Isoflurane inhibits NaChBac, a prokaryotic voltage-gated sodium channel

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ABBREVIATIONS: ANOVA, analysis of variance; HEK, human embryonic kidney; $I_{Na}$, transient NaChBac Na$^+$ current; $k$, slope factor of activation or inactivation; IC$_{50}$, concentration for 50% inhibition; $V_{1/2a}$, voltage of half-maximal activation; $V_{1/2in}$, voltage of half-maximal inactivation
Abstract

Volatile anesthetics inhibit mammalian voltage-gated Na\(^+\) channels, an action that contributes to their presynaptic inhibition of neurotransmitter release. We measured the effects of isoflurane, a prototypical halogenated ether volatile anesthetic, on the prokaryotic voltage-gated Na\(^+\) channel NaChBac from *Bacillus halodurans*. Using whole cell patch-clamp recording, HEK 293 cells transfected with NaChBac displayed large inward currents (\(I_{Na}\)) that activated at potentials \(\geq -60\) mV with a peak voltage of activation of 0 mV (from a holding potential of -80 mV) or -10 mV (from a holding potential of -100 mV). Isoflurane inhibited \(I_{Na}\) in a concentration-dependent manner over a clinically relevant concentration range; inhibition was significantly more potent from a holding potential of -80 mV (IC\(_{50}\)=0.35 mM) than from -100 mV (IC\(_{50}\)=0.48 mM). Isoflurane positively shifted the voltage dependence of peak activation and negatively shifted the voltage dependence of end steady-state activation. The voltage dependence of inactivation was negatively shifted with no change in slope factor. Enhanced inactivation of \(I_{Na}\) was 8-fold more sensitive to isoflurane than reduction of channel opening. In addition to tonic block of closed and/or open channels, isoflurane enhanced use-dependent block by delaying recovery from inactivation. These results indicate that a prokaryotic voltage-gated Na\(^+\) channel, like mammalian voltage-gated Na\(^+\) channels, is inhibited by clinical concentrations of isoflurane involving multiple state-dependent mechanisms. NaChBac should provide a useful model for structure-function studies of volatile anesthetic actions on voltage-gated ion channels.
Introduction

Despite significant recent advances, the mechanisms of volatile anesthetic action are still poorly understood (Hemmings et al., 2005a). Recent evidence implicates mammalian voltage-gated Na\(^+\) channels (Na\(_v\)) as targets for the presynaptic depression of neurotransmitter release by volatile anesthetics (OuYang et al., 2003, 2005; Wu et al., 2004; Hemmings et al., 2005b). Voltage-gated Na\(^+\) channels are critical for the initiation and conduction of action potentials in excitable cells (Hodgkin and Huxley 1952; Hille, 2001), and are important in regulating presynaptic excitability (Meir et al., 1999) and neurotransmitter release (Tibbs et al., 1989). Nine homologous subtypes of the 4 domain pore-forming Na\(_v\) \(\alpha\) subunit have been identified in mammals (Yu and Catterall, 2003). A single domain prokaryotic voltage-gated Na\(^+\) channel (NaChBac) with similar properties to mammalian voltage-gated ion channels has been isolated from *Bacillus halodurans* (Ren et al., 2001). The single domain of the presumably tetrameric NaChBac consists of 6 transmembrane \(\alpha\)-helical segments structurally homologous to the 6 transmembrane segments of each of the 4 domains of voltage-gated Na\(^+\) and Ca\(^{2+}\) channels.

NaChBac exhibits the basic kinetic features of Na\(_v\): it opens with depolarization in a voltage-dependent manner (activation) over a comparable voltage range, closes upon prolonged depolarization (inactivation), and returns to a resting, non-conducting state with repolarization (deactivation) (Ren et al., 2001). However, the kinetics of NaChBac are slower than those of eukaryotic Na\(_v\) channels including \(~\)6-fold slower activation, \(~\)17-fold slower inactivation, and \(~\)10-fold slower recovery from inactivation (Ren et al., 2001; Kuzmenkin et al., 2004). These conserved basic gating mechanisms with slower kinetics
provides a potential advantage in studying NaChBac as a model for Na_\text{v} because of the intrinsic demands of doing experiments with channels that have substantially faster gating. NaChBac activation is preceded by movement of a gating charge, an essential feature of voltage-gated ion channels (Kuzmenkin et al., 2004). The relatively simple amino acid sequence of NaChBac (274 amino acids, 31 kDa), its structural homology to Na_\text{v}, and the ease of producing large amounts in bacterial culture provide a potential opportunity for structure-function studies and 3-dimensional structure determinations by X-ray crystallography of a model anesthetic target. This led us to investigate the effects of isoflurane, a prototypical inhaled anesthetic ether, on the basic functional properties of heterologously expressed NaChBac. Isoflurane is representative of the modern family of halogenated ether anesthetics currently in widespread clinical use that are believed to share their essential molecular targets for the production of general anesthesia (Hemmings et al., 2005).
Methods

