α4β2 Neuronal Nicotinic Acetylcholine Receptors in the Central Nervous System Are Inhibited by Isoflurane and Propofol, but α7-type Nicotinic Acetylcholine Receptors Are Unaffected

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Background: The mechanisms of action of general anesthetics are not completely understood. Many general anesthetics are reported to potentiate gamma-aminobutyric acid (GABA) and glycine receptors in the central nervous system (CNS) and to inhibit the muscle-type nicotinic acetylcholine receptor (nAChR). The effects of general anesthetics on another family of ligand-gated ion channel in the CNS, the nAChRs, have not been defined.

Methods: Two types of CNS acetylcholine receptor, the α4β2 receptor or the α7 homomeric receptor, were expressed heterologously in Xenopus laevis oocytes. Using the standard two-microelectrode voltage-clamp technique, peak acetylcholine-gated current was measured before and after coapplication of isoflurane or propofol.

Results: Coapplication of either isoflurane or propofol with acetylcholine resulted in potent, dose-dependent inhibition of the α4β2 receptor current with median inhibitory concentrations of 85 and 19 μM, respectively. The inhibition of the α4β2 receptor by both isoflurane and propofol appears to be competitive with respect to acetylcholine. The α7 receptor current was not affected by either anesthetic.

Conclusions: The CNS-type nAChRs are differentially affected by isoflurane and propofol. The α4β2 receptor is affected by isoflurane more potently than the most sensitive GABA or glycine receptor that has been reported, whereas the α7 homomeric receptor is not affected by either anesthetic. Inhibition of specific subtypes of nAChRs in the CNS, along with potentiation of GABA and glycine receptors, may contribute to the effects and side effects of general anesthetics. (Key words: Anesthetic mechanism, general anesthetics, nicotinic acetylcholine receptors, isoflurane, propofol.)

It is accepted that general anesthetics of many types act specifically at ligand-gated ion channels such as the gamma-aminobutyric acid (GABA) and glycine receptors in the central nervous system (CNS). The diversity of effects and side effects of general anesthetics may be explained by their differential actions on these ligand-gated ion channels.

The neuronal nicotinic acetylcholine receptors (nAChRs) are homologous to the GABA, glycine, and muscle-type nAChRs. The nAChRs found in neurons, unlike those expressed by muscle, are composed of only two subunit types (α-type and β-type), each of which is encoded by a member of a diverse family of homologous genes. Although some neuronal α-type subunits are as much as 40% homologous with the muscle α sequence, sequences related to the muscle β, γ, δ, or ε type subunits have not been identified in neurons. Neuronal nAChRs are thought to be pentameric oligomers composed of both α- and β-type subunits, which form the channel/receptor complex. Although the nAChRs form a potentially large family, as the genes for α2–9 and β2–4 have been cloned, only a restricted number of the possible combinations seem to be able to form functional receptors for acetylcholine. Homomeric complexes of α7, 8, and 9 are expressed as functional receptor complexes; all other subunits must be expressed as α–β pairs to form functional receptors. The nAChR complexes expressed in various CNS regions differ from the muscle nAChR in composition.
agonist and antagonist pharmacology, and biophysical profile. The predominant nAChR subtype in the CNS includes α1- and β2-type subunits and has been implicated in both pre- and postsynaptic functions. The α7-type nAChRs, although more restricted in distribution, have been shown to influence both pre- and postsynaptic excitability.

Recent behavioral, molecular biological, and physiologic evidence points to the nAChRs as potential mediators of general anesthetic actions. The neuronal nAChRs are expressed throughout the CNS and in peripheral autonomic ganglia. Several nAChR subtypes are relatively restricted in their expression to limbic areas involved with memory and behavior. Neuronal nAChRs participate in synaptic transmission, both as postsynaptic mediators of fast synaptic responses and at presynaptic sites, where nAChR activation has been shown to modulate the release of norepinephrine, dopamine, serotonin, gamma-aminobutyric acid, acetylcholine, and, most recently, glutamate. Behavioral effects of nicotine and its antagonists suggest that the nAChRs may be targets of general anesthetic action. For example, nicotine administration can improve performance on short-term memory tasks, whereas the nicotinic antagonists (such as mecamylamine) worsen short-term memory performance.

