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Effects of Halothane and Isoflurane on Fast and Slow Inactivation of Human Heart hH1a Sodium Channels

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Background: **Cloning and heterologous expression of ion channels allow biophysical and molecular studies of the mechanisms of volatile anesthetic interactions with human heart sodium channels. Volatile anesthetics may influence the development of arrhythmias arising from cardiac sodium channel dysfunction. For that reason, understanding the mechanisms of interactions between these anesthetics and cardiac sodium channels is important. This study evaluated the mechanisms of volatile anesthetic actions on the cloned human cardiac sodium channel (hH1a) α subunit.**

Methods: **Inward sodium currents were recorded from human embryonic kidney (HEK293) cells stably expressing hH1a channels. The effects of halothane and isoflurane on current and channel properties were evaluated using the whole cell voltage-clamp technique.**

Results: **Halothane at 0.47 and 1.1 mM and isoflurane at 0.54 and 1.13 mM suppressed the sodium current in a dose- and voltage-dependent manner. Steady state activation was not affected, but current decay was accelerated. The voltage dependence of steady state fast and slow inactivations was shifted toward more hyperpolarized potentials. The slope factor of slow but not fast inactivation curves was reduced significantly. Halothane increased the time constant of recovery from fast inactivation. The recovery from slow inactivation was not affected significantly by either anesthetic.**

Conclusions: **In a heterologous expression system, halothane and isoflurane interact with the hH1a channels and suppress the sodium current. The mechanisms involve acceleration of the transition from the open to the inactivated state, stabilization of the fast and slow inactivated states, and prolongation of the inactivated state by delayed recovery from the fast inactivated to the resting state. (Key words: α Subunit; cardiac fast inward sodium current; I_{Na}; patch clamp; stable expression.)**

THE function of voltage-gated sodium channels in the heart that mediate rapid depolarization during the upstroke of the action potential is essential for the generation and coordinated propagation of the cardiac action potential. Structurally, these channels are heteromeric transmembrane proteins composed of a pore-forming α subunit and an auxiliary β_1 subunit. The α subunit is organized into four homologous domains (I–IV), each containing six membrane-spanning segments (S1–6) connected by intracellular and extracellular linkages.[1,2] When expressed in *Xenopus* oocytes or the established cell lines, the α subunit alone is sufficient to produce a channel with functional properties characteristic of cardiac cells.[1,3,4] Thus, cloning[5,6] and heterologous expression of the α subunit enable biophysical and molecular studies to identify the mechanisms of anesthetic actions on the human cardiac sodium channel.

Volatile anesthetics alter cardiac sodium channel function. At clinically relevant concentrations, halothane, isoflurane, and sevoflurane decrease the fast inward sodium current (I_{Na}) through native cardiac voltage-gated sodium channels.[7,8] The effects of halothane depend on the conformational state of the channel.[9] During the perioperative period, certain volatile anesthetics may influence arrhythmias arising from cardiac sodium channel dysfunction.[10] Therefore, for clinical reasons, it is important to establish the mechanisms and functional consequences of volatile anesthetic interactions with the human heart sodium channel.

To extend our understanding of the electrophysiologic actions of volatile anesthetics on the human cardiac sodium channel, we evaluated the effects of halothane and isoflurane on the sodium current recorded from HEK293 cells stably expressing the wild-type human cardiac sodium channel (hH1a) α subunits. Our results show that halothane and isoflurane suppress sodium

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current through hH1a channels by the mechanism involving preferential interaction with the inactivated state. Distinct effects on the fast and slow inactivation processes and the recovery from fast inactivation suggest a complex interaction of these anesthetics with the human cardiac sodium channel in the inactivated state.

Materials and Methods

Channel Expression and Cell Culture

The cDNA encoding the wild-type hH1a[6] α subunit was purified by alkaline lysis–polyethylene glycol precipitation and linearized at a nonessential site in the ampicillin gene of the pC-DNA3 vector (Invitrogen, Carlsbad, CA), to facilitate integration into host chromosomal DNA. The HEK293 cells were transfected with hH1a cDNA using lipofectin reagent (Gibco BRL, Life Technologies, Grand Island, NY) at a ratio of 10:1 (50 μg lipofectin, 5 μg hH1a DNA in 3 ml serum-free transfection medium per 1×10^6 cells). After 5 h of transfection, cells were returned to normal culture medium for an additional 48 h to allow expression of antibiotic resistance. A selection antibiotic, Geneticin (G418; Gibco BRL, Life Technologies, Gaithersburg, MD), was added to the culture medium at 500 μg/ml. After an additional 2.5 weeks in culture, single colonies of transfected cells were selected using cloning rings. HEK293 cells stably expressing hH1a channels were grown in Dulbecco modified Eagle medium supplemented with 25 mM glucose, 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 5% carbon dioxide humid atmosphere incubator at 37°C. For patch-clamp experiments, cells were lifted from the culture dishes and separated using an enzyme-free cell dissociation buffer (Gibco BRL, Life Technologies, Grand Island, NY).

Solutions

Modified Tyrode solution containing 150 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, at *p*H 7.4 adjusted with NaOH was used to wash and store the cells before patching. The pipette solution for recording the I_{Na} contained 1 mM NaF, 110 mM CsF, 10 mM EGTA, 1 mM $CaCl_2$, 2 mM MgATP, 10 mM HEPES, at *p*H 7.3 adjusted with CsOH. Fluoride, used as the main anion, helped to maintain stable whole-cell recordings with low access resistance and leak currents for more than 1 h. The external (bath) solution contained 10 mM NaCl, 135 mM CsCl, 10 mM HEPES, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 3 mM $CoCl_2$, at *p*H 7.4 adjusted with CsOH. The extracellular Na^+ was reduced to 10 mM to improve voltage control. Cesium (Cs^+) and cobalt (Co^{2+}) were used to block endogenous currents.

Anesthetics

Halothane (Fluothane; Ayerst Laboratories, Philadelphia, PA) or isoflurane (Forane; Ohmeda PPD, Liberty Corner, NJ) were dispersed in the bath solution by sonication and delivered to the recording chamber from glass-syringe reservoirs. Because commercially available halothane (Fluothane) contains 0.01% thymol as a stabilizer, we tested for its effects on hH1a channel current. In our experiments, the highest final concentration of thymol in the bath solution was 0.3 μM. At this concentration, thymol alone had no effect on the whole cell I_{Na} amplitude, conductance, and voltage dependence of steady state fast and slow inactivation (n = 3, data not shown). Concentrations of anesthetics in the bath solution sampled from the recording chamber were measured by gas chromatography (GC-8A; Shimadzu, Columbia, MD). Halothane was used at 0.47 ± 0.1 mM (0.72 vol% at 21°C) and 1.08 ± 0.03 mM (1.64 vol% at 21°C). Isoflurane concentrations were 0.54 ± 0.03 mM (1.10 vol% at 21°C) and 1.13 ± 0.1 mM (2.29 vol% at 21°C).