Heterologous expression of NaChBac

The cDNA for wild-type NaChBac in a modified pTracer expression vector (6.2 kb, containing GFP-Zeocin site, Invitrogen, Carlsbad, CA), generously provided by Dr. D. Clapham (Harvard University, Boston MA), was amplified in bacterial culture and purified. HEK 293 cells (ATCC, Manassas, VA) were transiently transfected using Lipofectamine 2000 (Invitrogen) and cultured in DMEM/F12 (Invitrogen) with 5% (v/v) fetal bovine serum (Biosource International, Camarillo CA) and 5% CO₂/95% O₂ (v/v) at 37°C. One to three days prior to electrophysiological recording, cells were plated on glass coverslips in 35-mm plastic dishes (Becton Dickinson, Franklin Lakes, NJ). Transfected cells were identified using a Nikon ECLIPSE TE300 inverted fluorescence microscope (Melville, NY).

Electrophysiological recording

The culture medium was changed and cells were superfused at 2-3 ml/min with extracellular solution containing (in mM): NaCl 140; KCl 4; CaCl₂ 1.5; MgCl₂ 1.5; HEPES 10; D-glucose 5; pH 7.30 with NaOH. Na⁺ currents (I_{NaChBac}) were recorded at room temperature (24-26°C) using the whole cell patch-clamp technique (Hamill et al., 1981). Patch electrodes were made from borosilicate glass capillaries (Drummond Scientific, Broomall, PA) using a 4-step protocol (P-97 micropipette puller, Sutter Instruments, Novato, CA), fire polished (Narishige microforge, Kyoto, Japan), and coated with SYLGARD (Dow Corning Corporation, Midland, MI) to reduce background noise and capacitance. The pipette electrode solution contained (in mM): CsF 80; CsCl 40; NaCl 15; HEPES 10; EGTA 10; pH 7.35 with CsOH. Cells <15 µm in diameter were selected for
recording using low resistance electrodes (1-3 MΩ). To determine contribution of steady-state inactivation to isoflurane inhibition on \(I_{NaChBac}\), two holding potentials (-80 mV or -100 mV) were used in present study. Because of the slow current decay observed with consecutive pulses, low frequency stimuli (0.2 or 2 Hz) were used to study use-dependent block of \(I_{Na}\). Currents were sampled at 10 or 20 kHz and filtered at 1-3 kHz using an Axon 200B amplifier, digitized with a Digidata 1321A interface, and analyzed using pClamp 8.2 software (Axon Instruments, Burlingame, CA). Capacitance and 60-85% series resistance were compensated, and leak current was subtracted using P/4 or P/5 protocols. Cells were held at -80 mV between recordings with pulse frequencies of 0.1-2 Hz.

Isoflurane (Abbott Laboratories, North Chicago, IL) was diluted from stock solutions in extracellular solution (10-12 mM, prepared by stirring at room temperature for 12-24 h) into airtight glass syringes, and applied locally to attached cells at 0.05 ml/min through a 0.15 mm diameter perfusion pipette located 30-40 µm from patched cells using an ALA-VM8 pressurized perfusion system (ALA Scientific, Westbury, NY). Isoflurane concentrations were determined by local sampling of the perfusate at the site of the recording pipette tip and analysis by gas chromatography following n-heptane extraction (Ratnakumari and Hemmings, 1998); measured isoflurane concentrations were ~85% of syringe concentrations.

**Data Analysis**

\(IC_{50}\) values (± SEM) for isoflurane were determined by least squares fitting of data to the Hill equation: 
\[
\frac{I_{Na}}{I_{Na \text{ Control}}} = \frac{1}{1+10^{(\log IC_{50}-X)\cdot h}}
\]

where \(X\) is isoflurane concentration, \(I_{Na}/I_{Na \text{ Control}}\) is normalized \(Na^+\) current, and \(h\) is Hill slope. Normalized activation data were fitted to a Boltzmann equation: 
\[
\frac{G}{G_{\text{max}}} = \frac{1}{1+\exp(V_{1/2_a}-V)/k}
\]

