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***n*-Alcohols Inhibit Voltage-Gated Na⁺ Channels Expressed in *Xenopus* Oocytes**

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Abstract

Voltage-gated sodium channels are essential for the initiation and propagation of action potentials in excitable cells and are known as a target of local anesthetics. In addition, inhibition of sodium channels by volatile anesthetics has been proposed as a mechanism of general anesthesia. The *n*-alcohols produce anesthesia, and their potency increases with carbon number until a “cut-off” is reached. In this study, we examined effects of a range of *n*-alcohols on Na_v1.2 subunits to determine the alcohol cut-off for this channel. We also studied the effect of a short-chain alcohol (ethanol) and a long-chain alcohol (octanol) on Na_v1.2, Na_v1.4, Na_v1.6, and Na_v1.8 subunits, and we investigated the effects of alcohol on channel kinetics. Ethanol and octanol inhibited sodium currents of all subunits, and the inhibition of the Na_v1.2 channel by *n*-alcohols indicated a cut-off at nonanol. Ethanol and octanol produced open-channel block, which was more pronounced for Na_v1.8 than for the other sodium channels. Inhibition of Na_v1.2 was due to decreased activation and increased inactivation. These results suggest that sodium channels may have a hydrophobic binding site for *n*-alcohols and demonstrate the differences in the kinetic mechanisms of inhibition for *n*-alcohols and inhaled anesthetics.

Introduction

Alcohols are known to affect the function of many ion channels, particularly ligand-gated channels. For example, ethanol enhances GABA_A and glycine receptor function at a 20–100 mM concentration that induces mild to severe intoxication (Harris et al., 1997; Mihic et al., 1997), inhibits glutamate receptor function (Wong et al., 1998), and potentiates neuronal nicotinic acetylcholine (ACh) receptor function (Godden et al., 2001). However, it is not known if actions of alcohols on these channels are sufficient to explain the behavioral effects of the *n*-alcohols.

Voltage-gated sodium channels play an essential role in action potential initiation and propagation in excitable cells of nerve and muscle. Nine distinct pore-forming alpha subunits, which are associated with auxiliary beta subunits, have been identified (Catterall, 2000; Catterall et al., 2005). Each α subunit has a different pattern of development and localization and demonstrates subtle differences in electrophysiological characteristics (Goldin, 2001). Moreover, these subunits have distinct physiological and pathophysiological roles. For example, multiple mutations of Na_v1.1 can cause epilepsy (Escayg et al., 2001), and mutations of Na_v1.5 are associated with arrhythmia, Brugada syndrome (Cordeiro et al., 2006), and long-QT syndrome (Vatta et al., 2006). The sodium channels expressed in dorsal root ganglion

(DRG) (Na_v1.7, Na_v1.8, Na_v1.9) play an important role in pain (Wood et al., 2004; Cummins et al., 2007).

Recent studies have shown that volatile anesthetics inhibit sodium channel function at clinical relevant concentrations in some neurons (Ratnakumari et al., 1998; Ouyang et al., 2003), and in some recombinant mammalian sodium channels (Rehberg et al., 1996; Shiraishi and Harris, 2004; Ouyang and Hemmings, 2007), suggesting sodium channels as a potential target for inhaled anesthetics. *n*-Alcohols can produce a state of general anesthesia, historically ethanol has been used for this purpose (Dundee et al., 1969), and there is evidence that ethanol and other alcohols inhibit sodium channel function. For example, a high concentration of ethanol (500 mM) inhibited sodium influx in synaptosomes isolated from rodent brain (Harris and Bruno, 1985), and Wu and Kendig (1998) showed that tetrodotoxin (TTX)-resistant and TTX-sensitive sodium channels in rat DRG neurons are inhibited by ethanol (50–200 mM) (Wu and Kendig, 1998). Haydon and Urban (1983) found that longer-chain *n*-alcohols (pentanol to decanol) inhibited the sodium current of the squid giant axon (Haydon and Urban, 1983). Some ion channels are affected by short, but not long, chain length *n*-alcohols and this alcohol “cut-off” has proven useful in characterizing alcohol action on ion channels (Wick et al., 1998). To extend these studies to sodium channels, we explored the effects of a series of *n*-alcohols on sodium channels.

Na_v1.1, Na_v1.2, Na_v1.3, and Na_v1.7 are TTX-sensitive and closely related by phylogenetic analysis, and all of their genes are located on human chromosome 2q23–24. Na_v1.5, Na_v1.8, and Na_v1.9 are TTX-resistant and also closely related, and their genes are located on chromosome 3p21–24. Na_v1.4 and Na_v1.6 are phylogenetically distant from the other two clusters. We selected four of these subunits for study, as follows. We examined Na_v1.2, which is expressed primarily in the central nervous system; Na_v1.4, which is expressed primarily in skeletal muscle; Na_v1.6, which is expressed primarily in the central nervous system and DRG; and Na_v1.8, which is expressed in DRG.

