003) Blockade of voltage-operated neuronal and skeletal muscle sodium channels by S(+) and R(-)-ketamine. *Anesth Analg* **96**:1019–1026, table of contents.
- Hara K, Eger EI 2nd, Laster MJ and Harris RA (2002) Nonhalogenated alkanes cyclopropane and butane affect neurotransmitter-gated ion channel and G-protein-coupled receptors: differential actions on GABAA and glycine receptors. *Anesthesiology* **97**:1512–1520.
- Hara K and Harris RA (2002) The anesthetic mechanism of urethane: the effects on neurotransmitter-gated ion channels. *Anesth Analg* **94**:313–318, table of contents.
- Harris RA, Mihic SJ, Dildy-Mayfield JE, and Machu TK (1995) Actions of anesthetics on ligand-gated ion channels: role of receptor subunit composition. *FASEB J* **9**:1454–1462.
- Hodgson PS, Liu SS, and Gras TW (1999) Does epidural anesthesia have general anesthetic effects? A prospective, randomized, double-blind, placebo-controlled trial. *Anesthesiology* **91**:1687–1692.
- Hollmann MW, Liu HT, Hoenemann CW, Liu WH, and Durieux ME (2001) Modulation of NMDA receptor function by ketamine and magnesium. Part II: interactions with volatile anesthetics. *Anesth Analg* **92**:1182–1191.
- Isvall J, Ahlgren I, Aronsen KR, and Stenberg P (1979) Ketamine infusions: pharmacokinetics and clinical effects. *Br J Anaesth* **51**:1167–1173.
- Ma JY, Catterall WA, and Scheuer T (1997) Persistent sodium currents through brain sodium channels induced by G protein betagamma subunits. *Neuron* **19**:443–452.
- Ma JY, Li M, Catterall WA, and Scheuer T (1994) Modulation of brain Na⁺ channels by a G-protein-coupled pathway. *Proc Natl Acad Sci USA* **91**:12351–12355.
- Mascia MP, Mihic SJ, Valenzuela CF, Schofield PR, and Harris RA (1996) A single amino acid determines differences in ethanol actions on strychnine-sensitive glycine receptors. *Mol Pharmacol* **50**:402–406.
- Mihic SJ, McQuilkin SJ, Eger EI 2nd, Ionescu P, and Harris RA (1994) Potentiation of gamma-aminobutyric acid type A receptor-mediated chloride currents by novel halogenated compounds correlates with their abilities to induce general anesthesia. *Mol Pharmacol* **46**:851–857.
- Novakovic SD, Eglén RM, and Hunter JC (2001) Regulation of Na⁺ channel distribution in the nervous system. *Trends Neurosci* **24**:473–478.
- Ouyang W, Wang G, and Hemmings HC Jr (2003) Isoflurane and propofol inhibit voltage-gated sodium channels in isolated rat neurohypophysial nerve terminals. *Mol Pharmacol* **64**:373–381.
- Patel MK, Mistry D, John JE 3rd, and Mounsey JP (2000) Sodium channel isoform-specific effects of halothane: protein kinase C co-expression and slow inactivation gating. *Br J Pharmacol* **130**:1785–1792.
- Ratnakumari L and Hemmings HC Jr (1998) Inhibition of presynaptic sodium channels by halothane. *Anesthesiology* **88**:1043–1054.
- Ratnakumari L, Vysotskaya TN, Duch DS, and Hemmings HC Jr (2000) Differential effects of anesthetic and nonanesthetic cyclobutanes on neuronal voltage-gated sodium channels. *Anesthesiology* **92**:529–541.
- Rehberg B and Duch DS (1999) Suppression of central nervous system sodium channels by propofol. *Anesthesiology* **91**:512–520.
- Rehberg B, Xiao YH, and Duch DS (1996) Central nervous system sodium channels are significantly suppressed at clinical concentrations of volatile anesthetics. *Anesthesiology* **84**:1223–1233; discussion 1227A.
- Roy ML and Narahashi T (1992) Differential properties of tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels in rat dorsal root ganglion neurons. *J Neurosci* **12**:2104–2111.
- Saidman LJ, Eger EI 2nd, Munson ES, Babad AA, and Muallem M (1967) Minimum alveolar concentrations of methoxyflurane, halothane, ether and cyclopropane in man: correlation with theories of anesthesia. *Anesthesiology* **28**:994–1002.
- Sanna E, Mascia MP, Klein RL, Whiting PJ, Biggio G, and Harris RA (1995) Actions of the general anesthetic propofol on recombinant human GABAA receptors: influence of receptor subunits. *J Pharmacol Exp Ther* **274**:353–360.
- Scholz A, Appel N, and Vogel W (1998a) Two types of TTX-resistant and one TTX-sensitive Na⁺ channel in rat dorsal root ganglion neurons and their blockade by halothane. *Eur J Neurosci* **10**:2547–2556.
- Scholz A, Kuboyama N, Hempelmann G, and Vogel W (1998b) Complex blockade of TTX-resistant Na⁺ currents by lidocaine and bupivacaine reduce firing frequency in DRG neurons. *J Neurophysiol* **79**:1746–1754.
- Scholz A and Vogel W (2000) Tetrodotoxin-resistant action potentials in dorsal root ganglion neurons are blocked by local anesthetics. *Pain* **89**:47–52.
- Smith C, McEwan AI, Jhaveri R, Wilkinson M, Goodman D, Smith LR, Canada AT, and Glass PS (1994) The interaction of fentanyl on the Cp50 of propofol for loss of consciousness and skin incision. *Anesthesiology* **81**:820–828; discussion 826A.
- Sonner JM, Antognini JF, Dutton RC, Flood P, Gray AT, Harris RA, Homanics GE, Kendig J, Orser B, Raines DE, et al. (2003) Inhaled anesthetics and immobility: mechanisms, mysteries and minimum alveolar anesthetic concentration. *Anesth Analg* **97**:718–740.
- Stadnicka A, Kwok WM, Hartmann HA, and Bosnjak ZJ (1999) Effects of halothane and isoflurane on fast and slow inactivation of human heart hH1a sodium channels. *Anesthesiology* **90**:1671–1683.
- Ueno S, Trudell JR, Eger EI 2nd, and Harris RA (1999) Actions of fluorinated alkanols on GABA(A) receptors: relevance to theories of narcosis. *Anesth Analg* **88**:877–883.
- Yamakura T, Bertaccini E, Trudell JR, and Harris RA (2001) Anesthetics and ion channels: molecular models and sites of action. *Annu Rev Pharmacol Toxicol* **41**:23–51.
- Yamakura T and Harris RA (2000) Effects of gaseous anesthetics nitrous oxide and xenon on ligand-gated ion channels. Comparison with isoflurane and ethanol. *Anesthesiology* **93**:1095–1101.
- Yoshimura M, Higashi H, Fujita S, and Shimoji K (1985) Selective depression of hippocampal inhibitory postsynaptic potentials and spontaneous firing by volatile anesthetics. *Brain Res* **340**:363–368.
- Zhou ZS and Zhao ZQ (2000) Ketamine blockage of both tetrodotoxin (TTX)-sensitive and TTX-resistant sodium channels of rat dorsal root ganglion neurons. *Brain Res Bull* **52**:427–433.

Address correspondence to: Dr. R. Adron Harris, Waggoner Center for Alcohol and Addiction Research, 1 University Station A4800, University of Texas at Austin, Austin, TX 78712-0159. E-mail: harris@mail.utexas.edu