where
G/G_{max} is normalized fractional conductance, G_{max} is maximum conductance, V_{1/2a} is voltage for half-maximal activation, and k is slope factor. Na\(^+\) conductance (G_{Na}) was calculated as G_{Na}=I_{Na}/(V_{t}-V_{r}), where I_{Na} is peak Na\(^+\) current, V_{t} is test potential and V_{r} is Na\(^+\) reversal potential (E_{Na}=67 mV). Inactivation curves were fitted to a Boltzmann equation: I_{Na}/I_{Na\ max}=1/(1+exp(V-V_{1/2in})/k), where I_{Na}/I_{Na\ max} is normalized current, I_{Na\ max} is maximum current, V_{1/2in} is voltage of half-maximal inactivation, V is test potential and k is slope factor. I_{Na} inactivation was analyzed by fitting to a mono-exponential equation: I_{Na}=A\cdot\exp(-t/\tau)+C, A is maximal I_{Na} amplitude, C is plateau I_{Na}, t is time, and \tau is the time constant of current decay. NaChBac current traces were also fitted to the conventional Hodgkin and Huxley m\(^3\)h model (Hodgkin and Huxley 1952) in modified form: I(t)=I_{o}+I_{max}\cdot(1-\exp(-(t-t_{o})/\tau_{act}))^{3}\cdot\exp(-(t-t_{o})/\tau_{inact}), where I(t) is I_{Na} as a function of time, I_{o} is I_{Na} at t=t_{o}, I_{max} is maximal current, t_{o} is initial time of I_{Na} rising phase, t is time during the pulse, and \tau_{act} and \tau_{inact} are activation and inactivation time constants, respectively. The time course of use-dependent decay of I_{Na} was analyzed by fitting to a mono-exponential equation: Normalized I_{Na}=\exp(-time\ constant\cdot\ t)+C, t is pulse number, C is plateau I_{Na}, and time constant is the time constant of used dependent of decay. Data were analyzed using pCLAMP 8.2, Prism v. 4.0 (GraphPad Software Inc., San Diego, CA), SigmaPlot v. 9.0 (Systat Software Inc., San Jose, CA), and Origin 7.5 SR4 (OriginLab Corp, Northampton, MA). Statistical significance (p<0.05) was assessed by ANOVA or paired t-test, as indicated.
Results

Basic properties of NaChBac

HEK 293 cells expressing NaChBac showed voltage-gated Na\(^+\) currents \(I_{Na}\) up to 10 nA (Fig. 1). Cells with endogenous Na\(^+\) currents (amplitude <0.25±0.08 nA, n=8), easily identified by their faster activation compared to \(I_{Na}\), were discarded. To avoid large series resistance and space clamp error, only cells with \(I_{Na}\) of 0.5-5 nA were analyzed. \(I_{Na}\) activated at ≥-60 mV. Peak activation was at 0 mV from a holding potential of -80 mV, and at -10 mV from a holding potential of -100 mV, where a large fraction of channels are available and in the resting state (Ren et al., 2001; Fig. 1B). Peak \(I_{Na}\) occurred 10-20 ms from the beginning of the depolarizing pulse, which is consistent with a report that NaChBac activation is several-fold slower compared to Na\(_v\) (Ren et al., 2001). \(I_{Na}\) reversed at +70 mV (Fig. 1B), near the calculated Nernst equilibrium potential for Na\(^+\) for the conditions used (+67 mV). \(I_{Na}\) inactivation developed over 700-850 ms (Fig. 2).

Isoflurane inhibits NaChBac

Isoflurane reversibly inhibited \(I_{Na}\) with no change in the voltage of peak activation or reversal potential (Figs. 1 and 2). The effects of isoflurane on \(I_{Na}\) were similar from a holding potential of -80 or -100 mV. The concentration-effect curves were well fitted by the Hill equation (Fig. 2B), and yielded IC\(_{50}\) values near the clinically effective concentration of isoflurane (aqueous concentration equivalent to MAC [minimum alveolar concentration]=0.35 mM in rat; Taheri et al., 1991): 0.35±0.03 mM from a holding potential of -80 mV, and 0.48±0.02 mM from a holding potential of -100 mV (p<0.01, n=4-12).
Onset of inhibition of $I_{Na}$ by local pipette application of isoflurane was rapid (<1 min), and reversed within minutes of washout (Fig. 3). Inactivation of $I_{Na}$ was highly sensitive to isoflurane (Figs. 2A and 3). Inactivation was best fitted by a mono-exponential function with a time constant ($\tau$) that was markedly reduced by isoflurane, consistent with facilitation of the inactivation process (see below). Isoflurane binding affinity during channel inactivation ($K_D=0.06$ mM; Fig. 2C) was 8-fold higher than the IC$_{50}$ for peak current amplitude (IC$_{50}=0.48\pm 0.02$ mM, from a holding of -100 mV, Fig 2B), which reflects binding affinity to open channels. The marked reversible increase in current decay induced by isoflurane was also observed with repetitive stimulation (Fig. 3C). The linear relationship between blocking rate ($1/\tau$) and isoflurane concentration (Fig. 2C) reflects a bimolecular interaction between isoflurane and its binding site (Ramos and O’Leary, 2004).