Methods

Materials

Adult female *Xenopus laevis* frogs were obtained from Xenopus Express, Inc. (Plant City, FL). Methanol and ethanol were purchased from AAPER Alcohol (Shelbyville, KY), 1-propanol was obtained from Fisher Scientific (Pittsburgh, PA), and the other *n*-alcohols were purchased from Sigma-Aldrich (St. Louis, MO). We thank the following researchers for providing the subunit clones: cDNA for rat Na_v1.2 α subunit (gift from Dr. W. A. Catterall, University of Washington, Seattle, WA), cDNA for human Na_v1.4 α subunit (gift from Dr. A. L. George, Vanderbilt University, Nashville, TN), cDNA for rat Na_v1.6 α subunit (gift from Dr. A. L. Goldin, University of California, Irvine, CA), cDNA for rat Na_v1.8 α subunit (gift from Dr. A. N. Akopian, University of Texas Health Science Center, San Antonio, TX), and cDNA for human β_1 subunit (gift from Dr. A.L. George, Vanderbilt University).

Site-Directed Mutagenesis

Mutations were created using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Briefly, polymerase chain reaction was performed using the cDNA of wild type Na_v1.2 and Na_v1.4 α subunit clone and the mutagenesis primers designed with the desired mutation at the appropriate place. Then, *Escherichia coli*-competent cells (XL1-Blue or One-shot TOP10/P3) were transformed with the polymerase chain reaction product digested with *DpnI*.

cRNA Preparation and Oocyte Injection

After linearized cDNA with *Cla*I ($\text{Na}_v1.2$ α subunit), *Eco*RI ($\text{Na}_v1.4$ α subunit, β_1 subunit), and *Not*I ($\text{Na}_v1.6$ α subunit), *Xba*I ($\text{Na}_v1.8$ α subunit), cRNA were transcribed using T6 ($\text{Na}_v1.4$, 1.8 α , β_1 subunit) or T7 ($\text{Na}_v1.2$, 1.6 α subunit) RNA polymerase of mMESSAGE mMACHINE kit (Ambion, Austin, TX). Preparation of *Xenopus laevis* oocytes and microinjection of the cRNA was performed as described previously (Shiraishi and Harris, 2004). Briefly, stage IV–VI oocytes were manually isolated from a removed portion of ovary. The oocytes were then treated with collagenase (0.5 mg/ml) for 10 min and placed in modified Barth's solution (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO_3 , 10 mM HEPES, 0.82 mM MgSO_4 , 0.33 mM Ca $(\text{NO}_3)_2$, and 0.91 mM CaCl_2 , adjusted to pH 7.5), supplemented with 10,000 units of penicillin, 50 mg of gentamicin, 90 mg of theophylline, and 220 mg of sodium pyruvate per liter (incubation medium). Na_v α subunit cRNA were co-injected with β_m subunit cRNA at a ratio of 1:10 (total volume was 20 ~ 40 ng/50 nl) into *Xenopus* oocytes (all α subunits were co-injected with β_1 subunit). The injected oocytes were incubated at 19 °C in incubation medium, and 2–6 days after injection, they were used in electrophysiological recordings.

Electrophysiological recordings

All electrical recording was performed at room temperature (23 °C). The oocytes were placed in a rectangular chamber (approximately 100 μ l volume) and perfused at 2 ml/min with Frog Ringer solution containing NaCl 115 mM, KCl 2.5 mM, HEPES 10 mM, CaCl_2 1.8 mM, at pH 7.2 using a peristaltic pump (Cole-Palmer Instrument Co., Chicago, IL). Recording electrodes were prepared with borosilicate glass using a puller (P-97, Sutter Instruments Company, Novato, CA), and microelectrodes were filled with 3M KCl/0.5% low-melting-point agarose and had resistances between 0.3 and 0.5 M Ω . The whole-cell voltage clamp was achieved through these two electrodes using a Warner Instruments model OC-725C (Hamden, CT).

The amplitude of expressed sodium currents was typically 2–15 μ A, and currents were recorded and analyzed using pCLAMP 7.0 software (Axon Instruments, Foster City, CA). The transients and leak currents were subtracted using the P/N procedure. Capacitance and 60–80 % series resistance were compensated, and leak current was subtracted using P/4 protocols. For the poorly water-soluble alcohols (heptanol-dodecanol), stocks were prepared in dimethylsulphoxide (DMSO) and diluted and sonicated in Frog Ringer solution to a final DMSO concentration not exceeding 0.05%. *n*-Alcohols were then perfused for 3 min to reach equilibrium. The loss of concentration from vial to bath was approximately 50–70 % for long-chain alcohols from heptanol to dodecanol (Dildy-Mayfield et al., 1996).

The voltage dependence of activation was determined by 50-ms depolarizing pulses from a holding potential of –90 mV to 50 mV in 10-mV increments or from a holding potential causing half-maximal current ($V_{1/2}$) (approximately from –50 mV to –60 mV) to 50 mV in 10-mV increments. Activation curves were fitted to a Boltzmann equation: $G/G_{\max} = 1/(1 + \exp((V_{1/2} - V)/k))$, where G is the voltage-dependent sodium conductance, $G_{\max}$ is the maximal sodium conductance, $G/G_{\max}$ is the normalized fractional conductance, $V_{1/2}$ is the potential at which activation is half maximal, and k is the slope factor. The G value for each oocyte was calculated using the formula $G = I/(V_t - V_r)$, where I is the peak sodium current, V_t is the test potential, and V_r is the reversal potential. V_r for each oocyte was estimated by extrapolating the linear ascending segment of the current voltage relationship (I – V) curve to the voltage axis. To measure steady-state inactivation, currents were elicited by a 50-ms test pulse to –20 mV after 200-ms prepulses ranging from –140 mV to 0 mV in 10-mV increments from a holding potential of –90 mV. Steady-state inactivation curves were fitted to a Boltzmann equation: $I/I_{\max} = 1/(1 + \exp((V_{1/2} - V)/k))$, where $I_{\max}$ is the maximal sodium current, $I/I_{\max}$ is the normalized current, $V_{1/2}$ is the voltage of half-maximal inactivation, and k is the slope factor.