Electrophysiologic Experiments

The inward I_{Na} was recorded in the whole-cell configuration of the patch-clamp technique[11] using a List EPC7 patch-clamp amplifier (Adams & List, Westbury, NY) interfaced to a standard personal computer *via* a TL-1 DMA board (Axon Instruments, Foster City, CA). Voltage protocols and data acquisition were controlled by the pCLAMP6 software (Axon Instruments). Heat-polished patch electrodes with resistances of 1 to 2 MΩ were prepared from borosilicate capillary tubing (Garner Glass, Claremont, CA) with a Sutter P-87 puller (Sutter Instrument Co., Novato, CA). All experiments were performed at room temperature (20–22°C).

The cells were patched in a 1-ml flow-through chamber mounted on the stage of an inverted Olympus IMT-2 microscope (Olympus America, Inc., Melville, NY). After gigaohm seal formation and establishment of the whole cell conditions, each cell was allowed to stabilize for 20 min before data were acquired to minimize the effects of a time-dependent shift. Previous studies by Wang *et al.*[12] showed time-dependent negative shifts in the voltage dependence of activation and inactivation of human cardiac and skeletal muscle sodium channels expressed in the mammalian cell line. To evaluate these effects for our

experimental conditions, in a separate set of control experiments (n = 30 cells, data not shown) the current–voltage relation, activation, inactivation, and recovery from inactivation were monitored for 60 min at 5-min intervals. We determined that changes in peak current and small negative shifts in the steady state inactivation curves, if present, were seen mainly during the first 15 min after the whole cell voltage-clamp was established.

Data Analyses

Data were analyzed and plotted using pClamp6 (Axon Instruments, Foster City, CA), Origin 4.1 (Microcal, Northampton, MA), and Excel software (Microsoft, Buffalo, NY). The statistical significance of data from two groups was determined using paired or unpaired Student *t* tests. One-way analysis of variance was used to compare the differences derived from three or more groups. The level of significance was set at $P < 0.05$. Data are expressed as the mean ± SEM.

To evaluate the voltage dependence of the steady state activation, channel conductance was determined from the whole-cell inward I_{Na} elicited by 30-ms test pulses from −100 mV to +50 mV from a holding potential of −120 mV in 5- or 10-mV increments. The following equation was used:

$$G = I_{Na}/(V_t - E_{rev}) \quad (1)$$

where G is conductance, I_{Na} is the peak sodium current, V_t is the test pulse voltage, and E_{rev} is the measured reversal potential. The conductance–voltage relation was fit by a Boltzmann function:

$$G/G_{max} = 1/(1 + \exp(V_{1/2} - V_t)/k) \quad (2)$$

where G/G_{max} is normalized conductance, $V_{1/2}$ is the voltage for 50% activation, V_t is the test pulse voltage, and k is the slope factor.

Macroscopic current decay after channel opening was determined as the time constant (τ) derived from fitting a single exponential function to the decaying phase of individual currents according to the function

$$I(t) = I_{SS} + \alpha_1 \exp(-t/\tau) \quad (3)$$

where I(t) is the current amplitude as a function of time, I_{SS} is asymptote or plateau amplitude, α_1 is the amplitude at time = 0 (peak current), and τ is the time constant measured in milliseconds.

The voltage dependence of channel availability was evaluated by differentiating between fast and slow inactivation processes. To measure the steady state fast inactivation, 50-ms prepulses, ranging from −140 mV to +20 mV were applied in 10 mV increments from a holding potential of −140 mV. After each prepulse, a 20-ms test pulse to peak current voltage (−30 mV to −40 mV) assayed the current to determine the fraction of available channels. To measure the steady state slow inactivation, the cells were held for 10 s at variable holding potentials (−140 mV to +20 mV in 20-mV increments) to induce slow inactivation. Each prepulse was followed by a 20-ms hyperpolarizing pulse to −140 mV to remove fast inactivation and thus kinetically isolate slow inactivation. A 20-ms test pulse to −30 mV was then applied to assay the current. Townsend and Horn,[13] and more recently Richmond *et al.*,[14] used 1- or 2-min prepulses to develop slow inactivation. Such long prepulses would considerably increase the duration of the slow inactivation voltage protocol. Therefore, in our study, the time for induction of slow inactivation was optimized to 10 s, and cumulative inactivation was used where channels are not allowed to recover from slow inactivation between consecutive inactivating holding potentials.[13] Peak current amplitude determined from both inactivation protocols was normalized and plotted *versus* either prepulse voltages (fast inactivation) or holding potentials (slow inactivation). Data distribution was fit by a Boltzmann function

$$I/I_{max} = 1/(1 + \exp(V_p - V_{1/2})/k) \quad (4)$$

where I/I_{max} is the normalized current amplitude, V_p is the prepulse for fast and the holding potential for slow inactivation, $V_{1/2}$ is the voltage at which 50% of the channels are available for opening, and k is the slope factor of the Boltzmann fit.

Recovery from fast inactivation was determined using a two-pulse protocol. A 50-ms conditioning prepulse to predetermined peak current voltage (−30 mV) was applied to induce fast inactivation of the channels. The prepulse was followed by a variable-duration recovery interval (measured in milliseconds) at a holding potential of −120 mV before a 20-ms test pulse to −30 mV assayed the recovered current. To measure recovery from slow inactivation, the prepulse was increased to 10 s, and after a variable-duration recovery interval at −120 mV, a 20-ms pulse to −140 mV was applied to ensure that only recovery from slow inactivation was measured by a 20-ms test pulse to −30 mV. Between individual episodes, cells were allowed to rest for 30 s at a holding potential of −120 mV to prevent accumulation of recovery from slow inactivation.[14] The recovery was monitored for 20 s in 2-s increments, and for 5 s in 500-ms increments. The normalized peak current ampli-