**Effects of isoflurane on channel gating**

The effects of isoflurane on NaChBac channel gating kinetics were analyzed from the calculated conductance and conductance ratios at each of 2 points (peak and end) during a 30 ms depolarizing pulse (Fig. 4). Conductance and normalized conductance activation data were best fitted by a simple Boltzmann function. Inhibition at peak current and at the end of the depolarizing pulse more accurately reflect channel kinetic changes induced by isoflurane (see Fig. 1 and Fig. 4a). Isoflurane (~0.8 mM) produced a depolarizing shift in the voltage dependence of peak $I_{Na}$ activation and a hyperpolarizing shift in the voltage dependence of end $I_{Na}$ activation (Fig. 4b and Table 1). The shift in $V_{1/2_{aend}}$ by isoflurane was greater from a holding potential of -100 mV, and of $V_{1/2_{apeak}}$ from -80 mV (Table 1). This could be due to a greater fraction of inactivated channels at a holding potential of -80 mV (Fig. 5), and more newly inactivated channels at the end of the pulse at a holding
potential of -100 mV, respectively. The net effect of isoflurane was to narrow the current window of activation for either holding potential, thus decreasing the peak current.

Isoflurane reduced channel availability as reflected in the marked hyperpolarizing shift in the voltage dependence of inactivation from a holding potential of -80 mV with no change in slope factor (Fig. 5, Table 1). With a 2 second prepulse from -80 mV, ~26% of channels were inactivated, consistent with greater isoflurane effect early (5 ms, data not shown) in the depolarizing pulse. From a holding potential of -100 mV, greater block of $I_{\text{Na}}$ by isoflurane occurred later in the depolarizing pulse (End of pulse at 29 ms, Table 1) due to greater current decay from enhanced channel inactivation.

The effects of isoflurane on NaChBac channel gating were also modeled by fitting to Hodgkin and Huxley $m^3h$ kinetics. This approach provides only a phenomenological description, but is useful in evaluating relative effects on activation and inactivation kinetics. Increasing isoflurane shifted the time to peak $I_{\text{Na}}$ to earlier times (inset Fig 6A, circles), revealing an accelerating effect on current time course. The time to peak $I_{\text{Na}}$ was reduced ~25% by 0.83 mM isoflurane (n=2). From the fit of the complete current time course, $\tau_{\text{act}}/\tau_{\text{ctl}}$ decreased ~30% while $\tau_{\text{inact}}/\tau_{\text{ctl}}$ decreased ~95% for 0.83 mM isoflurane (n=2, Fig. 6B). Both effects contributed to the reduction of peak $I_{\text{Na}}$ by isoflurane (Fig. 6B, inset). The time to half activation was less sensitive to isoflurane than the time to peak $I_{\text{Na}}$ (inset Fig. 6A). This suggests rapid tonic block of open channels during activation before current reaches peak amplitude and/or tonic block of closed channels, which cannot be distinguished by these data.
Use-dependent block of NaChBac by isoflurane

NaChBac exhibited greater use-dependent current decay at 2 Hz vs. 0.2 Hz stimulus frequency (Table 2), which reflects the slow recovery of NaChBac from inactivation. Isoflurane enhanced the use-dependent decay of $I_{Na}$ and increased the time constant (Table 2). Isoflurane also produced tonic block evident in reduced normalized $I_{Na}$ amplitudes (Fig. 7, Table 2). The enhanced use-dependent reduction of residual current, expressed as normalized $I_{Na}$ plateau, by isoflurane was greater from a holding potential of -80 mV ($\Delta C=-0.31$) than from -100 mV ($\Delta C=-0.14$; Table 2), consistent with enhanced channel inactivation and slower recovery from inactivation at a holding potential of -80 mV.
Discussion

Volatile anesthetics inhibit native neuronal and recombinant mammalian voltage-gated Na⁺ channels in a voltage-dependent manner by enhanced inactivation and tonic block (Rehberg et al., 1996; Ratnakumari et al., 2000; Ouyang et al., 2003; Ouyang and Hemmings, 2005; Shiraishi & Harris, 2004). We now show that isoflurane inhibits the prokaryotic voltage-gated Na⁺ channel NaChBac at concentrations that are comparable to those that block neuronal (Ratnakumari et al., 2000; Ouyang et al., 2003; Ouyang and Hemmings, 2005) and heterologously expressed mammalian Na⁺ channels (Rehberg et al., 1996; Stadnicka et al., 1999; Shiraishi and Harris, 2004). The simple single domain structure, homologous pore region, and slower gating kinetics compared with eukaryotic channels make NaChBac an excellent model for structure-function studies of the pharmacology and ion channel binding sites of volatile anesthetics. The similar pharmacological sensitivity to isoflurane suggests that the site(s) of volatile anesthetic interaction with voltage-gated channels is conserved from bacteria to mammals.

