Inhibition of Human $\alpha_4\beta_2$ Neuronal Nicotinic Acetylcholine Receptors by Volatile Aromatic Anesthetics Depends on Drug Hydrophobicity

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BACKGROUND: Volatile aromatic compounds such as benzene are general anesthetics that cause amnesia, hypnosis, and immobility in response to noxious stimuli when inhaled. Although these compounds are not used clinically, they are frequently found in commercial items such as solvents and household cleaning products and are abused as inhalant drugs. Volatile aromatic anesthetics are useful pharmacological tools for probing the relationship between chemical structure and drug activity at putative general anesthetic targets. Neuronal nicotinic acetylcholine (nACh) receptors are ligand-gated ion channels widely expressed in the brain, which are thought to play important roles in learning and memory. In this study, we tested the hypothesis that aromatic anesthetics reversibly inhibit $\alpha_4\beta_2$ neuronal nACh receptor function and sought to determine the structural correlates of receptor inhibition.

METHODS: Electrophysiological techniques were used to quantify the effects of 8 volatile aromatic anesthetics on currents elicited by 1 mM ACh and mediated by human $\alpha_4\beta_2$ nACh receptors expressed in *Xenopus* oocytes.

RESULTS: All of the volatile aromatic anesthetics used in this study reversibly inhibited $\alpha_4\beta_2$ nACh receptors with IC_{50} values ranging from 0.00091 atm for 1,2-difluorobenzene to 0.045 atm for hexafluorobenzene. With the exception of hexafluorobenzene, all of the compounds had IC_{50} values less than minimum alveolar concentration. Inhibitory potency correlated poorly with the cation-π binding energies of the compounds ($r^2 = 0.48$, $P = 0.059$). However, there was a good correlation between inhibitory potency and the octanol/gas partition coefficient ($r^2 = 0.87$, $P = 0.0008$).

CONCLUSIONS: Volatile aromatic anesthetics potently and reversibly inhibit human $\alpha_4\beta_2$ neuronal nACh receptors. This inhibition may play a role in producing amnesia. In contrast to *N*-methyl-D-aspartate receptors, the inhibitory potencies of aromatic anesthetics for $\alpha_4\beta_2$ neuronal nACh receptors seem to be dependent on drug hydrophobicity rather than electrostatic properties. This implies that the volatile aromatic anesthetic binding site in the $\alpha_4\beta_2$ neuronal nACh receptor is hydrophobic in character and differs from the nature of the binding site in *N*-methyl-D-aspartate receptors.

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Volatile aromatic compounds such as benzene are general anesthetics that produce amnesia, hypnosis, and immobility in response to noxious stimuli when inhaled.[1] Although these compounds are not used clinically, they are frequently found in commercial items such as solvents and household cleaning products and are abused as inhalant drugs. They are also used as pharmacological tools for assessing the importance of structural properties in defining anesthetic potency on relevant molecular targets. In a previous study, Raines et al.[2] demonstrated that the inhibitory potencies of volatile aromatic anesthetics for human NR1/NR2B *N*-methyl-D-aspartate (NMDA) receptors correlate strongly with the abilities of the drugs to form cation-π binding interactions. Anesthetics with high π-electron density (such as benzene) were nearly 100-fold more potent than highly fluorinated compounds with low π-electron density (such as hexafluorobenzene), suggesting that aromatic anesthetics

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bind to an NMDA receptor site that contains a positive charge, leading to subsequent inhibition of receptor function. Recent studies have shown that volatile aromatic anesthetics also produce γ-aminobutyric acid type A ($GABA_A$) receptor enhancement[3] and voltage-gated sodium channel inhibition,[4] which may also contribute to the behavioral effects of these drugs.