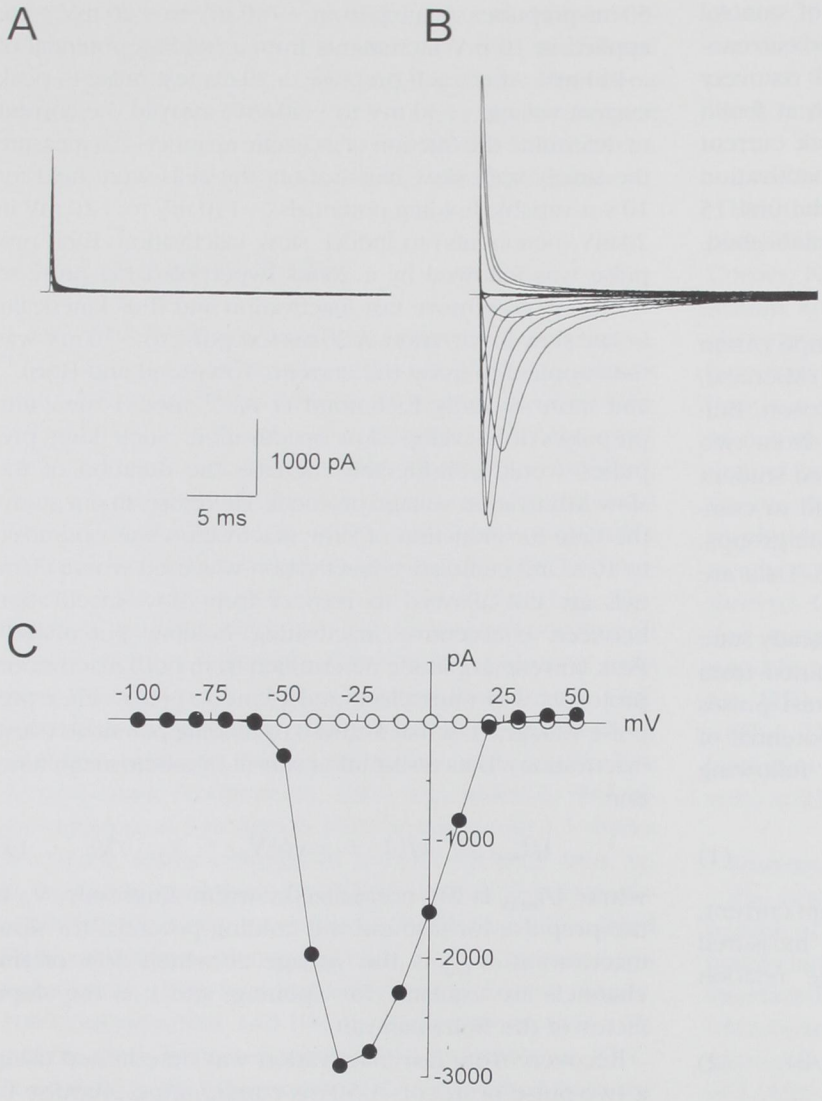

Fig. 1. Functional expression of the hH1a channel α subunit in the HEK293 cells. (*A*) The absence of the fast inward sodium current (I_{Na}) in a control nontransfected HEK293 cell is shown. (*B*) Family of currents recorded in the presence of 10 mM $[Na^+]_{out}$ from an HEK293 cell stably expressing hH1a channels is shown. The whole-cell inward I_{Na} was elicited by 30-ms depolarizing pulses from −100 mV to +50 mV in 10-mV increments from a holding potential of −120 mV. (*C*) Current–voltage relations for recordings in *A* (○) and *B* (●).

tude determined with both protocols was plotted *versus* the duration of the recovery interval. The results were quantified by fitting single exponentials to the data distribution to determine the time constant of recovery from fast or slow inactivation.

Results

Sodium Currents in HEK293 Cells Stably Expressing Wild-type hH1a Sodium Channels

Under the experimental conditions of our study, no measurable sodium current (I_{Na}) was detected in 10 nontransfected HEK293 cells (see the example given in figure 1A). In contrast, evoked by step depolarization from −100 mV to +50 mV from a holding potential of −120 mV, I_{Na} with fast activation and inactivation kinetics was recorded from the HEK293 cells stably expressing hH1a (fig. 1B). Current was activated at a threshold between −70 mV and −60 mV. The peak inward current occurred at −30 mV to −40 mV. The averaged peak current density was −78.6 ± 6.1 pA/pF, and the averaged cell capacitance was 24.5 ± 1.3 pF (n = 41).

Halothane and Isoflurane Decrease Amplitude of Inward Sodium Current in a Dose- and Voltage-dependent Manner

When applied in the superfusing bath solution, anesthetics reduced the inward I_{Na} amplitude (fig. 2A). Concentration-dependent, steady state effects were observed within 1 or 2 min of exposure to anesthetics (fig.