Data Analysis

All values are presented as the mean $\pm$ SEM. The n values refer to the number of oocytes studied. Each experiment was performed with oocytes from at least two different frogs. Statistical analyses were performed using a one-way analysis of variance (ANOVA) for multiple comparisons and a t test using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). We also calculated Hill slope and IC_{50} values using GraphPad Prism.

Results

Effects of ethanol and octanol on the peak Na^+ inward currents

The effects of ethanol and octanol on the peak Na^+ inward currents (I_{Na}) were examined at concentrations corresponding to the EC_{50} for producing loss of righting reflex in tadpoles (ethanol, 190 mM; octanol, 0.057 mM) (Alifimoff et al., 1989). Currents were elicited by a 50-ms depolarizing pulse to -20 mV applied every 10 s from -90 mV (V_{max}) or a holding potential of $V_{1/2}$. It was found that ethanol inhibited I_{Na} induced by $Na_v1.2$ at V_{max} , and the inhibitory effect of ethanol was more potent at $V_{1/2}$ (Fig. 1A, B). Octanol also inhibited I_{Na} induced by $Na_v1.2$ at both V_{max} and $V_{1/2}$, but more effectively at $V_{1/2}$ (Fig. 1C, D). Ethanol and octanol were also tested on the other α subunits: $Na_v1.4$, $Na_v1.6$, or $Na_v1.8$. Ethanol reduced the peak I_{Na} induced by $Na_v1.2$, $Na_v1.4$, $Na_v1.6$, and $Na_v1.8$ by $19 \pm 1\%$, $19 \pm 4\%$, $14 \pm 1\%$, and $28 \pm 1\%$ at V_{max} , respectively, and $29 \pm 1\%$, $35 \pm 2\%$, $25 \pm 1\%$, and $30 \pm 3\%$ at $V_{1/2}$, respectively (Fig. 1E). Octanol reduced the peak I_{Na} induced by $Na_v1.2$, $Na_v1.4$, $Na_v1.6$, and $Na_v1.8$ by $17 \pm 1\%$, $19 \pm 3\%$, $17 \pm 1\%$, and $13 \pm 1\%$ at V_{max} , respectively, and $36 \pm 2\%$, $46 \pm 2\%$, $38 \pm 4\%$, and $19 \pm 1\%$ at $V_{1/2}$, respectively (Fig. 1E). Thus, for $Na_v1.2$, $Na_v1.4$, and $Na_v1.6$, ethanol and octanol inhibited the peak I_{Na} more effectively at $V_{1/2}$ than at V_{max} . On the other hand, for $Na_v1.8$, ethanol similarly suppressed the peak I_{Na} at $V_{1/2}$ and V_{max} (Fig. 1E).

Effects of n -alcohols on $Na_v1.2$ currents

Increases in carbon chain length shifted the concentration-response curves to lower alcohol concentrations, but these shifts became very small as the chain length increased from octanol to nonanol to decanol, indicating a cut-off approximately at nonanol (Fig. 2). The IC_{50} values and slopes calculated from dose-response data are shown in Table 1. The IC_{50} value for dodecanol was estimated by extrapolation because the inhibitory effect at highest concentration of dodecanol was less than 50%.

Effects of ethanol and octanol on activation and inactivation of sodium Currents

The voltage dependence of activation was determined by 50-ms depolarizing pulses from a holding potential of -90 mV (V_{max}) to 50 mV in 10-mV increments or from a holding potential of $V_{1/2}$ to 50 mV in 10-mV increments for $Na_v1.2$, $Na_v1.4$, $Na_v1.6$, or $Na_v1.8$. The activation curves were derived from the I - V curves (see Methods), and the peak I_{Na} was reduced by ethanol (190 mM) and octanol (0.057 mM) at V_{max} and $V_{1/2}$ holding potentials with all subunits (Fig. 3). Ethanol and octanol shifted the midpoint of steady-state activation in a depolarizing direction by 2.7 and 3.3 mV, respectively, at V_{max} for $Na_v1.2$ (Fig. 4A, Table 2). These changes were small but significant statistically.

Ethanol and octanol also had a tendency to shift the $V_{1/2}$ of $Na_v1.6$ at V_{max} in the depolarizing direction (2.2 and 2.7 mV, respectively), but these shifts were not statistically significant (Fig. 4A, Table 2). The alcohols did not affect the activation kinetics of $Na_v1.4$ or $Na_v1.8$ at V_{max} . At $V_{1/2}$, ethanol and octanol showed significant shifts of the $V_{1/2}$ of $Na_v1.2$ (3.0 and 2.7 mV, respectively) (Fig. 4B, Table 2). Octanol shifted the $V_{1/2}$ of $Na_v1.6$ (3.2 mV), and ethanol produced a smaller, nonsignificant shift (Fig. 4B, Table 2). In oocytes expressing $Na_v1.4$ or

Na_v1.8, both alcohols shifted $V_{1/2}$ slightly, and, on the whole, both alcohols were more effective on activation at $V_{1/2}$ than at V_{max} , although even the effects at $V_{1/2}$ were small.