Nicotinic receptor dysfunction has been implicated in Alzheimer's disease: Certain nAChR subtypes are reduced in the brains of patients with Alzheimer's dementia, and, as such, nicotine is currently in clinical trial for treatment of these dementias. Neurologists have noted an acute worsening of dementia in Alzheimer's patients, who have changes in both muscarinic and nicotinic transmission, after general anesthesia. This finding may be due to an action of the general anesthetic on neuronal nicotinic receptors involved in cognition, in addition to muscarinic effects. Neuronal nicotinic receptors may be involved in aspects of the analgesia provided by some general anesthetics, such as isoflurane. The specific nicotinic agonist epibatidine is a potent analgesic, 200 times more potent than morphine, but without action at opioid receptor sites. Although general anesthetics have been found to be inhibitory in bovine chromaffin cells, the actions of general anesthetics on nAChRs expressed in the CNS have not been reported.

General anesthesia is a complex state with different qualities depending on the drugs that are used. Because the drugs used to induce general anesthesia have been shown to act specifically and potently at several members of the ligand-gated ion channel family, the homologous CNS nAChRs are likely targets of general anesthetics in vivo. The goal of these studies was to determine whether isoflurane, an inhalational anesthetic, and propofol, an intravenous agent, alter the activity of the nAChRs expressed in the CNS.

Materials and Methods

The nAChR subunit cRNAs were prepared from chicken cDNAs (kindly provided by M. Ballivet, U. of Geneva) in a PGH19 vector using standard techniques. The vector was linearized and used as a template for run-off transcription from the T7 promoter. Xenopus laevis oocytes were surgically removed from the female and defolliculated with collagenase. After incubation overnight in L-15 oocyte medium, 5 ng each of α4 and β2 cRNA or 10 ng α7 cRNA were injected in each oocyte with a “Nanoject Variable” automatic injector (Drummond Scientific, Broomall, PA). The oocytes were incubated for 24–72 h in L-15 oocyte medium before physiologic assay.

Whole oocyte currents were recorded using a GeneClamp 500 two-microelectrode voltage-clamp amplifier with an active ground (Axon Instruments, Foster City, CA). The voltage, current, and active ground electrodes were filled with a 3 M KCl solution, such that voltage and current electrodes had a resistance of 1–5 MΩ. Extracellular recording solution consisted of 82.5 mm NaCl, 2 mm KCl, 1 mm MgCl₂, 10 mm HEPES, and 1 mm CaCl₂, pH = 7.2. Experiments were performed at room temperature (20–24°C). Propofol (2,6-disopropylphenol, 97% purity without vehicle: Aldrich Chemical, Milwaukee, WI) or isoflurane (Abbott Laboratories, North Chicago, IL) were added to the extracellular recording solution, vigorously mixed on a vortex machine, and stored in sealed glass bottles to equilibrate for at least 2 h. This procedure produced anesthetics in solution rather than in suspension and yields solutions of anesthetic with concentrations within 20% of the calculated concentration, as measured by gas chromatography for isoflurane and high-performance liquid chromatography for propofol (data not shown). The perfusion system was closed such that the solutions were not exposed to the open atmosphere until the moment of application. The clinical median effective concentration (EC₅₀) or minimum alveolar concentration (MAC) equivalent for producing general anesthesia in aqueous solution at room temperature are 320 μM isoflurane and 0.4 μM propofol.

Oocytes were held at a membrane potential of −50
Fig. 1. Current from α4β2-expressing oocytes and inhibition by isoflurane and propofol. Inhibition of macroscopic currents by propofol and isoflurane. (A) An oocyte expressing α4β2 receptors, 2.4 μA of current in response to 1 μM acetylcholine. The same application of acetylcholine coapplied with 320 μM isoflurane reduced the macroscopic current to 1.1 μA (46% of control). (B) An oocyte expressing α4β2 receptors, as in A, produces 1.4 μA of current in response to 1 μM acetylcholine. The same application in the presence of 400 μM propofol reduces the current to 0.3 μA (21% of control).

mV, and peak current was measured in response to acetylcholine with and without anesthetic. The drug solutions were placed in a closed syringe at the time of application and injected into a closed loop with a volume of 400 μl. The drug containing solution replaced the bath perfusate, when activated by a manual switch, in a specially prepared recording dish with a 125-μl cylindrical channel to minimize the time and surface area exposed to the atmosphere. Drugs were applied for an approximately 1-s pulse of known volume and concentration. Three minutes were allowed to elapse between repeated agonist application, to minimize the contribution of nAChR desensitization. A baseline control response to acetylcholine was measured before each acetylcholine-anesthetic coapplication. Each anesthetic response is expressed relative to this internal control (n = 3 for each data point).