Neuronal nicotinic acetylcholine (nACh) receptors are cation-selective ligand-gated ion channels that are widely expressed in the brain. Postsynaptic neuronal nACh receptors mediate excitatory synaptic transmission, whereas presynaptic receptors modulate the release of several neurotransmitters including GABA, dopamine, and glutamate.[5–7] Neuronal nACh receptors are thought to mediate a variety of important brain functions such as learning and memory.[8–10] One of the most common neuronal nACh receptor subtypes found in the brain is a heteromer composed of α_4 and β_2 subunits,[11] and $\alpha_4\beta_2$ nACh receptors have been shown to be highly sensitive to a wide range of inhaled and IV general anesthetics.[12–17] Some inhalation anesthetics such as cyclopropane and butane, which do not appreciably enhance the function of $GABA_A$ receptors at clinically relevant concentrations, are potent inhibitors of $\alpha_4\beta_2$ nACh receptors.[18] Although nACh receptors do not mediate anesthetic-induced immobility,[19,20] nonimmobilizing halogenated alkanes such as F6 have been shown to inhibit $\alpha_4\beta_2$ nACh receptors at concentrations that cause memory impairment in rats,[18,21] suggesting that neuronal nACh receptors may play a role in mediating anesthetic-induced amnesia.

Because neuronal nACh receptors are inhibited by many inhaled and IV general anesthetic drugs, we hypothesized that they are also reversibly inhibited by volatile aromatic anesthetics. We used electrophysiological techniques to assess the inhibitory potencies of 8 aromatic compounds for human $\alpha_4\beta_2$ nACh receptors and used a similar approach from the previous study with NMDA receptors[2] to establish the structural correlates of $\alpha_4\beta_2$ nACh receptor inhibition.

METHODS

This study was approved by the Massachusetts General Hospital Subcommittee on Research Animal Care. Ovary lobes were harvested from adult female *Xenopus laevis* frogs (Xenopus One, Ann Arbor, MI) through a small laparotomy incision after frogs were anesthetized with 0.2% tricaine (ethyl-*m*-aminobenzoate) and hypothermia. Lobes were placed in OR-2 solution (82 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, adjusted to pH 7.4 using NaOH) containing 1 mg/mL collagenase D for 3 h to remove connective tissue.

Messenger RNA encoding the human α_4 and β_2 neuronal nACh receptor subunits were synthesized from linearized cDNA templates generously provided by Jon Lindstrom, PhD (University of Pennsylvania, Philadelphia, PA), using the mMessage mMachine SP6 kit (Applied Biosystems/Ambion, Austin, TX). Stage 4 and 5 oocytes were selected and injected with approximately 5 ng of a 1:1 mixture of α_4 and β_2 subunit mRNA. Injected oocytes were kept at 18°C in an incubating buffer (96 mM NaCl, 2 mM KCl, 10 mM HEPES, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 U/mL penicillin, and 5 μg/mL streptomycin, adjusted to pH 7.4 using NaOH) for at least 18 h before electrophysiological studies.

All electrophysiological recordings were performed at room temperature (22°C–24°C) using the 2-electrode voltage-clamp technique. Capillary glass electrodes filled with 3 M KCl and possessing open tip resistances <5 MΩ were used to clamp oocytes at a membrane potential of −50 mV using a GeneClamp 500B amplifier (Molecular Devices, Sunnyvale, CA). Oocytes were continuously perfused in a 0.04-mL recording chamber with ND-96 buffer (96 mM NaCl, 2 mM KCl, 10 mM HEPES, 1.0 mM $CaCl_2$, and 0.8 mM $MgCl_2$, adjusted to pH 7.4 using NaOH) at a rate of 4–6 mL/min. Perfusion was controlled with a 6-channel valve controller (Warner Instruments, Hamden, CT) interfaced with a Digidata 1322A data acquisition system (Molecular Devices). The perfusion apparatus was constructed using gas-tight glass syringes and Teflon tubing to minimize absorptive and evaporative loss of anesthetic compounds. Using gas chromatography, we have found that volatile anesthetic loss using this technique is <15%. Current responses were recorded using Clampex v9.0 software (Molecular Devices) and filtered using a Bessel (8-pole) low-pass filter with a −3 dB cutoff at 1.56 Hz using Clampfit v9.0 software (Molecular Devices) before analysis.