The effect of ethanol and octanol on the steady-state inactivation was also investigated. Ethanol weakly (and insignificantly) shifted the $V_{1/2}$ in the hyperpolarizing direction by 1.0, 2.2, 1.2, and 2.1 mV in Na_v1.2, Na_v1.4, Na_v1.6, and Na_v1.8, respectively (Fig. 5, Table 2). On the other hand, octanol shifted the $V_{1/2}$ in the hyperpolarizing direction significantly by 3.4, 3.6, and 3.5 mV in Na_v1.2, Na_v1.4, and Na_v1.6, respectively, although the shift with Na_v1.8 was not significant (1.7 mV) (Fig. 5, Table 2). On the whole, octanol was more effective than ethanol with the steady-state inactivation of Na_v1.2, Na_v1.4, or Na_v1.6, but the effect of octanol on Na_v1.8 was smaller than the effect of ethanol.

Effects of ethanol and octanol on mutated Na_v1.2 and Na_v1.4 channels

We found that the inhibitory effect of ethanol on Na_v1.8 was similar at V_{max} and at $V_{1/2}$, but was greater at $V_{1/2}$ than at V_{max} for Na_v1.2, Na_v1.4, and Na_v1.6 (Fig. 1E). To explore the molecular differences that might account for this distinction between Na_v1.8 and other α subunits, we introduced single-point mutants into Na_v1.2 and Na_v1.4 to see if these mutations would switch the alcohol sensitivity of these subunits. We selected three amino acids that were different in Na_v1.8 from Na_v1.2, Na_v1.4, and Na_v1.6 in domains I and II (Fig. 6), and we hypothesized that these amino acid differences could be functionally important because of the polarity differences of these amino acids between Na_v1.8 and other α subunits. We introduced the amino acids found in Na_v1.8 into Na_v1.2 and Na_v1.4 (M271K, M900K, and M965T in Na_v1.2, and M273K, M719K, and M784T in Na_v1.4). At $V_{1/2}$, the mutations in Na_v1.2 did not alter the inhibitory effect of ethanol, hexanol and octanol, but at V_{max} the M900K mutant increased the inhibitory effect of these alcohols significantly compared with Na_v1.2 wild type (Fig. 7). Ethanol inhibited Na_v1.2 wild type and M900K by $14 \pm 1\%$ and $20 \pm 2\%$, respectively; hexanol inhibited Na_v1.2 wild type and M900K by $7 \pm 1\%$ and $13 \pm 2\%$, respectively; and octanol inhibited Na_v1.2 wild type and M900K by $11 \pm 1\%$ and $16 \pm 2\%$, respectively. There were no differences in alcohol inhibition between Na_v1.4 wild type and its mutants at V_{max} or $V_{1/2}$, although at V_{max} the inhibitory effect of these three alcohols on M719K (equivalent to M900K of Na_v1.2) increased slightly (data not shown).

Discussion

Alcohols inhibit sodium currents

We found that anesthetic concentrations of both ethanol and octanol suppressed I_{Na} of four α subunits by 30–40% at $V_{1/2}$ and by 13–30% at V_{max} . This is consistent with previous studies of other anesthetics. For example, anesthetic concentrations (0.25–0.4 mM) of isoflurane inhibited I_{Na} in isolated rat neurohypophyseal nerve terminal by around 40% (Ouyang et al., 2003), suppressed sodium currents of Na_v1.2 in Chinese hamster ovary cells (Rehberg et al., 1996), and inhibited currents from Na_v1.2, Na_v1.4, and Na_v1.6 by about 30% in an oocyte expression system (Shiraishi and Harris, 2004).

Two questions that remain are (1) how much inhibition of sodium currents is required to alter neuronal function, and (2) can the observed alcohol effects contribute to anesthesia? Scholz and colleagues (Scholz et al., 1998a; Scholz and Vogel, 2000) demonstrated that firing frequency was reduced from 35 Hz to 21 Hz by 30 μ M lidocaine in DRG neurons. They also found that the number of action potentials decreased from 21 to 10 in 750 ms by 10% inhibition of sodium currents, suggesting that even 10% inhibition may reduce action potential propagation. These previous reports, together with our present results, support the idea that alcohol actions on sodium channels could reduce neuronal firing and potentially result in anesthesia.

***n*-Alcohol cut-off and hydrophobic binding sites**

Alifimoff et al. (1989) demonstrated a cut-off at about C13 for anesthesia using tadpoles, and this raised the possibility that *n*-alcohols produce anesthesia by acting on a protein cavity that can accommodate as large as C12, but not larger (Alifimoff et al., 1989). One of the first proteins systematically studied with the *n*-alcohols was firefly luciferase. Franks and Lieb (1984, 2004) showed a remarkable correlation between luciferase inhibition and anesthetic potency (Franks and Lieb, 1984, 2004) and suggested the existence of a binding pocket based on the *n*-alcohol cut-off of luciferase (Franks and Lieb, 1985). More recent studies have reported alcohol cut-offs for ligand-gated ion channels. For example, cut-offs of GABA ρ , NMDA, GABA $_A$, glycine, and neuronal nicotinic acetylcholine receptors are C7, C8, C10, C10, and C12, respectively (Wick et al., 1998; Peoples and Weight, 1995; Dildy-Mayfield et al., 1996; Zuo et al., 2001). The sodium channels show a cut-off at C9 in the present study, yet tadpoles are sensitive to C12 (and rats to at least C10, Dildy-Mayfield et al., 1996), raising the question of which of these channels is relevant, or clearly not relevant, for the anesthetic actions of long chain alcohols. We propose that the cut-offs for GABA ρ and NMDA are probably too short, but it is difficult to absolutely exclude GABA $_A$, glycine and sodium channels based on the cut-off.