Isoflurane and other volatile anesthetics affect multiple molecular targets critical to neuronal signaling in addition to Na⁺ channels, in particular GABA_A and glycine receptors, the principal inhibitory ligand-gated ion channels (Hemmings et al., 2005). Resolution of the relative contributions of various anesthetic-sensitive targets to the spectrum of pharmacological effects has been greatly facilitated by the design of anesthetic-insensitive mice harboring site-specific mutations in GABA_A receptors identified by structure-function studies (Jurd et al., 2003). This approach in the much larger four domain voltage-gated ion channels will be greatly facilitated by models such as NaChBac. Although NaChBac differs in some respects from eukaryotic Na⁺ channels, elucidation of anesthetic mechanisms.
involving eukaryotic channels will be facilitated by looking at a simpler channel. The observations that NaChBac is inhibited by isoflurane at similar concentrations as Na\textsubscript{v} and that the mechanisms are similar to those observed for Na\textsubscript{v} validates this approach. Because NaChBac is selective for Na\textsuperscript{+}, it is reasonable to assume that features of the pore structure will be similar between NaChBac and other Na\textsuperscript{+} channels. Similarities and differences between the mechanisms of block of NaChBac relative to other Na\textsuperscript{+} channel blockers will be informative to understanding the pharmacology of both channel types. Because NaChBac is presumed to be a homotetramer (by analogy with K\textsuperscript{+} channels), it is easier to mutate regions of interest for structure-function studies in this smaller protein than it is with the eukaryotic Na channels (Zhao et al., 2004). NaChBac can also be expressed in bacteria and hence large amounts of the protein can be produced that will facilitate finer structural studies. NaChBac expresses well in mammalian cells so mutants can be easily tested.

The effect of isoflurane on NaChBac activation differs from its effects on mammalian Na\textsubscript{v} (Ouyang et al., 2003; Rehberg et al., 1996; Shiraishi and Harris, 2004; Stadnicka et al., 1999), which exhibit no change in channel activation kinetics or activation curves. The rising and falling faces of NaChBac currents could be reproduced reasonably well using a Hodgkin-Huxley m\textsuperscript{3}h kinetic model for classical nerve type Na\textsuperscript{+} channels (Hodgkin and Huxley 1952), even though some nerve type Na\textsuperscript{+} channels are not fit well by the m\textsuperscript{3}h kinetic model (Herzog et al., 2001). Although m\textsuperscript{3}h kinetics do not accurately describe the activation time course because of transitions between multiple closed states before channel opening in neuronal Na\textsuperscript{+} channels (Baranauskas and Martina, 2006) that are also present in NaChBac (Kuzmenkin et al., 2004), the m\textsuperscript{3}h model allows a simple phenomenological
comparison of relative effects on activation and inactivation. While a change in activation kinetics was apparent it was not as large as the speeding of inactivation. However, a significant shift in the voltage-dependence of activation and of inactivation was observed. Since NaChBac activates ~6-fold slower than mammalian Na\textsubscript{v} (Ren et al., 2001), the mechanism of tonic block of NaChBac by isoflurane might differ from its effects on mammalian Na\textsubscript{v} by a greater contribution of open channel block during activation. Analysis of the mechanisms underlying isoflurane effects on NaChBac activation will require detailed gating current and single channel studies.

Isoflurane dissociates quickly from mammalian Na\textsuperscript{+} channels, which require rapid repetitive depolarization to develop use-dependent block (unpublished observations). The slower inactivation of NaChBac compared to Na\textsubscript{v} resulted in enhanced use-dependent block by isoflurane at relatively low stimulus frequency. These results are consistent with the modulated receptor hypothesis of Na\textsuperscript{+} channel block (Hille, 1977; Hondeghem and Katzung, 1977) in which the inactivated state is selectively stabilized by isoflurane. Open channel block by isoflurane is also likely due to the slower activation and inactivation kinetics of NaChBac. The NaChBac tail current was inhibited by isoflurane and the tail conductance ratio overlapped with the pseudo steady-state conductance ratio suggesting that enhancement of inactivation is the predominant mechanism of isoflurane inhibition. More detailed mechanistic analysis of the mechanisms of isoflurane block will require more refined gating and single channel studies.