Concentration response curves were prepared plotting the fraction of current obtained after the coapplication of acetylcholine and increasing concentrations of propofol or isoflurane as compared with the current response to acetylcholine alone. These data were fit to a Hill equation, \( P_4 + ((P_2 - P_4)/(1 + (IC_{50}/x)^n)) \), where \( P_4 \) is the minimal current obtained and \( P_2 \) is the maximal current obtained in the presence of antagonist. The Hill coefficient \( n \) is the dose at which 50% of available receptors are inhibited, and \( n \) is the Hill coefficient. A Ks for inhibition by each anesthetic was determined that is independent of agonist concentration by plotting the peak currents in a modified Schild plot that compares log [ACH] with current produced at different anesthetic concentrations (see Appendix 1). All fitting algorithms and graphs were produced with Microcal Origin 4.0 software (Microcal Software, Northhampton, MA).

Results

Both isoflurane (fig. 1a) and propofol (fig. 1b) inhibit macroscopic current induced by acetylcholine gating of the α4β2 receptor. The propofol inhibition is less potent. The inhibition by isoflurane and propofol was readily reversible with washout of the drug.

Inhibition by isoflurane of the α4β2 nAChR is concentration dependent (fig. 2). When the concentration response of isoflurane to 1 μM acetylcholine is fit by the Hill equation, the IC50 is determined to be 85 μM, and the Hill coefficient is 1.3. To determine whether isoflurane’s inhibition of the α4β2 nAChR response is competitive or noncompetitive, a Schild-like plot was generated (fig. 3a, b). The linear portion of the dose response (fig. 3a, 1 vs log [ACH]) shifts parallel to the control response with increasing concentrations of antagonist. The shift in acetylcholine concentration, X’, is used to calculate the dose ratio (r = X’/X), where X

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Fig. 2. Isoflurane inhibits the α4β2 current in a dose-dependent manner. Concentration-response curve of the inhibition of α4β2 currents by increasing concentrations of isoflurane. Peak currents were measured in response to increasing concentrations of isoflurane coapplied with 1 μM acetylcholine. Data are fit to the Hill equation, and 95% confidence limits are expressed as dashed lines. The Hill coefficient is 1.3 and the median inhibitory concentration IC50 is 85 (± 7) μM. P1 is 34 (± 4.1). Data are expressed as means ±SD.
= 1/Kf. The dose ratio, plotted against concentration of antagonist, has a slope of 0.99, suggesting competitive antagonism when one molecule of isoflurane binds to the α4β2 nAChR. The Kf is 11 μM. When isoflurane at MAC is applied to oocytes expressing α4β2 nAChRs without agonist, a current is gated that is approximately 5% of that gated by 1 μM acetylcholine (data not shown). This current is not present in uninjected oocytes, and like the current gated by acetylcholine, it reverses at a holding potential of 0 mV and is inhibited by 1 μM curare.

Propofol’s inhibition of the α4β2 nAChR is also concentration dependent. A concentration response curve with propofol and 1 μM acetylcholine as an agonist, fit by the Hill equation as above, yields an IC50 of 19 μM propofol and a Hill number of 2.9 (Fig. 4). As is the case with isoflurane, propofol acts as a competitive inhibitor at the α4β2 nAChR. When the propofol data are plotted by the method described above, the linear portion of the dose-response curve yields parallel lines (Fig. 5a), and the dose ratio yields a K1 of 6 μM and a K12 of 248 μM propofol (Fig. 5b). An adequate fit of the propofol data requires the assumption that two molecules contribute to the inhibition by propofol (n = 2). Negative cooperativity is apparent between the binding of the first and second molecules. Propofol at high concentration applied to oocytes expressing the α4β2 nAChR without agonist, also gates a small current (data not shown). This response is not present in uninjected oocytes, reverses at 0 mV, and is inhibited by 1 μM curare.