Volatile aromatic anesthetic stock solutions were prepared by weighing the amount of drug necessary to make 250 mL of a concentrated anesthetic solution in a glass bottle. After quickly adding 250 mL of ND-96 buffer, the bottle was sealed with a polytetrafluoroethylene-coated cap, and the mixture was stirred overnight with a polytetrafluoroethylene-coated stir bar. The amount of air in the bottle was kept at a minimum (<5% of the total volume). The resulting stock solution was diluted with ND-96 buffer (with or without acetylcholine) in gas-tight glass syringes to obtain the final desired anesthetic concentrations. The anesthetizing concentrations of volatile aromatic anesthetics were defined as the concentrations corresponding to minimum alveolar concentration (MAC) in rats[1] because human MAC data are unavailable for these drugs. MAC (in atm) was converted to aqueous concentrations using the equation:

$$C_{aq} = 44.614(\alpha)(P)$$

where C_{aq} is the aqueous concentration of anesthetic (in mM), α is the aqueous/gas partition coefficient at

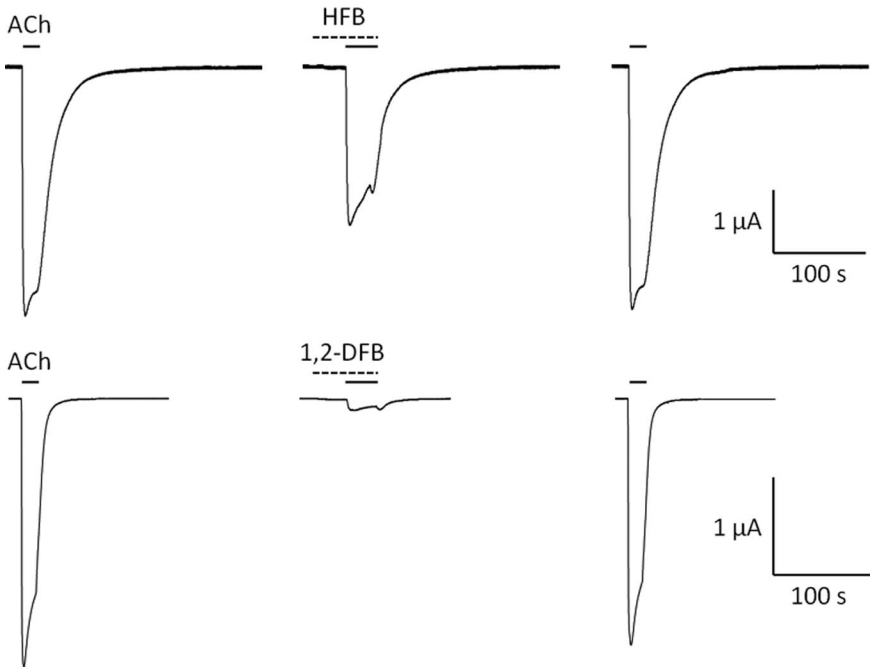

Figure 1. Representative electrophysiological traces demonstrating reversible inhibition of acetylcholine (ACh)-mediated currents by volatile aromatic anesthetics. The top and bottom traces were each obtained from a single oocyte expressing human $\alpha_4\beta_2$ nicotinic acetylcholine (nACh) receptors. The solid lines above the traces represent application of 1 mM ACh, and the dashed lines represent application of anesthetic. The concentrations of hexafluorobenzene (HFB) and 1,2-difluorobenzene (1,2-DFB) used in these experiments were 0.0048 atm (3 minimum alveolar concentration [MAC]) and 0.0064 atm (1 MAC), respectively. For each experiment, the second control trace is shown to demonstrate the reversibility of anesthetic-induced current inhibition.

37°C, and P is the gaseous partial pressure of anesthetic in atmospheres.[22] All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

For each experiment, an initial control current was elicited by perfusing the oocyte with 1 mM ACh for 15 s. After a recovery period in buffer for 5 min, the test anesthetic was preapplied to the oocyte for 30 s, followed immediately by a 30-s application of anesthetic with 1 mM ACh. After another recovery period of 5 min, a second control current was elicited by perfusing the oocyte with 1 mM ACh for 15 s to ensure reversibility of any drug-induced change in current response. Anesthetic-induced inhibition of peak current amplitude was quantified using the average of the 2 control experiments before and after anesthetic exposure.