In current models of Na\textsubscript{v} gating, the S4 helix, which contains highly conserved positively charged amino acids, plays a critical role as voltage sensor for activation (Chahine et al., 2004). Like Na\textsubscript{v}, NaChBac undergoes several gating transitions involving
gating charge movement (Kuzmenkin et al., 2004), the kinetics of which closely follow those of macroscopic activation. A neutralizing mutation in S4 (R114C) produced similar effects on NaChBac as isoflurane: a positive shift in the voltage dependence of activation and a negative shift in voltage dependence of inactivation (Chahine et al., 2004). The positive shift in the voltage dependence of NaChBac activation and the net decrease in channel conductance by isoflurane suggest a reduction in voltage sensitivity via direct or indirect interaction with the voltage sensor. Since there are no interdomain linkers and the N-terminus of NaChBac is too short to achieve N-type tethered ball-chain inactivation, NaChBac is predicted to undergo C-type inactivation (Catterall, 2001; Ren et al., 2001) similar to slow inactivation in Na_v (Balser et al., 1996a; Fukuda et al., 2005) and K_v channels (Lopez-Barneo et al., 1993; Cordero-Morales et al., 2006). This was confirmed by a recent report in which the pore S6 linker was found to be important in NaChBac inactivation (Pavlov et al., 2005). Our results indicate that NaChBac availability was reduced by isoflurane, evident in the negative shift in the inactivation curve as well as marked enhancement of current decay upon opening. The slope factor for inactivation was unaffected, consistent with no effect of isoflurane on the voltage sensor during channel inactivation. Since NaChBac lacks fast inactivation as seen in Na_v, use-dependent block is likely more complicated in Na_v with additional coupling between channel closing and recovery.

There are similarities between the effects of isoflurane and local anesthetics on Na_v. Voltage-dependent block by isoflurane of NaChBac (this study) and of Na_v (Stacnicka et al., 1999; Ouyang et al., 2003; Ouyang and Hemmings, 2005) is consistent with greater anesthetic affinity for the inactivated state, similar to local anesthetic and anticonvulsant
block of Na\textsubscript{v} (Ragsdale et al., 1994; Nau and Wang, 2004). Multiple state-dependent mechanisms are involved in local anesthetic block. Lidocaine slows gating transitions during activation (Vedantham and Cannon, 1999; Wang et al., 2004), allosterically modifies S4 of domains III and IV (Sheets and Hanck, 2003), and interacts with the extracellular loop of domain IV (E1555; Li et al., 2002) and with the S4 voltage sensor (Hanck et al., 2000; Sheets and Hanck, 2003). These findings and our observation that isoflurane shifts NaChBac activation indicate that isoflurane, similar to local anesthetics, acts via multiple mechanisms to affect channel gating. The outer pore vestibule is a pivotal determinant of local anesthetic binding in Na\textsubscript{v} such that the W1531C mutation in domain IV of Na\textsubscript{v,1.4} eliminates local anesthetic block (Li et al., 2002; Tsang et al., 2005). Similar target sites for volatile anesthetics could exist in the NaChBac pore region and should be identifiable by site-directed mutagenesis.

Upon prolonged depolarization, mammalian Na\textsubscript{v} channels open and subsequently enter fast and slow inactivated states (Hille, 2001; Nau and Wang, 2004). The mechanism of slow inactivation differs from that of fast inactivation by involving rearrangement of the selectivity filter (Balser et al., 1996a; Hille, 2001), as seen in C-type inactivation of K\textsubscript{v} channels (Lopez-Barneo et al., 1993; Cordero-Morales et al., 2006). According to the modulated receptor model (Hille, 1977; Hondeghem and Katzung, 1977), use-dependent block during repetitive stimulation results from accumulation of fast-inactivated channels and slowed recovery of channel availability from mostly fast inactivation. Local anesthetics also produce use-dependent block by modulating slow inactivation (Balser et al., 1996b; Vedantham and Cannon, 1999; Fukuda et al., 2005) and/or inhibiting activation (Vedantham and Cannon, 1999; Wang et al., 2004). Since the inactivation kinetics of
NaChBac resemble C-type inactivation of Kv channels and slow inactivation of Nav channels, isoflurane might also reduce Nav availability via effects on activation and inactivation (enhancing inactivation and delaying recovery). The relative contribution of fast and slow inactivation to block of Nav by isoflurane is an interesting area for further investigation. Structure-function studies to compare volatile anesthetic effects on NaChBac and Nav channels should provide a useful approach in dissecting the mechanisms of anesthetic modulation of voltage-gated Na+ channels.

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References


Footnotes

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**Legends for Figures**

**Fig. 1.** Effects of isoflurane on NaChBac current-voltage (I-V) relationships. A. Isoflurane (~0.8 mM, equivalent to ~2.3 times MAC in rat) significantly inhibited $I_{Na}$ from a holding potential of either -80 mV or -100 mV. Effects were analyzed at various phases of the test pulse: a, 5 ms after depolarization; b, Peak $I_{Na}$ at 10-20 ms; c, End $I_{Na}$ 29 ms after depolarization; and d, Tail current at repolarization. B. Voltage for peak inward current ($V_{max}$) was 0 mV from a holding potential of -80 mV (left) and -10 mV from a holding potential of -100 mV (right). $I_{Na}$ was activated by 30 ms test pulses of -60 mV to +70 mV in 10 mV steps. Mean isoflurane concentrations in B. were 0.79±0.06 mM for holding potential of -80 mV and 0.82±0.05 mM for holding potential of -100 mV (mean±SEM, n=7-8).