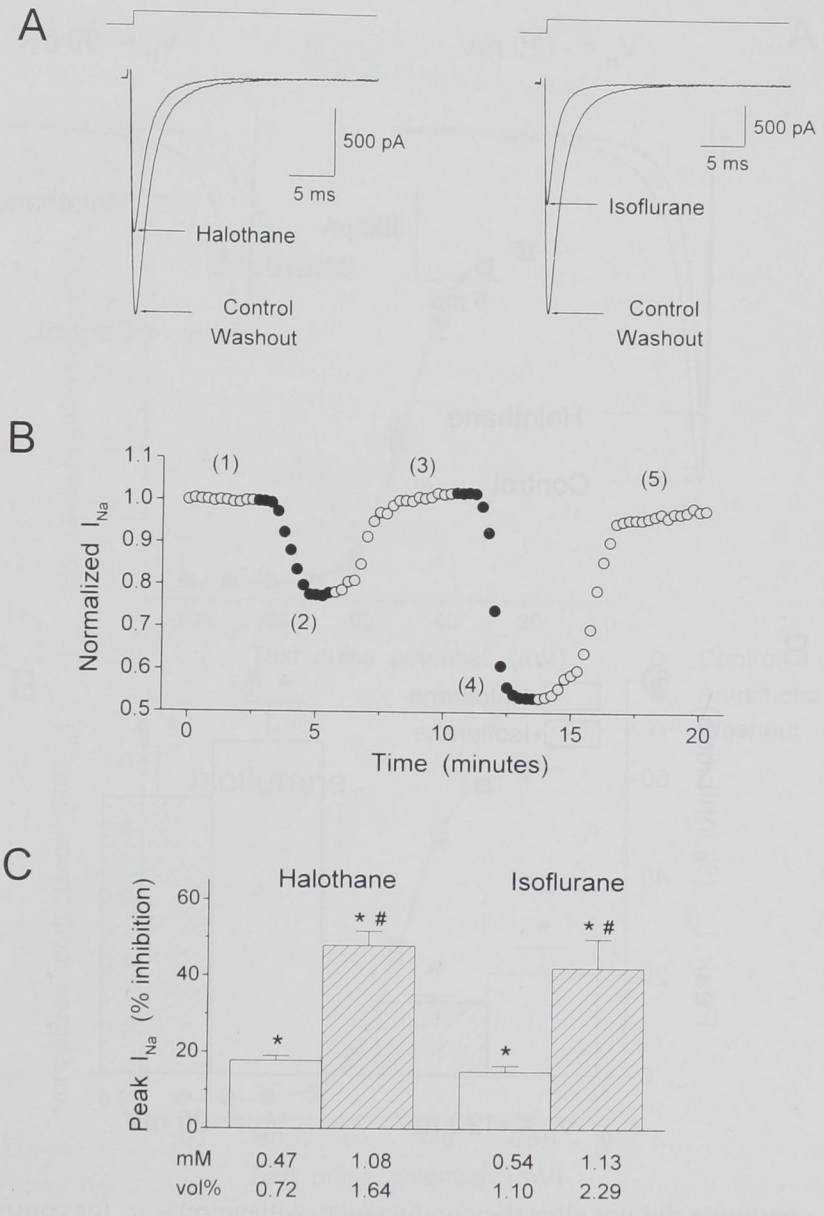

Fig. 2. Halothane and isoflurane decrease the fast inward sodium current (I_{Na}) through hH1a channels expressed stably in HEK293 cells. (*A*) Traces of currents evoked by a 30-ms depolarizing pulse to −30 mV from a holding potential of −120 mV in the absence (control/washout) and presence of 1 mM halothane and 1.1 mM isoflurane. Decreases in the peak current amplitude by anesthetics was reversible upon washout. (*B*) The time course of halothane effects on I_{Na}. Current was elicited by a 30-ms depolarizing pulse to −30 mV applied every 15 s from a holding potential of −120 mV. The normalized current is plotted *versus* time: (1) control, (2) 0.5 mM halothane, (3) washout, (4) 1 mM halothane, (5) washout. (*C*) The effects of halothane and isoflurane are dose-dependent. The I_{Na} was elicited by 30-ms depolarizations to −30 mV from −120 mV, and the peak current amplitude was measured in the absence and presence of anesthetics. Inhibition was determined as a percentage reduction in current amplitude relative to anesthetic-free controls. Data are the mean ± SEM (n = 10 in each group). All changes are significantly different from respective controls (*) at $P < 0.05$. The effects of high doses of anesthetics are significantly different from low doses (#) at $P < 0.05$.

2B). The degree of suppression of the current amplitude was similar with both anesthetics (fig. 2C), and the effect was reversible upon washout. The peak current amplitude decreased by 18 ± 1.2% and 48 ± 3.7% in the presence of 0.47 and 1.1 mM halothane (n = 10), respectively, and by 15 ± 1.5% and 55 ± 7.6% in the presence of 0.54 and 1.13 mM isoflurane (n = 10), respectively.

Inhibition of the peak current amplitude depended on the membrane holding potential. When the membrane potential was held at −120 mV, where most channels are in the resting state, 0.5 mM halothane or isoflurane blocked the current by 21 ± 5.2% and 15 ± 1.4%, respectively. However, at the membrane holding potential of −90 mV, at which a certain fraction of channels (10–15%) is already in the inactivated state, the block increased to 67.5 ± 7.8% and 56 ± 10%, respectively (figs. 3A and B). This suggests a voltage dependence of current block and preferential interaction of anesthetics with the channel in the inactivated state.

Steady State Activation Is Not Affected by Halothane or Isoflurane

Figure 4 summarizes the effects of anesthetics on steady state activation. Suppression of peak current

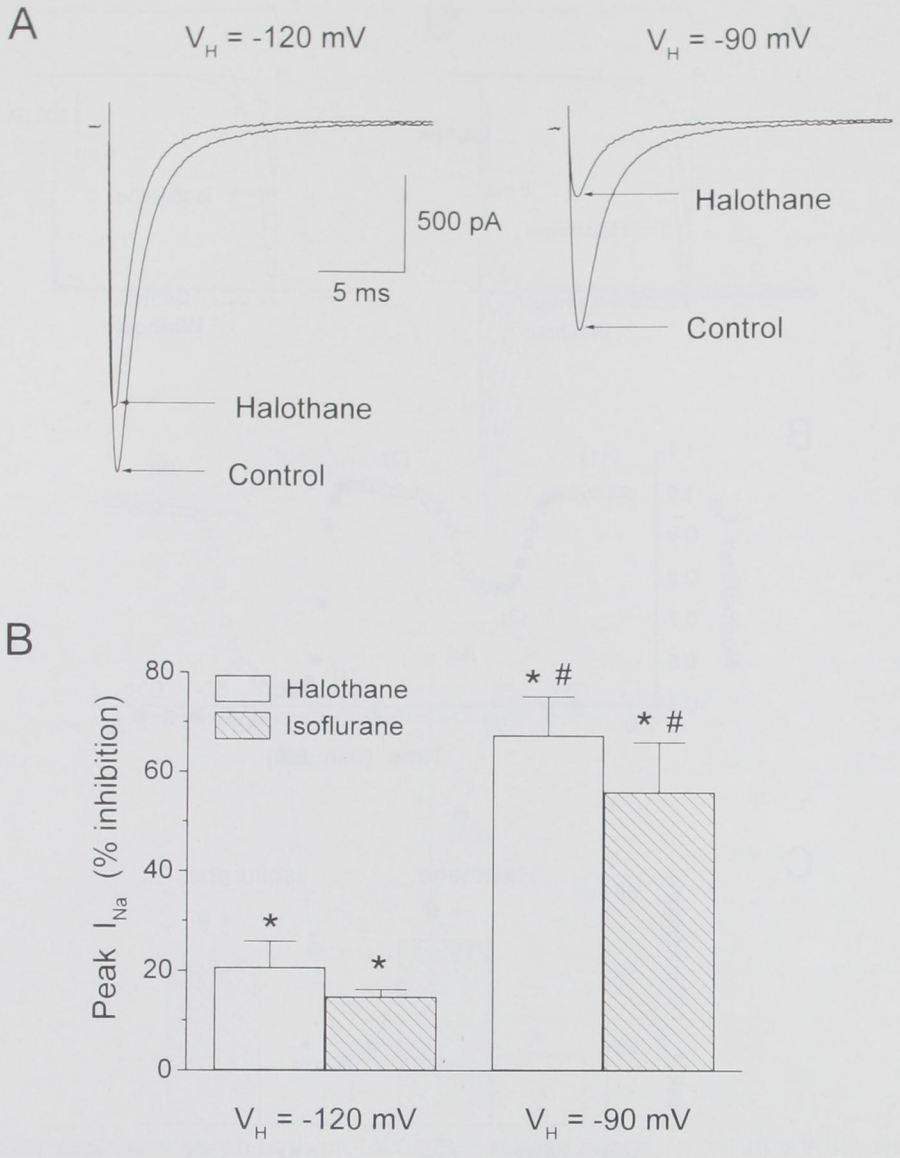

Fig. 3. The inhibition of the fast inward sodium current (I_{Na}) by anesthetics depends on the membrane holding potential. (*A*) Traces of I_{Na} evoked by 30-ms depolarizations to −30 mV from the holding potentials (V_H) of −120 mV and −90 mV in the absence and presence of 0.5 mM halothane are shown. The inhibition of I_{Na} by halothane increases at a more positive holding potential. (*B*) A summary of the V_H-dependent effects of 0.5 mM halothane and 0.5 mM isoflurane on the peak current amplitude is shown. All changes are significantly different from controls (*); the current block measured from V_H −90 mV is significantly larger from that at V_H −120 mV (#). Data are the mean ± SEM (n = 4 in each group).