Testing a series of *n*-alcohols also allows estimation of the free energy change that occurs upon transfer of the alcohol from water to its site of action. This is commonly calculated as the free energy change obtained by adding each CH $_2$ group, which gives a measure of the hydrophobicity of the site of action. For our sodium channel, we calculated a value of $\Delta\Delta G = -835 \text{ cal/mol/CH}_2 = -3.49 \text{ kJ/mol/CH}_2$. We compared $\Delta\Delta G$ from our study with results from other studies of *n*-alcohols (Figure 8B, Table 3). Na $_v$ 1.2 is similar to tadpole anesthesia in approach to cut-off and $\Delta\Delta G$ values, although tadpole anesthesia shows a gradual change at C10–C12, whereas the sodium channel shows a more abrupt cut-off. In contrast, one ligand-gated ion channel, the NMDA receptor, shows a cut-off at much shorter chain lengths (Peoples and Weight, 1995). Thus, *n*-alcohol inhibition of brain sodium channel function is close to anesthetic potency *in vivo*, suggesting the possibility of existence that binding sites for anesthetics on sodium channels (Fig 8A).

Effects of alcohol on activation and inactivation

The actions of ethanol and octanol on channel activation and inactivation demonstrated some common characteristics but also some differences among the four α subunits we studied. We found that suppression of sodium currents by alcohols was voltage insensitive in the hyperpolarizing potential range and that inhibition was seen at a holding potential that produces a maximal current. These results suggest that one mechanism of alcohol action is inhibition of open channels. Although ethanol and octanol shifted the $V_{1/2}$ of activation of Na $_v$ 1.2 in a depolarizing direction, the shift was small ($\sim 4 \text{ mV}$), and the $V_{1/2}$ of activations of the other subunits were not significantly changed, suggesting that alcohol effects on channel activation are not sufficient to account for inhibition of channel function. Octanol shifted the $V_{1/2}$ of inactivation of the Na $_v$ 1.2, Na $_v$ 1.4, and Na $_v$ 1.6 subunits in a hyperpolarizing direction ($\sim 4 \text{ mV}$), and ethanol produced a small shift, which was not statistically significant. Neither ethanol nor octanol altered inactivation of Na $_v$ 1.8.

From these analyses of activation and inactivation, the most consistent component of alcohol-induced suppression for the four different α subunits is suppression of open channels. For Na $_v$ 1.2, Na $_v$ 1.4, and Na $_v$ 1.6, increased inactivation also contributes to inhibition of sodium currents for octanol, but for Na $_v$ 1.8 there is only a small change in inactivation kinetics. Thus, the main component of ethanol-induced inhibition of Na $_v$ 1.8 is suppression of open channels. Wu and Kendig (1998) concluded that the open state of TTX-resistant sodium channels is more sensitive to ethanol than those of TTX-sensitive sodium channels of DRG neurons (Wu and

Kendig, 1998). In addition, they showed small effects of ethanol on activated state and inactivated state in TTX-sensitive and TTX-resistant sodium channels of DRG neurons (Table 4). The TTX-resistant current in DRG neurons is probably due to $\text{Na}_v1.8$, and our results with recombinant channels are in agreement with the results from DRG neurons.

Inhaled anesthetics also inhibit sodium channels, but the mechanism of inhibition may be different from those shown for alcohols. For example, suppression of sodium currents (HEK293 cells, $\text{Na}_v1.5$) by halothane and isoflurane involves acceleration of the transition from the open to the inactivated state and stabilization of inactivated states (Stadnicka et al., 1999). Ouyang et al. (2003) suggested that general anesthetics inhibit presynaptic voltage-gated Na^+ channels through enhanced inactivation (Ouyang et al., 2003). We also concluded that isoflurane stabilizes the inactivated state of several α subunits of sodium channels expressed in oocytes (Shiraishi and Harris, 2004). Compared with inhaled anesthetics, alcohols appear to be more effective as open-channel blockers, less effective at stabilizing the inactivated state, and capable of producing effects (albeit weak) on the activated state that are not found with anesthetics (Table 4).

Recently, Ouyang and Hemmings (2007) demonstrated different mechanisms of inhibition by isoflurane for several sodium channel α subunits expressed in Chinese hamster ovary cells (Ouyang and Hemmings, 2007). They showed that isoflurane is more effective on $\text{Na}_v1.2$ than on $\text{Na}_v1.4$ or $\text{Na}_v1.5$ for the resting and open states, and is more effective on $\text{Na}_v1.4$ or $\text{Na}_v1.5$ than on $\text{Na}_v1.2$ for the fast-inactivating state. That study, together with our results, indicates that α subunits differ in the mechanisms of channel inhibition by volatile anesthetics and alcohol.