Isoflurane and propofol are most potent in their inhibition of the α4β2 nAChRs current gated by lower concentrations of acetylcholine, as would be expected with a competitive inhibitor. Isoflurane at MAC reduces the amplitude of the current gated by 1 μM acetylcholine to 45% of the control value. Inhibition persists, however, with concentrations of acetylcholine as high as 1 mM, which is three orders of magnitude greater than the EC50 of acetylcholine for α4β2 nAChRs (0.8 μM acetylcholine)15 and likely to approximate the concentrations of released neurotransmitter within the synap-
Fig. 5. Modified Schild plot showing competitive inhibition with propofol. (A) A $K_i$ determined by plotting the voltage-clamp results for propofol inhibition displayed in figure 4, with a modified Schild plot comparing log[ACh] with current produced at different anesthetic concentrations. (B) Modified Schild dose ratio for the binding of two antagonist molecules (see appendix for derivation): $(3r^2/1 + 2r - 1)$ versus [propofol]. Fitting equation,

$$\frac{3r^2}{1 + 2r} = \frac{[P]}{K_{p_1}} + \frac{[P]}{K_{p_2}}.$$  

$k_{p_1} = 5.8 \mu M$, $k_{p_2} = 248 \mu M$. $\text{Chi}^2 = 4.7$.

Discussion

Two general anesthetics, isoflurane and propofol, potently inhibit the $\alpha_4\beta_2$, but not $\alpha_7$-type, nAChRs in a clinically relevant range. Inhibition by propofol is unlikely to be clinically relevant, whereas the inhibition by isoflurane reported here is the most potent effect of a general anesthetic on a CNS receptor described to date. The inhibition of the $\alpha_4\beta_2$ nAChR response by both isoflurane and propofol appears to be competitive with acetylcholine according to our modified Schild analysis. In addition, the modified Schild analysis and a Hill coefficient of 1.3 suggest that one molecule of isoflurane binds competitively with acetylcholine to each $\alpha_4\beta_2$ receptor. Similarly, analysis of the propofol data predicts two molecular-binding steps, the first competitive with ACh and the second showing negative cooperativity. This is consistent with the derived Hill coefficient of 2.9 for propofol inhibition. However, the appearance of competitive inhibition based on whole-cell experiments may not represent simple competition for the agonist binding site. These results may reflect a site of action for these antagonists near the ACh binding site, destabilizing acetylcholine binding, an allosteric change effecting ACh binding affinity, or an alteration in rate constants for channel gating or desensitization. Preliminary examination of single channel currents favors the latter explanation (P. Flood, J. Ramirez-Latorre, unpublished observations). Thus the binding of general anesthetic may slow the opening-rate constant or speed the closing-rate constant of the $\alpha_4\beta_2$ nAChR in response to acetylcholine, mimicking competitive inhibition in a whole-cell analysis. Confirmation of this mechanistic analysis will re...
quire the analysis of activity on single nAChRs and also rapid perfusion techniques.

The inhibition of α4β2 nAChRs by isoflurane and propofol is not generalizable to all CNS nAChRs, because the α7 receptor is not inhibited by either anesthetic. Manipulation of the nAChR subunit sequences by chimeric analysis or with site-directed mutagenesis will permit a more detailed molecular dissection of the structure of interaction of these drugs with the various nAChR subtypes. In this regard, chimeras of the α7 N-terminal domain, including the presumed homooligomeric assembly sequences, with the channel-forming regions of the α3 or α4 subunit would be of particular interest in comparing the competitive antagonist with the channel blocking activities of the general anesthetics.