amplitude did not alter the conductance–voltage relation. The activation curves calculated from conductances in the absence and presence of anesthetics overlapped. Boltzmann fits to mean control data (n = 10 in each group) gave the voltage for half-maximal activation ($V_{1/2}$) and the slope factors of −48 ± 0.4 mV and 5.7 ± 0.3 mV, respectively. The voltage dependence of activation was not altered by anesthetics. The $V_{1/2}$ and slope factor values were unchanged in the presence of 1.08 mM halothane (−48.7 ± 0.4 and 5.7 ± 0.5 mV) or 1.13 mM isoflurane (−48.6 ± 0.4 mV and 5.2 ± 0.4 mV). Means of individual data were −47.8 ± 1.8 mV and 5.0 ± 0.5 mV for control, −48.3 ± 1.6 mV and 5.1 ± 0.4 mV for 1.1 mM halothane, and −48.6 ± 1.6 mV and 5.2 ± 0.4 mV for 1.13 mM isoflurane, respectively. The voltage dependence of activation was also unaffected by 0.47 mM halothane and 0.54 mM isoflurane (data not shown).

Current Decay Is Accelerated by Halothane and Isoflurane

To characterize macroscopic decay, traces of currents evoked by 30-ms test pulses to −30 mV from a holding potential of −120 mV were fitted with a single exponential function (see Materials and Methods) to

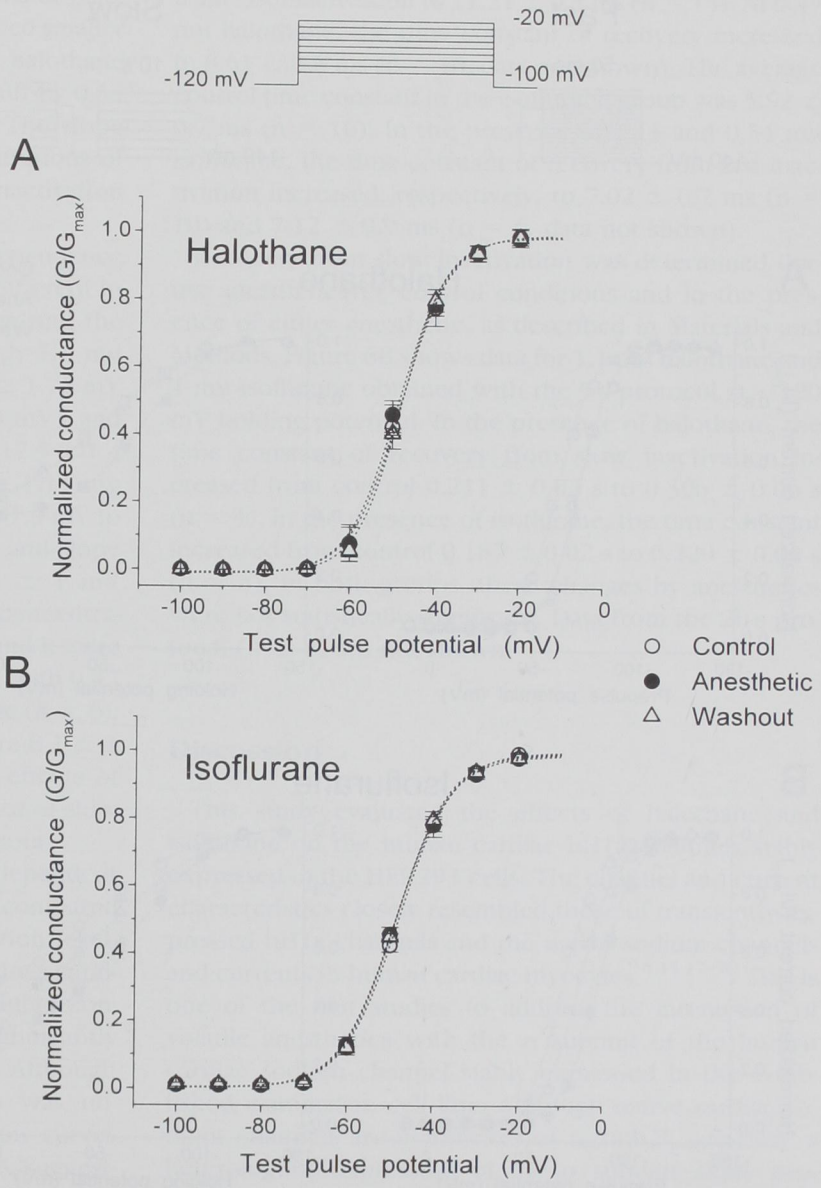

Fig. 4. The steady state activation of hH1a channels is not affected by halothane and isoflurane. Conductance was determined as described in Materials and Methods. Normalized conductance and voltage relations for controls, anesthetics, and washouts were fit to the Boltzmann function. Data are the mean ± SEM (n = 10 cells in each group). No statistical differences were found between anesthetic-free controls, anesthetics, and washouts.

derive the time constant of current decay. From average control of 1.98 ± 0.08 ms, the time constant of decay was decreased by 9.5 ± 0.7% with 0.47 mM halothane (n = 6) and 15.3 ± 2.4% with 1.1 mM halothane (n = 15). In isoflurane group, the time constant of decay decreased by 8.9 ± 0.4% (n = 6) with 0.54 mM isoflurane, and 18.8 ± 2.2% with 1.13 mM isoflurane (n = 9) from the average control of 1.92 ± 0.07 ms. These changes indicate that both anesthetics accelerate the conformational transition of the hH1a channel from the open to the inactivated state.

Steady State Fast and Slow Inactivations Are Modulated Differentially by Halothane and Isoflurane

The inactivation of cardiac voltage-gated sodium channels is a complex, nonuniform process.[15] Two distinct types of inactivation, fast and slow, can be distinguished by the kinetics of their development and recovery. Brief depolarizations induce fast inactivation, recovery from which is voltage-dependent and completed in milliseconds. Fast inactivation contributes to termination of the action potential upstroke by limiting channel availability as a function of initial membrane potential, and it deter-