Effects of alcohol on mutant $\text{Na}_v1.2$

A previous report suggested that $\text{Na}_v1.8$ was insensitive to isoflurane and concluded that one possible explanation for the insensitivity of $\text{Na}_v1.8$ to anesthetics lies in the differences in channel gating between $\text{Na}_v1.8$ and other channels (Shiraishi and Harris, 2004). In the present study, we found $\text{Na}_v1.8$ to be more sensitive than other subunits to ethanol inhibition of the open state. To explore possible molecular mechanisms, we made three single mutants based on amino acid differences between $\text{Na}_v1.8$ and other subunits. One $\text{Na}_v1.2$ mutant, M900K, showed increased inhibition by ethanol, hexanol, and octanol at V_{max} . These experiments suggest that lysine 806 of segment 5 in domain II of $\text{Na}_v1.8$ has a role in the high sensitivity of $\text{Na}_v1.8$ to open-channel blockage by alcohol.

Conclusions

Alcohols inhibited sodium currents induced by $\text{Na}_v1.2$, $\text{Na}_v1.4$, $\text{Na}_v1.6$, and $\text{Na}_v1.8$ at anesthetic concentrations and demonstrated a cut-off for long-chain *n*-alcohols. These results raise the possibility that alcohol and anesthetic inhibition of sodium channel function may be important for anesthesia, but further study is needed to clarify the relevance of sodium channels to anesthesia.

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Abbreviations

***n*-alcohols**

normal alcohols

Na_v

	voltage-gated Na ⁺ channel(s)
GABA_A	γ-amino butyric acid A
ACh	acetylcholine
NMDA	<i>N</i> -methyl-D-aspartate
TTX	tetrodotoxin
DRG	dorsal root ganglion
DMSO	dimethylsulphoxide
ANOVA	one-way analysis of variance
I_{Na}	the peak Na ⁺ inward currents
HEK	human embryonic kidney

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Figure 1.

Inhibitory effects of ethanol (C2) (190 mM) and octanol (C8) (0.057 mM) on sodium channels at different holding potentials. (A) Traces of sodium currents evoked by a 50-ms depolarizing pulse to -20 mV from a holding potential of -90 mV ($V_{\max}$) and to -20 mV from a holding potential which induced half maximal current ($V_{1/2}$), in the absence and presence of ethanol in an oocyte expressing Na_v1.2. (B) Time course of ethanol effects on sodium currents of Na_v1.2. Currents were elicited by 50-ms depolarizing pulses to -20 mV applied every 10 s from a $V_{1/2}$ holding potential. The current is normalized to the initial values. Filled circles represent control and washout, and open circles represent currents during ethanol treatment. Ethanol was applied for 3 min. (C) Traces of sodium currents evoked by a 50-ms depolarizing

pulse to -20 mV from $V_{\max}$ and $V_{1/2}$ in the absence and presence of octanol in an oocyte expressing $\text{Na}_v1.2$. (D) Time course of octanol effects on sodium currents of $\text{Na}_v1.2$. (E) Percent inhibition of sodium current by ethanol and octanol in oocytes expressing $\text{Na}_v1.2$, $\text{Na}_v1.4$, $\text{Na}_v1.6$, and $\text{Na}_v1.8$. Open columns indicate the effect at $V_{\max}$, and closed columns indicate the effect at $V_{1/2}$. $V_{1/2}$ value of $\text{Na}_v1.2$, $\text{Na}_v1.4$, $\text{Na}_v1.6$, and $\text{Na}_v1.8$ were 55.8 ± 0.5 , 57.5 ± 2.0 , 59.3 ± 0.4 and 43.9 ± 1.4 mV, respectively. Data are mean $\pm$ S.E.M. ($n = 4-6$). Differences between $V_{\max}$ and $V_{1/2}$ for each condition are indicated as *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ (one-way ANOVA).

Figure 2.

Effects of *n*-alcohols on sodium currents in oocytes expressing Na_v1.2. Concentration-response curves for *n*-alcohols (methanol to dodecanol) on sodium currents elicited by a 50-ms depolarizing pulse to -20 mV from $V_{1/2}$ holding potential. $V_{1/2}$ value of Na_v1.2 was 54.7 ± 0.3 mV. Data are represented as means $\pm$ S.E.M. ($n = 5-6$). The data were fit by a logistic equation to give IC_{50} s and Hill slopes shown in Table 1.

Figure 3.

Effects of ethanol (190 mM) and octanol (0.057 mM) on I-V curves of sodium currents in oocytes expressing $\text{Na}_v1.2$ and $\text{Na}_v1.8$. (A) Representative I_{Na} traces from oocytes expressing $\text{Na}_v1.2$ in the absence and presence of ethanol or octanol. Currents were elicited by 50-ms depolarizing steps between -80 and 50 mV in 10-mV increments from holding potentials of -90 mV. (B) Effects of ethanol and octanol on representative I-V curves elicited from V_{max} holding potential for $\text{Na}_v1.2$. (C) Effects of ethanol and octanol on representative I-V curves elicited from $V_{1/2}$ holding potential in oocytes expressing $\text{Na}_v1.2$. (D) Representative I_{Na} traces in oocytes expressing $\text{Na}_v1.8$ in the absence and presence of ethanol and octanol. (E) Effects of ethanol and octanol on representative I-V curves elicited from a V_{max} holding potential in

oocyte expressing $\text{Na}_v1.8$. (F) Effects of ethanol and octanol on representative I–V curves elicited from a $V_{1/2}$ holding potential in oocyte expressing $\text{Na}_v1.8$.