**Fig. 2.** Isoflurane reversibly inhibits NaChBac at clinically relevant concentrations. A. Representative current traces showing reversible inhibition by isoflurane of $I_{Na}$ from a holding potential of -100 mV. B. Concentration-effect curves for inhibition of normalized peak $I_{Na}$ by isoflurane from holding potentials of -80 mV or -100 mV. Data (mean±SEM, n=4-12) were fitted to the Hill equation. ***, p<0.01 by Student unpaired t test. C. Isoflurane-facilitated current decay was fitted to a mono-exponential equation (see Methods). The blocking rate ($1/\tau \cdot 1000$) plotted against isoflurane concentration was fitted to a linear equation to yield the isoflurane binding affinity ($k_D=k_{off}/k_{on}=0.06$ mM); the slope and y-intercept yielded the association rate ($k_{on}=98.4$ s$^{-1}$mM$^{-1}$) and dissociation rate ($k_{off}=5.5$ s$^{-1}$), respectively. Currents were evoked by 850 ms test pulses to $V_{max}$ (-20 to 0 mV).
Fig. 3. **Reversible inhibition of NaChBac by isoflurane with repetitive stimulation.** $I_{Na}$ was activated from a holding potential of -100 mV by 850 ms test pulses to -10 mV at 8.5 sec intervals between sweeps. **A.** Representative traces showing time-course of $I_{Na}$ inhibition over 106 pulses. **B.** Normalized peak $I_{Na}$ amplitudes showing reversible inhibition of $I_{Na}$ by 0.41 mM isoflurane. The black line indicates the period of isoflurane application (1.5 min). **C.** Reduction and recovery of time-constant of current inactivation ($\tau$) by isoflurane.

Fig. 4. **Effects of isoflurane on NaChBac activation.** **A.** NaChBac conductance was calculated (see Methods) and plotted for two time points during the depolarizing pulse as shown in Fig. 1A: a, Peak at 10-20 ms and b, End of pulse at 29 ms. Conductance was plotted vs. the 30 ms depolarizing test voltage (-60 mV to +40 mV). **B.** Conductance was normalized to its maximal control value and fitted to a Boltzmann equation. Mean isoflurane concentrations were 0.79±0.06 mM for holding potential of -80 mV and 0.82±0.05 mM for holding potential of -100 mV (mean±SEM, n=7-8).

Fig. 5. **Effect of isoflurane on NaChBac inactivation.** **A.** Representative curves for steady-state inactivation before and after application of 0.78 mM isoflurane. Inactivation was measured using a two-pulse protocol in which cells were held at -80 mV and exposed to a 2 sec conditioning pulse from -100 mV to 0 mV, followed by a 30 ms test pulse to -20 mV or to -10 mV to determine channel availability. **B.** Normalized $I_{Na}$ was fit to a Boltzmann equation to yield voltage of 50% maximal inactivation ($V_{1/2in}$). Isoflurane
significantly shifted the voltage dependence of steady-state inactivation in the negative direction, with no effect on slope factors. Mean isoflurane concentration was 0.83±0.06 mM. Data expressed as mean±SEM, n=5. ***, p<0.001 by paired t test.

**Fig. 6. Differential effect of isoflurane on NaChBac activation and inactivation kinetics.** A. Representative currents elicited by pulses to -20 mV from a holding potential of -100 mV. Superimposed on each trace (in grey) is the fit of the current data to $I(t)=I_o+I_{\max}(1-\exp(-(t-t_o)/\tau_{act}))^3\cdot\exp(-(t-t_o)/\tau_{inact})$ (see Methods). **Inset:** Time to half activation ($I_{midpt}$; △, ▲) and the time to $I_{peak}$ (○, ●) as a function of isoflurane concentration. Data from two separate experiments are indicated by open vs. closed symbols. $\tau_{midpt}$ and $\tau_{peak}$ returned to control values after wash (data not shown). B. Activation and inactivation time constants ($\tau_{act}$ and $\tau_{inact}$, respectively) relative to control (ctl) plotted vs. isoflurane concentration. Data from each of two independent experiments are indicated by open vs. closed symbols. **Inset:** Fractional maximal current amplitude ($II_{ctl}$) vs. isoflurane concentration fitted by linear regression to $y=-0.40x+0.99$ (set 1, ●) or $y=-0.52x+1.04$ (set 2, △).