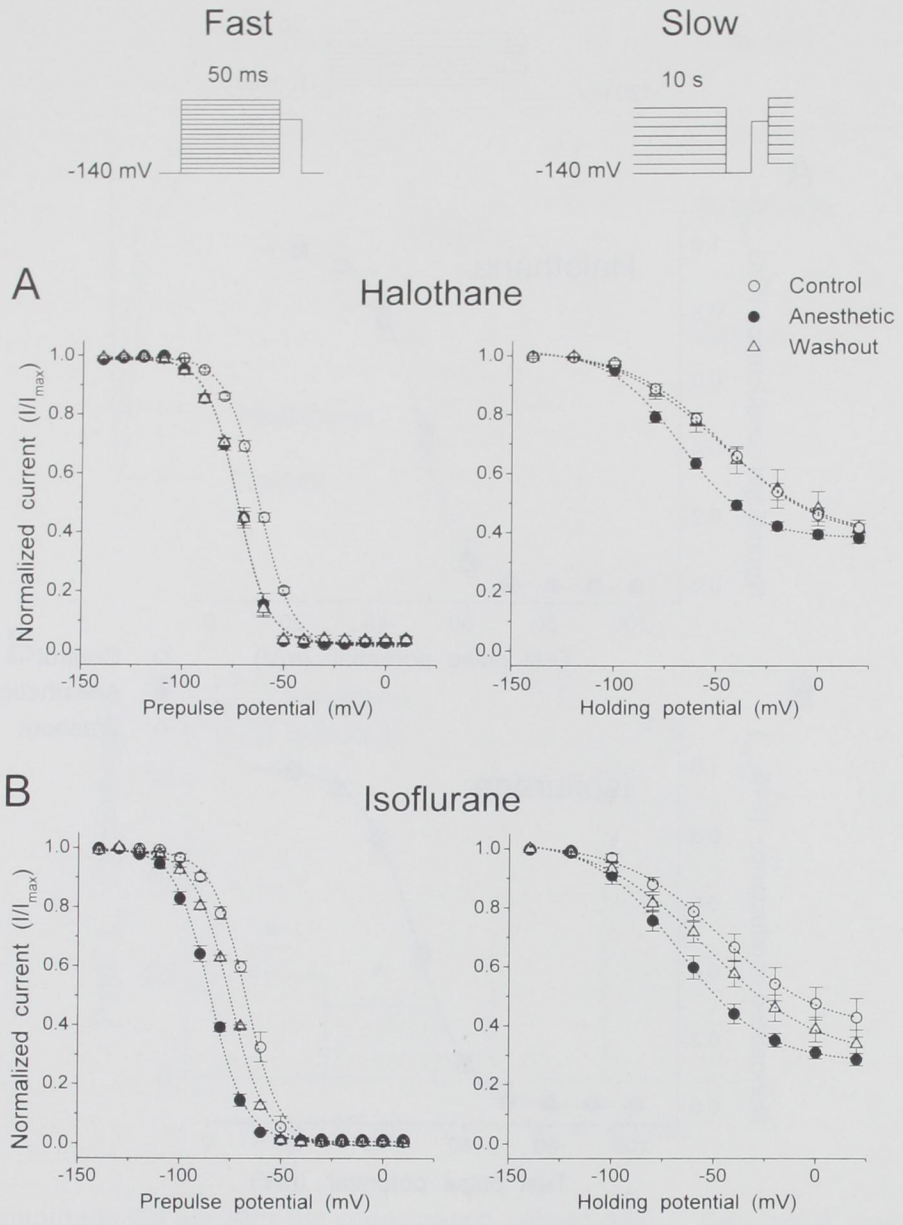

Fig. 5. The steady state fast and slow inactivation of hH1a channels is altered markedly by 1.1 mM halothane and 1 mM isoflurane. Steady state fast inactivation (Fast) was measured after 50-ms prepulses to the indicated voltages from −140 mV holding potentials. The average, normalized currents were plotted against prepulse voltages and fitted to the Boltzmann function (see Materials and Methods). $V_{1/2}$ and slope factor data are reported in Results. Data points are the mean ± SEM for 10 cells. Steady state slow inactivation (Slow) was measured after holding the cells for 10 s at potentials ranging from −140 mV to +20 mV. The average, normalized currents were plotted against holding potentials and fitted to the Boltzmann function (see Materials and Methods). $V_{1/2}$ and slope factor data are reported in Results. Data points are the mean ± SEM for 7–10 cells.

mines the immediate pool of channels available for subsequent action potentials. Slow inactivation develops during sustained or repetitive depolarizations. In cardiac sodium channels, the recovery from slow inactivation may take seconds. Slow changes in the availability of the channels for activation are important for regulation of the repetitive, long-duration cardiac action potentials and contribute to long-term modulation of membrane excitability.[16] We investigated the actions of halothane and isoflurane on the hH1a channel availability by evaluating fast and slow inactivation independently (see Materials and Methods). Figure 5 shows that anesthetics shifted the steady state fast inactivation curves toward more hyperpolarized potentials. Halothane, at 1.1 mM (n = 10), shifted $V_{1/2}$ from control −62.7 ± 0.6 mV to −73.1 ± 0.6 mV (average shift, 10.4 ± 1.1 mV), but the slope factor was not significantly altered (8.5 ± 0.5 mV to 8.9 ± 0.6 mV). Isoflurane, at 1.13 mM (n = 10), shifted $V_{1/2}$ from −67.1 ± 0.7 mV to −84.8 ± 0.4 mV (average shift, 17.7 ± 1.6 mV) without changing the slope factor (8.5 ± 0.6 mV to 8.7 ± 0.3 mV). The effects of anesthetics on fast inactivation were reversed only in part and

were even irreversible at higher concentrations of halothane. Lower doses of both anesthetics produced smaller shifts. The average shift in $V_{1/2}$ by 0.47 mM halothane was 6.7 ± 1.1 mV (n = 9), and the average shift by 0.54 mM isoflurane was 6.0 ± 0.9 mV (n = 6). The slope factors were not changed. For lower concentrations of anesthetics, the reversibility of shifts in fast inactivation curves was also incomplete.

The effects of anesthetics on the voltage dependence of cumulative slow inactivation curves were different in that hyperpolarizing shifts in $V_{1/2}$ were larger, and the slope factor was significantly decreased. With 1.1 mM halothane (n = 10), $V_{1/2}$ shifted from −46.5 ± 1.72 mV to −67.8 ± 1.5 mV (average shift, 21.3 ± 1.3 mV), and slope factor decreased from 23.2 ± 1.8 mV to 17.5 ± 1.4 mV (average decrease, 5.7 ± 0.6 mV). With 1.13 mM isoflurane (n = 10), $V_{1/2}$ shifted from −47.0 ± 1.9 mV to −67 ± 1.1 mV (average shift, 20 ± 3.7 mV), and slope factor decreased from 24 ± 1.2 mV to 20 ± 1 mV (average decrease, 4.1 ± 0.8 mV). At lower concentrations of anesthetics, the average shifts in $V_{1/2}$ and k were 12.4 ± 1.6 mV and 4.2 ± 0.4 mV, respectively, with 0.47 mM halothane (n = 6). With 0.54 mM isoflurane (n = 6), the average shifts in $V_{1/2}$ and slope factor were 8.1 ± 1 mV mV and 3.5 ± 1 mV, respectively. The effects of anesthetics on shifts in $V_{1/2}$ and the slope factor of slow inactivation curves were reversible with washout.