Figure 4.

Effects of ethanol (190 mM) and octanol (0.057 mM) on channel activation in oocytes expressing $\text{Na}_v1.2$, $\text{Na}_v1.4$, $\text{Na}_v1.6$, and $\text{Na}_v1.8$ from V_{max} holding potential (A panels) or $V_{1/2}$ holding potential (B panels). $V_{1/2}$ value of $\text{Na}_v1.2$, $\text{Na}_v1.4$, $\text{Na}_v1.6$, and $\text{Na}_v1.8$ were 54.0 ± 0.9 , 55.3 ± 1.2 , 58.7 ± 0.9 and 42.0 ± 1.2 mV, respectively. Data are shown as mean $\pm$ S.E.M. ($n = 4-6$). Activation curves fitted to a Boltzmann equation and $V_{1/2}$ are shown in Table 2.

Figure 5.

Effects of ethanol and octanol on inactivation curves in oocytes expressing $\text{Na}_v1.2$ (A), $\text{Na}_v1.4$ (B), $\text{Na}_v1.6$ (C), and $\text{Na}_v1.8$ (D). Currents were elicited by a 50-ms test pulse to -20 mV ($\text{Na}_v1.8$: 10 mV) after 200-ms prepulses ($\text{Na}_v1.8$: 500-ms) ranging from -140 to 0 mV in 10 -mV increments. Data shown as mean $\pm$ S.E.M. ($n = 4-6$). Inactivation curves were fitted to a Boltzmann equation and the $V_{1/2}$ values are shown in Table 2.

Domain I, Segment 5

Na _v 1.2	250	DVMILTVUCLSVFALIGLQLE	MGNL	274	→	M271K
Na _v 1.4	252	DVMILTVUCLSVFALVGLQLE	MGNL	276	→	M273K
Na _v 1.6	253	DVMILTVUCLSVFALIGLQLE	MGNL	277		
Na _v 1.8	248	DV T IL T TUCLSVFALVGLQLE	KGNL	272		

Domain II, Segment 5

Na _v 1.2	282	GNLTLVLAIIVFIFAVVGMOLF	903	→	M900K
Na _v 1.4	701	GNLTLVLAIIVFIFAVVGMOLF	722	→	M719K
Na _v 1.6	874	GNLTLVLAIIVFIFAVVGMOLF	895		
Na _v 1.8	788	GNL T IL T LAIIV F V F ALV G KOLL	809		

Domain II, Segment 6

Na _v 1.2	959	CLTVFM M VMVIGNLVVLNLFALLL	983	→	M965T
Na _v 1.4	778	CLTVF I MMVMVIGNLVVLNLFALLL	802	→	M784T
Na _v 1.6	951	CLIVFM M VMVIGNLVVLNLFALLL	965		
Na _v 1.8	866	CL I L F I T VMV L GNLVVLNLF I ALLL	880		

Figure 6.

Sequence alignment of Na_v1.2, Na_v1.4, Na_v1.6, and Na_v1.8 for segment 5 in domain I, segment 5 in domain II, and segment 6 in domain II. Three amino acids were selected in these areas because these amino acids are different in Na_v1.8 from those in Na_v1.2, Na_v1.4, and Na_v1.6 and are in trans-membrane regions close to the extracellular surface. The amino acids of Na_v1.8 were introduced into Na_v1.2 and Na_v1.4, giving the single mutants: M271K, M900K, and M965T (Na_v1.2 mutants), and M273K, M719K, and M784T (Na_v1.4 mutants).

Figure 7.

The inhibitory effect of ethanol (190 mM), hexanol (0.57 mM), and octanol (0.057 mM) on Na_v1.2 wild type and three mutants. Currents were elicited by a 50-ms depolarizing pulse to -20 mV applied every 10 s from V_{max} and V_{1/2} holding potential, and each alcohol was applied for 3 min. V_{1/2} value of Na_v1.2 WT, M271K, M900K, and M965T were 51.0 ± 1.0 , 43.8 ± 0.6 , 56.2 ± 1.1 and 48.6 ± 0.9 mV, respectively. Data are mean $\pm$ S.E.M. (n = 4–6). **, $p < 0.05$ by one-way ANOVA.

Figure 8.

Comparison of IC_{50} (EC_{50}) and ΔG values for *n*-alcohols between published studies and data from the present study. (A) Relationship between the logarithms of IC_{50} and carbon number. Filled circles, EC_{50} s for tadpole anesthesia (Alifimoff et al., 1989); open circles, IC_{50} s for $Na_v1.2$ (present study); open triangles, IC_{50} s for squid axon sodium channel (Haydon and Urban, 1983); crosses, IC_{50} s for NMDA receptors (Peoples and Weight, 1995). (B) Gibb's free energy change for partitioning from the water phase to the site of action. The free energy change contributed by each methylene group ($\Delta\Delta G$) was calculated from slope of Figure 8B and is given in Table 3.