Anesthetic concentration-response relationships were obtained by plotting the percent of control current in the presence of anesthetic against anesthetic concentration. Concentration-response data were fitted to a Hill equation in the form:

$$I_{peak} = [\text{anesthetic}]^n/(IC_{50}{}^n + [\text{anesthetic}]^n)$$

where I_{peak} is the normalized peak current amplitude evoked by 1 mM ACh in the presence of anesthetic, IC_{50} is the concentration of anesthetic that inhibits the peak current amplitude to one-half of control, and n is the Hill coefficient. For curve fitting, the maximal and minimal y axis values were constrained to 100% and 0%, respectively. All curve fitting, linear regression, and statistical analyses were performed using Prism v4.03 (GraphPad, San Diego, CA). Multiple regression analysis was performed using Instat v3.10 (GraphPad). Cation-π binding energy, octanol/gas partition coefficient, and MAC (in rats) for each of the 8 compounds used in this study have been determined previously,[1,2] and these published values were used for data analysis.

RESULTS

All 8 aromatic anesthetics used in this study reversibly inhibited currents induced by 1 mM ACh in a concentration-dependent manner. Figure 1 shows representative electrophysiological traces obtained when ACh was applied in the absence and presence of 0.0048 atm hexafluorobenzene (3 MAC) and 0.0064 atm 1,2-difluorobenzene (1 MAC). The second control traces from both experiments are shown to demonstrate the reversibility of anesthetic-induced current inhibition. In this set of experiments, hexafluorobenzene and 1,2-difluorobenzene produced 35.8% and 95.7% inhibition, respectively.

A plot of normalized peak current amplitude versus anesthetic concentration is shown in Figure 2 for hexafluorobenzene (the least potent compound) and 1,2-difluorobenzene (the most potent), demonstrating a 50-fold difference in potency between these 2 drugs. IC_{50} (in atm) and IC_{50}/MAC for all 8 compounds are listed in Table 1. All of the compounds had similar Hill coefficients ranging from −1.0 to −1.3. With the exception of hexafluorobenzene, all of the compounds produced half-maximal inhibition at concentrations below MAC.

Figure 3 plots inhibitory potency against cation-π binding energy for the 8 anesthetics. Linear regression analysis yielded a correlation coefficient (r^2) of 0.48 and a slope that did not significantly deviate from 0 ($P = 0.06$). Figure 4 plots inhibitory potency against log (octanol/gas partition coefficient). Linear regression analysis yielded $r^2 = 0.87$, with a slope that deviated significantly from 0 ($P = 0.0008$). Multiple regression analysis yielded $r^2 = 0.87$, with log

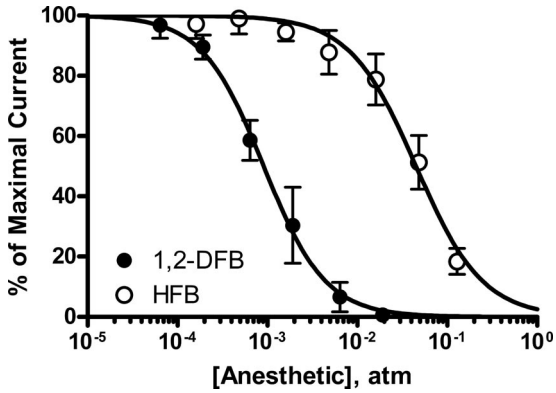

Figure 2. Anesthetic concentration-response relationships for 1,2-difluorobenzene (1,2-DFB, closed circles) and hexafluorobenzene (HFB, open circles). Each data point represents the average of at least 3 separate experiments performed in different oocytes, and the error bars represent SEM.