**Fig. 7. Use-dependent block of NaChBac by isoflurane.** Isoflurane increased use-dependent decay of $I_{Na}$ more from a holding potential of -80 mV (left) than from -100 mV (right). Graphs show peak amplitude of each pulse normalized to that of the first pulse (mean±SEM; n=6-11), plotted against stimulus number, and fitted to a mono-exponential function (see Methods). Protocol: 30 ms, 2 Hz test pulses from holding potential (-80 mV or -100 mV) to peak activation voltage (-20 mV to 0 mV) in 10 mV steps.
Table 1. Effects of isoflurane on NaChBac activation and inactivation

<table>
<thead>
<tr>
<th></th>
<th>Holding</th>
<th>-80 mV</th>
<th></th>
<th>Holding</th>
<th>-100 mV</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Isoflurane</td>
<td>(0.79 ± 0.06 mM)</td>
<td>Control</td>
<td>Isoflurane</td>
<td>(0.82 ± 0.05 mM)</td>
</tr>
<tr>
<td>V_{1/2apeak} (mV)</td>
<td>-21.4 ± 2.6</td>
<td>-16.0 ± 3.1**</td>
<td>-29.0 ± 1.9††</td>
<td>-27.2 ± 4.2†††</td>
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<tr>
<td>A_{peak}</td>
<td>0.94 ± 0.04</td>
<td>0.30 ± 0.02***</td>
<td>0.95 ± 0.03</td>
<td>0.40 ± 0.03***†††</td>
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<tr>
<td>k_{peak}</td>
<td>12.4 ± 2.19</td>
<td>16.4 ± 2.37</td>
<td>10.2 ± 1.65</td>
<td>16.4 ± 3.87**</td>
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<tr>
<td>V_{1/2aend} (mV)</td>
<td>-23.9 ± 2.4</td>
<td>-26.4 ± 4.5</td>
<td>-31.1 ± 1.9††</td>
<td>41.6 ± 4.9***†††</td>
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<tr>
<td>A_{end}</td>
<td>0.92 ± 0.04</td>
<td>0.16 ± 0.01***</td>
<td>0.93 ± 0.03</td>
<td>0.19 ± 0.02***</td>
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<tr>
<td>k_{end}</td>
<td>11.2 ± 2.02</td>
<td>13.7 ± 3.91</td>
<td>9.25 ± 1.64</td>
<td>9.98 ± 4.54</td>
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</tbody>
</table>

V_{1/2apeak}, V_{1/2aend}, voltage of half-maximal peak current and end steady-state current activation respectively; V_{1/2in}, voltage of half-maximal inactivation; A, relative amplitude; k, slope factor. **p<0.01; ***p<0.001 vs. respective control by unpaired t-test. ††p<0.01; †††, p<0.001 for -80 mV holding vs. -100 mV holding by unpaired t test. Values are mean±SEM (n=6-8).
Table 2. Isoflurane enhances use-dependent decay of NaChBac

| f, stimulation frequency; C, normalized $I_{Na}$ amplitude plateau (SEM<0.01). Mean isoflurane concentrations were 0.80±0.04 mM for a holding potential of -80 mV and 0.82±0.05 mM for a holding potential of -100 mV. *, p<0.05; **, p<0.01; ***, p<0.001 vs. respective control by one-way ANOVA with Newman-Keuls post hoc test (mean±SEM, n=6-11). †, p< 0.05; ††, p<0.01; †††, p<0.001 for -100 mV holding vs. -80 mV holding by unpaired t test. |
Figure 1

A

-80 mV
Control

-100 mV
Control

+70 mV
30 ms

Isoflurane 0.77 mM

Isoflurane 0.81 mM

B

Voltage (mV)
-60 -40 0 20 40 60 80

\[ \text{Voltage (mV)} \]

\[ \text{I}_N (\text{nA}) \]

\[ \text{I}_N (\text{nA}) \]

Control

Isoflurane

Control

Isoflurane
Figure 3

A

B

C

 Isoflurane

$\frac{I_{\text{Na}}}{I_{\text{Na control}}}$

$\tau$ (ms)

Pulse number

Pulse number

1 nA

-10 mV

850 ms

-100 mV

0.00

0.25

0.50

0.75

1.00

$\frac{I_{\text{Na}}}{I_{\text{Na control}}}$

0

50

100

150

0

50

100

150

Isoflurane

$\tau$ (ms)

Pulse number
Figure 4

A

-80 mV

-100 mV

B

Control at Peak  ■ Isoflurane

Control at End  ▲ Isoflurane

Voltage (mV)

Voltage (mV)

G (pS)

G (pS)

G/G_{max}

G/G_{max}

Volatges (mV)

Volatges (mV)
Figure 7

-80 mV
Control

-100 mV
Control

2 nA
10 ms

0 mV 30 ms
-20 mV
-10 mV

-80 mV
-100 mV

Isoflurane 0.78 mM

Isoflurane 0.81 mM

Normalized $I_Na$

Control

Isoflurane 0.80 mM

Control

Isoflurane 0.82 mM

Pulse Number

Pulse Number