Table 1Inhibitory Effect of *n*-Alcohols on Na_v1.2 Calculated From Concentration Response Curves Shown in Figure 2

<i>n</i> -Alcohol	Concentration range (mM)	IC ₅₀	Hill slope
Methanol	30–5000	1418 ± 203 mM	1.42 ± 0.04
Ethanol	10–3000	361 ± 6 mM	1.64 ± 0.07
Propanol	3–1000	78.8 ± 2.6 mM	1.60 ± 0.05
Butanol	1–300	19.5 ± 0.8 mM	1.66 ± 0.06
Pentanol	0.3–100	5.00 ± 0.13 mM	1.73 ± 0.06
Hexanol	0.03–30	1.15 ± 0.06 mM	1.62 ± 0.02
Heptanol	0.01–10	0.28 ± 0.01 mM	1.55 ± 0.04
Octanol	0.003–3	72.1 ± 4.5 pM	1.45 ± 0.04
Nonanol	0.001–1	44.2 ± 3.0 pM	1.51 ± 0.04
Decanol	0.0003–0.3	42.7 ± 11.6 pM	0.71 ± 0.05
Dodecanol	0.00001–0.001	44.6 ± 12.6 pM	0.61 ± 0.14

Table 2

Effect of Ethanol (C2, 190 mM) and Octanol (C8, 0.057 mM) on Activation and Inactivation of Four Different Sodium Channels (See Figures 4 and 5 for Details)

Activation	Holding -90 mV			Holding $V_{1/2}$		
	Control	C2	C8	Control	C2	C8
	$V_{1/2}(\text{mV})$					
Na _v 1.2	-32.2 ± 0.4	-29.5 ± 1.0 [*]	-28.9 ± 0.8 ^{**}	-25.2 ± 0.4	-22.2 ± 0.4 ^{**}	-22.5 ± 0.4 ^{**}
Na _v 1.4	-42.6 ± 0.4	-41.8 ± 0.9	-42.5 ± 0.1	-32.7 ± 1.1	-29.5 ± 3.0	-27.8 ± 2.3
Na _v 1.6	-33.0 ± 0.7	-30.8 ± 1.1	-30.3 ± 1.6	-26.8 ± 0.5	-24.6 ± 0.9	-23.6 ± 0.7 ^{**}
Na _v 1.8	4.2 ± 0.4	6.0 ± 0.8	5.1 ± 0.8	5.0 ± 0.6	7.8 ± 0.5	6.2 ± 1.4
Inactivation	Control	C2	C8			
	$V_{1/2}(\text{mV})$					
Na _v 1.2	-55.3 ± 0.6	-56.3 ± 0.8	-58.7 ± 0.9 ^{**}			
Na _v 1.4	-55.7 ± 0.3	-57.9 ± 0.7	-59.3 ± 1.0 ^{**}			
Na _v 1.6	-59.6 ± 0.6	-60.8 ± 1.0	-63.1 ± 0.5 [*]			
Na _v 1.8	-53.6 ± 1.4	-55.7 ± 2.5	-55.3 ± 2.6			

* $p < 0.05$;

** $p < 0.01$, compared with control by one-way analysis of variance (mean ± S.E.M.; $n = 4-6$).

Table 3

Comparison of Gibb's Free Energy Change ($\Delta\Delta G$) for Transfer of Each Methylene Group of a Series of *n*-Alcohols From Water to the Site of Action for the Present Study With Published Results. Values for the Present Study Were Calculated From Figure 8B

	$\Delta\Delta G$		Reference
	cal/mol/CH ₂	kJ/mol/CH ₂	
Tadpole	-800	-3.35	Alifimoff et al. (1989)
Na _v 1.2	-835	-3.49	Present study
Squid axon	-726	-3.04	Haydon and Urban (1983)
NMDA receptor	-478	-2.00	Peoples and Weight (1995)

Table 4

Comparison of Changes in Activation and Inactivation (as the shift in $V_{1/2}$) Produced by Ethanol (C2), Octanol (C8) And Isoflurane for Results From the Present Study and Previous Publications *

	Activation		
	C2	C8	Isoflurane
	mV		
Na _v 1.2	+2.7 (190 mM)	+3.3 (0.057 mM)	+0.3 (0.6 mM)
Na _v 1.8	+1.8 (190 mM)	+0.9 (0.057 mM)	−0.7 (0.6 mM)
DRG (TTX-S)	+0.23 (200 mM)		
DRG (TTX-R)	+0.35 (200 mM)		
	Inactivation		
	C2	C8	Isoflurane
	mV		
Na _v 1.2	−1.0 (190 mM)	−3.4 (0.057 mM)	−8.9 (0.6 mM)
Na _v 1.8	−2.1 (190 mM)	−1.7 (0.057 mM)	−0.8 (0.6 mM)
DRG (TTX-S)	−2.3 (200 mM)		
DRG (TTX-R)	−2.1 (200 mM)		

* Effects of isoflurane on Na_v1.2 and Na_v1.8 are from Shiraishi and Harris (2004), and the effects of ethanol on TTX-S and TTX-R (DRG) are from Wu and Kendig (1998). Values in parentheses represent the concentrations of alcohols or isoflurane used in the studies. Holding potentials for activation curves were −90 mV in Na_v1.2 and Na_v1.8, and −80 mV in DRG neurons.