Thus, with both anesthetics, concentration-dependent shifts in $V_{1/2}$ were significantly larger for slow compared with the fast inactivation. At each concentration level, the shifts by halothane and isoflurane were not significantly different from each other, except for higher concentrations of isoflurane, which caused a significantly larger shift in $V_{1/2}$ compared with halothane. Although the slope factor of fast inactivation curves was unchanged, the slope factor of slow inactivation curves was significantly decreased by both anesthetics, suggesting a distinct alteration of voltage sensitivity.

Recovery from Fast Inactivation

Recovery from inactivation underlies the conformational transition of channel from the inactivated to the resting state during membrane repolarization. In cardiac sodium channels, recovery from fast inactivation occurs within milliseconds, whereas recovery from slow inactivation may take seconds. Under anesthetic-free control conditions, the hH1a channels recovered from fast inactivation with an average time constant of 6.16 ± 0.4 ms (n = 25). As shown in figure 6A, 1.1 mM halothane significantly increased the time constant of recovery from fast inactivation to 11.21 ± 0.5 ms (n = 15). At 0.47 mM halothane, the time constant of recovery increased to 8.61 ± 0.6 ms (n = 10, data not shown). The average control time constant in the isoflurane group was 5.92 ± 0.7 ms (n = 16). In the presence of 1.13 and 0.54 mM isoflurane, the time constant of recovery from fast inactivation increased, respectively, to 7.02 ± 0.7 ms (n = 10) and 7.12 ± 0.9 ms (n = 6, data not shown).

Recovery from slow inactivation was determined during anesthetic-free control conditions and in the presence of either anesthetic, as described in Materials and Methods. Figure 6B shows data for 1.1 mM halothane and 1 mM isoflurane obtained with the 5-s protocol at −120 mV holding potential. In the presence of halothane, the time constant of recovery from slow inactivation increased from control 0.211 ± 0.02 s to 0.306 ± 0.06 s (n = 4). In the presence of isoflurane, the time constant increased from control 0.187 ± 0.02 s to 0.220 ± 0.04 s (n = 4). In both groups, these changes by anesthetics were not statistically significant. Data from the 20-s protocol (n = 8) are not shown.

Discussion

This study evaluated the effects of halothane and isoflurane on the human cardiac hH1a channels stably expressed in the HEK293 cells. The channel and current characteristics closely resembled those of transiently expressed hH1a channels and the native sodium channels and currents in human cardiac myocytes.[6,14,17–20] This is one of the first studies to address the interaction of volatile anesthetics with the α subunit of the human cardiac sodium channel stably expressed in the established mammalian cell line. Although native cardiac sodium channels are complexes of α and β_1 subunits, a heterologous expression of the α subunit alone produces a functional channel.[3,4,21] Therefore, at clinically relevant concentrations, volatile anesthetics were expected to alter the current through hH1a channels in a manner similar to native sodium channels. However, the suppression of peak I_{Na} amplitude by halothane and isoflurane was larger for hH1a channels. Furthermore, the degree of block was similar at equianesthetic concentrations of anesthetics. This is in contrast to native channels, in which current block by halothane was larger than isoflurane or sevoflurane.[8] The reasons for these dissimilarities are not clear, but they may be caused by species differences and microenvironmental differences between native cells and the expression sys-

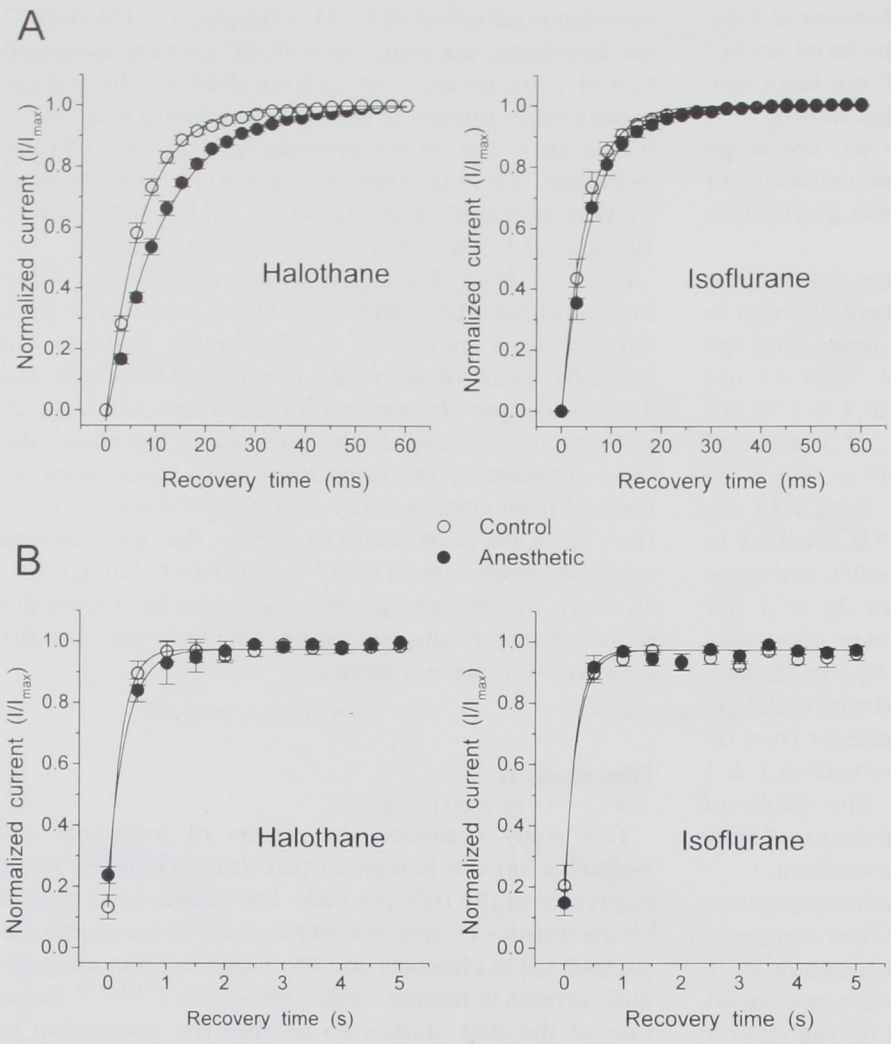

Fig. 6. The effects of halothane and isoflurane on the recovery from inactivation. Standard two-pulse protocols were used to measure the recovery from (*A*) fast and (*B*) slow inactivation (see Materials and Methods). The normalized current was plotted *versus* the recovery interval. Solid lines are single exponential fits giving the time constant (τ) of recovery. (*A*) Recovery from fast inactivation is shown. The time constant was significantly increased by 1.1 mM halothane, but not by 1 mM isoflurane. Data are the mean ± SEM (n ranged from 10 cells to 15 cells). (*B*) Recovery from slow inactivation is shown. The time constant of the recovery from slow inactivation was not affected by halothane and isoflurane. Data are the mean ± SEM (n = 4 in each group).

tem cells regarding the availability of complete second-messenger systems. The absence of an auxiliary β_1 subunit in the expression system also may contribute to these differences, since other researchers have shown that the hH1a channel block by lidocaine may be altered significantly when the β_1 subunit is coexpressed with the α subunit.[22] Further, in the absence of all components of the intact voltage-dependent cardiac sodium channel, the configuration of the α subunit may be more accessible to volatile anesthetics.