Table 1. IC_{50}, MAC, and IC_{50}/MAC for Each of the 8 Volatile Aromatic Anesthetics Used in This Study

Anesthetic	IC_{50} (atm)	MAC (atm)	IC_{50}/ MAC
Benzene	0.0024	0.0101	0.24
Fluorobenzene	0.0070	0.0112	0.63
1,2-Difluorobenzene	0.00091	0.0061	0.15
1,4-Difluorobenzene	0.0020	0.0064	0.31
1,2,4-Trifluorobenzene	0.0022	0.0097	0.23
1,3,5-Trifluorobenzene	0.0090	0.0222	0.41
Pentafluorobenzene	0.011	0.0125	0.88
Hexafluorobenzene	0.045	0.0161	2.8

Values for MAC (in rats) were established by Fang et al.[1]

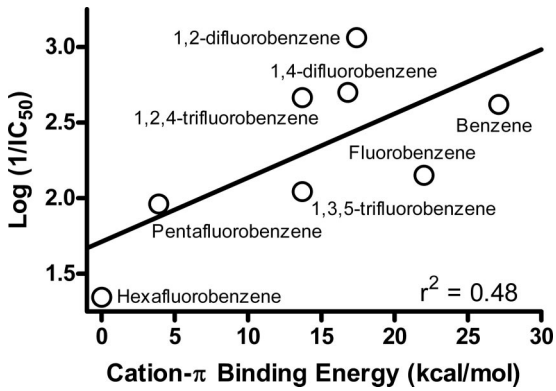

Figure 3. Correlation between the $\alpha_4\beta_2$ nicotinic acetylcholine (nACh) receptor inhibitory potencies of aromatic anesthetics and their abilities to engage in cation-π binding interactions. The line was derived from linear least-squares analysis. The correlation coefficient (r^2) was 0.48, and the slope of the line did not deviate significantly from 0 (P = 0.06).

(octanol/gas partition coefficient) making a significant contribution to the correlation (P = 0.01), whereas cation-π binding energy did not (P = 0.80).

For the conventional inhalation anesthetics isoflurane and halothane, published values for octanol/gas and aqueous/gas partition coefficients[23,24] and IC_{50} for $\alpha_4\beta_2$ nACh receptors expressed in *Xenopus* oocytes[12] were used to plot log (1/IC_{50}) against log

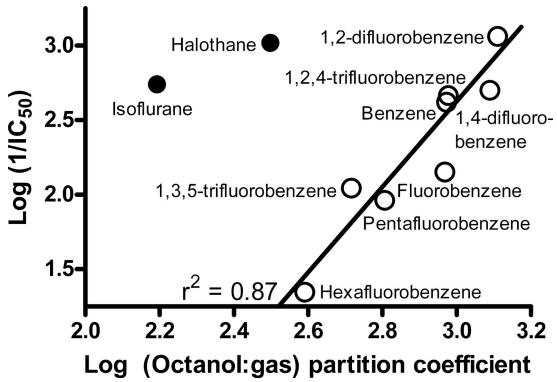

Figure 4. Correlation between the $\alpha_4\beta_2$ nicotinic acetylcholine (nACh) receptor inhibitory potencies of aromatic anesthetics (open circles) and their octanol/gas partition coefficients. The line was derived from linear least-squares analysis. The correlation coefficient (r^2) was 0.87, with a slope that deviated significantly from 0 (P = 0.0008). Published values for partition coefficients[23,24] and IC_{50}[12] were used to plot the conventional anesthetics isoflurane and halothane (closed circles) on the same graph.

(octanol/gas partition coefficient). As Figure 4 demonstrates, the correlation observed for aromatic anesthetics did not hold for isoflurane and halothane.

DISCUSSION

Although they are not used clinically, volatile aromatic anesthetics are useful pharmacological tools for assessing how chemical properties define anesthetic potency for relevant molecular targets. In addition, although conventional inhalation anesthetics such as isoflurane and halothane produce immobility in the spinal cord,[25–27] 1,2-difluorobenzene is capable of producing immobility in the brain,[28] suggesting that some volatile aromatic compounds may produce general anesthesia by mechanisms that differ from those of conventional inhalation anesthetics. Therefore, understanding the mechanisms underlying aromatic anesthetic actions may prove valuable in the design of novel anesthetics.