Previous studies performed in our laboratory showed that volatile anesthetics shift the steady state activation of the native cardiac sodium channel to more negative membrane potentials.[8] In contrast, the steady state activation of hH1a channels was not affected. Conductance–voltage relations and the slope factors were not altered by anesthetics. This might suggest that, in the expression system, volatile anesthetics do not interact with the region of the α subunit that is involved in voltage-dependent activation. Alternatively, the absence of a β_1 subunit may change the accessibility to a site where anesthetics affect the modulation of conductance in the intact channel. However, some similarities exist between these two systems. As in the native channels,[9] the anesthetic effects on hH1a channel current were modulated by the membrane holding potential, with block increasing when the membrane was held at a more depolarized potential. The reversibility of I_{Na} block by halothane at a more depolarized holding potential was partial, as in the native channels.[9] To achieve a complete

recovery from block, hyperpolarization of the membrane to −150 mV was necessary (data not shown). These effects indicate voltage dependence and strong binding of halothane to the channel in the inactivated state. Furthermore, a decrease in the time constant of macroscopic current decay suggests that halothane and isoflurane facilitate the conformational transition of the hH1a channel from the open to the inactivated state.

The steady state inactivation of the hH1a channels was markedly affected by halothane and isoflurane. Both anesthetics, at higher and lower concentrations, produced hyperpolarizing shifts in fast inactivation curves without changing the slope factor. This effect, reported also for other sodium channel blocking agents, suggests preferential binding to the channel in the inactivated state. The reversibility of anesthetic effects on fast inactivation was incomplete. In contrast, large hyperpolarizing shifts in the steady state slow inactivation curves and significantly reduced slope factors were reversible with anesthetic washout. Thus, anesthetics appear to differentially alter the steady state fast and slow inactivation of hH1a channels.

Fast and slow inactivation gates are structurally independent.[23] A conserved isoleucine–phenylalanine–methionine cluster in the intracellular linker between domains III and IV is considered an essential part of the fast inactivation gate.[24] The slow inactivation gate includes multiple sites in the pore and the intra- and extracellular linkers.[13,14] Considering these structural differences, the results of our study suggest, as one possibility, that the fast and slow inactivation gates may be altered by anesthetics *via* multiple sites of interaction, which also may include the channel voltage sensor, the S4 segment. Positively charged residues in specific regions of the S4 segments of domains I–IV are involved in activation and inactivation gating and contribute differentially to the voltage dependence of fast and slow inactivation.[25] Our study showed that the voltage sensitivity of activation and fast inactivation of the hH1a channel is not changed by anesthetics, whereas the voltage sensitivity of slow inactivation is altered. Thus, the regions of the voltage sensor that contribute to the voltage dependence of slow inactivation may be modulated selectively by anesthetics, whereas those that contribute to the voltage dependence of fast inactivation or activation are not affected.

However, multiple differential effects by anesthetics also could result from interaction with a single site on the channel. This type of interaction is well-established for the actions of the site 3 toxins, such as scorpion α toxins and the sea anemone venoms. Acting specifically on cardiac sodium channels, a sea anemone venom, anthopleurin B, retards fast inactivation by binding to a site located on the extracellular surface of the channel and is structurally not associated with the intracellular fast inactivation gate.[26] A similar mechanism could be adopted for volatile anesthetic interaction with the inactivation processes of the hH1a channel. The Modulated Receptor Hypothesis for the interaction of local anesthetics and antiarrhythmic drugs with the cardiac sodium channel assumes different affinities for drug binding depending on the conformational state of the channel (resting, open, inactivated), but it emphasizes a single receptor site.[27,28] Such effects of volatile anesthetics on the hH1a channel as interaction with the inactivated state and altered recovery from fast inactivation may imply a common site or overlapping sites. However, this hypothesis does not adequately explain the distinct effects of anesthetics on fast and slow inactivation and recovery from fast inactivation. Clearly, our results cannot differentiate between multiple sites or a single site of interaction of volatile anesthetics with the cardiac sodium channel. Further experiments, including site-directed mutagenesis, are needed to solve this problem.

Differences in the reversibility of anesthetic effects on slow *versus* fast inactivation further emphasize the complex nature of interactions between the volatile anesthetics and the cardiac sodium channel. Partial reversibility or irreversibility of anesthetic effects on fast inactivation is in sharp contrast to a complete reversibility of anesthetic effects on slow inactivation. These differences suggest a selective and long-lasting alteration of the fast inactivation by volatile anesthetics or, alternatively, a permanent change to selective regions of the channel. Sodium channel blocking agents such as fatty acids and various antiarrhythmic drugs cause long-lasting shifts in the voltage dependence of fast inactivation, and the washout of these drugs does not recover completely channel availability. These effects, frequently attributed to time-dependent shifts in the voltage dependence of channel gating, are demonstrated for the native and cloned cardiac sodium channel and the cloned brain sodium channels.[9,18,29,30] For our experimental conditions, the irreversibility of anesthetic effects on fast inactivation remains unsolved. The time-dependent shifts do not explain these effects because of individual cell selection *via* the stabilization period included in our protocols.

Cardiac excitability and conductivity depend on the availability of sodium channels for voltage-dependent

activation. The available pool of voltage-gated sodium channels is regulated by molecularly and kinetically distinct fast and slow inactivation processes.[14] Although fast inactivation determines the number of channels for consecutive action potentials, slow inactivation contributes to long-lasting modulation of channel availability for repetitive, long-duration action potentials, characteristic of normal cardiac function.[14] Our study shows that halothane and isoflurane differentially alter fast and slow inactivation and recovery from the fast inactivation of hH1a channels, and suggests that the short-term channel availability may be affected more drastically by volatile anesthetics than the long-term modulation of cardiac cell excitability. A delayed recovery of sodium channel availability by anesthetics may predispose the cell membrane to electric instability.

The results of our electrophysiologic studies of single cells using a heterologous channel expression system cannot be related directly to the clinical state and are of uncertain clinical relevance. Still, the model described herein allows more insight into the molecular interaction of volatile anesthetics with the human cardiac sodium channel.

In conclusion, the results of this study show that halothane and isoflurane suppress sodium currents through stably expressed human cardiac hH1a sodium channels by a mechanism involving preferential interaction with the inactivated state. Anesthetics accelerate conformational transition from the open to inactivated state and stabilize the inactivated state of the channel by acting differentially on fast and slow inactivation processes. In addition, halothane prolongs the fast inactivated state by slowing the recovery from fast inactivated to the closed resting state.

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