Previous work has demonstrated that volatile aromatic anesthetics inhibit NMDA receptors[2] and voltage-gated sodium channels,[4] and enhance the function of $GABA_A$ receptors.[3] In this study, we tested the hypothesis that volatile aromatic anesthetics reversibly inhibit human $\alpha_4\beta_2$ nACh receptors, and found this to be true for all of the compounds tested. Although nACh receptors do not mediate anesthetic-induced immobility,[19,20] potent inhibition of $\alpha_4\beta_2$ nACh receptors may mediate certain behavioral effects of both conventional and aromatic volatile anesthetics, such as amnesia.[18,21]

We also tested the hypothesis that the inhibitory potencies of volatile aromatic anesthetics for $\alpha_4\beta_2$ nACh receptors depend on the ability of the drug to engage in cation-π interactions, by studying 8 aromatic agents whose electrostatic properties vary dramatically. Benzene has a dense π-electron cloud that can interact strongly with positive charges at the

binding site. Substitution of fluorine atoms onto the ring reduces the density of the π-electron cloud and the strength of the interaction. We did not find a strong correlation between the $\alpha_4\beta_2$ nACh receptor inhibitory potencies of aromatic anesthetics and their abilities to engage in cation-π interactions. Therefore, we conclude that such interactions do not contribute significantly to aromatic anesthetic action on the $\alpha_4\beta_2$ nACh receptor.

However, we found a strong correlation ($r^2 = 0.87$) between inhibitory potency and octanol/gas partition coefficient that was not improved by accounting for cation-π binding energy using a multiple regression analysis. Although our results do not prove that all volatile aromatic anesthetics bind to the same site on $\alpha_4\beta_2$ nACh receptors, they suggest that hydrophobic interactions are important determinants of volatile aromatic anesthetic inhibition of $\alpha_4\beta_2$ nACh receptors. Such interactions are also important for the actions of other volatile agents, because structurally diverse volatile clinical anesthetics and nonimmobilizers inhibit $\alpha_4\beta_2$ nACh receptors with potencies that correlate with their hydrophobicities.[18] However, the same correlation between potency and hydrophobicity for volatile aromatic anesthetics was not observed for isoflurane and halothane, suggesting that conventional inhalation anesthetics and volatile aromatic anesthetics may bind to different inhibitory sites in $\alpha_4\beta_2$ nACh receptors.

Although we found no evidence to support a role for cation-π interactions in defining aromatic anesthetic inhibitory potency on $\alpha_4\beta_2$ nACh receptors, such interactions are thought to define inhibitory potency on NMDA receptors.[2] Cation-π interactions presumably enhance NMDA receptor inhibitory potency by stabilizing aromatic anesthetic binding. However, we previously found that highly fluorinated aromatic anesthetics such as pentafluorobenzene and hexafluorobenzene (which are weak NMDA receptor inhibitors at MAC[29]) potently enhance $GABA_A$ receptor function at the same concentrations, whereas the opposite is true for aromatic anesthetics with dense π-electron clouds (such as benzene and fluorobenzene).[3] In this study, we found that anesthetic hydrophobicity is a much better predictor of $\alpha_4\beta_2$ nACh receptor inhibition than the abilities of the drugs to participate in cation-π interactions. Taken together, our previous and current results suggest that the molecular determinants of volatile aromatic anesthetic potency for NMDA receptor inhibition, $GABA_A$ receptor enhancement, and neuronal nACh receptor inhibition are distinct. These data may be valuable in the future design of novel, target-specific general anesthetic drugs.

In summary, electrophysiological techniques were used in this study to demonstrate that volatile aromatic anesthetics potently and reversibly inhibit the function of human $\alpha_4\beta_2$ nACh receptors. For all but 1 of the compounds (hexafluorobenzene), IC_{50} was a fraction of MAC, supporting the role of the $\alpha_4\beta_2$ nACh receptor in mediating anesthetic-induced amnesia for those drugs. We found a poor correlation between the inhibitory potencies and the cation-π binding energies of the compounds, suggesting that the molecular determinants of inhibition for NMDA receptors and $\alpha_4\beta_2$ nACh receptors are distinct. The strong correlation between anesthetic potency and hydrophobicity suggests that these compounds bind to a hydrophobic domain in the $\alpha_4\beta_2$ nACh receptor to produce inhibition, and that the electrostatic properties of these drugs do not play a significant role in determining receptor binding and subsequent inhibition.

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