These anesthetics act as partial agonists in that they can gate a small nicotinic current in addition to their inhibition of the acetylcholine response. The currents gated by isoflurane and propofol are very small at clinical concentrations and may not influence synaptic transmission by nAChRs. Isoflurane-gated currents may be important, however, at presynaptic nAChRs, where gating of even a few receptors could induce sufficient calcium entry to enhance transmitter release. The agonist activity of general anesthetics may be more substantial at other types of nAChRs, just as nicotine has been found to be a potent agonist at α7 or α5-containing nAChRs at the submicromolar concentrations detected after smoking.6,17

Inhibition of the nAChR may be particularly important at presynaptic sites, where, in addition to augmentation of GABA and glycine receptor responses, the anesthetics may act to inhibit synaptic transmission. Inhibition of the α4β2 nAChRs by general anesthetics may contribute to the diminution in synaptic transmission seen in the presence of these drugs, as α4β2 nAChRs have been implicated in presynaptic facilitation of transmission at various sites of cholinergic innervation, including the ventral striatum, nucleus accumbens, and amygdala.1 The combined inhibition of nAChR-mediated synaptic facilitation and nicotinic transmission in the CNS may lead to the clinical effects of anesthetics, such as unconsciousness, analgesia, and amnesia, and to some of the side effects, such as nausea and temperature and autonomic instability. Autonomic inhibition in particular may be of central or peripheral origin: different nAChR subtypes are represented in each. The fact that isoflurane but propofol inhibits the α4β2-type nAChR suggests that the net clinical effect of this inhibition may be an activity of isoflurane that does not occur with propofol (e.g., analgesia, nausea). The high potency of isoflurane in the inhibition of nAChRs suggests a possible mechanism for some of the side effects experienced by patients on emergence when only low concentrations of isoflurane remain. Experiments in whole animals evaluating competition with general anesthetics by nicotinic agonists may clarify these questions.

Our findings add the CNS α4β2-type nAChR to the small family of homologous ligand-gated ion channels affected by general anesthetics in a clinically relevant range. The evidence that α7 homomeric nAChR is unaffected suggests that inhibition is subtype specific. Clinical effects of general anesthetics may be explained by their selective potency at various ligand-gated receptors in different brain regions.

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References

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Appendix 1

Derivation of modified Schild equation from mechanistic assumptions:
Assuming the following kinetic scheme for isoflurane,

\[
\begin{align*}
2k_1 & \quad k_2 & \quad \beta \\
A+R & \rightarrow AR+\alpha & \rightarrow ARA \rightarrow O^*
\end{align*}
\]

\[
\begin{align*}
k_0 \quad k_0 & \quad k_1 & \quad k_0 \quad k_1 & \quad 2k_{-2} & \quad \alpha \\
R-\text{Iso} & \rightarrow AR-\text{Iso}
\end{align*}
\]

Where R is the receptor, A is the ligand, ARA is the diliganded receptor, O* is the open receptor, and R-Iso is the receptor complexed to isoflurane. The desensitized states are ignored because our analysis includes only peak current. According to the laws of mass action,

\[
I_P = \frac{(\frac{\beta}{\alpha})K_1K_2X^2}{(1 + 2K_1X)(1 + K_0[\text{iso}]) + K_1K_2X^2\left(1 + \frac{\beta}{\alpha}\right)}
\]

where \(K_0 = k_0/k_1\) and \(\text{iso}\) is the isoflurane concentration. \(K_1\) and \(K_{-2}\) are the forward and backward rate constants for binding of the first molecule of agonist. \(K_2\) and \(K_{-1}\) are the forward and backward rate constants for binding of the second agonist molecule. If we set equal the peak current in the presence and absence of isoflurane, \(I_P(X, [\text{iso}] = 0) = I_P(X, [\text{iso}] = 0)\), where \(X\) is the new acetylcholine concentration necessary to achieve the same current as in the absence of isoflurane (\([\text{iso}] = 0\), then:

\[
\frac{(\frac{\beta}{\alpha})K_1K_2X^2}{(1 + 2K_1X')(1 + K_0[\text{iso}]) + K_1K_2X'^2\left(1 + \frac{\beta}{\alpha}\right)} = \frac{(\frac{\beta}{\alpha})K_1K_2X^2}{(1 + 2K_1X + K_0[\text{iso}]) + K_1K_2X^2\left(1 + \frac{\beta}{\alpha}\right)}
\]

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This relationship can be simplified to:

\[
\frac{X^2(1 + 2K_1X)}{K^2(1 + 2K_1X')} = 1 + \frac{[\text{iso}]}{K_i}
\]

setting \(r = X'/X\) (or the dose ratio as per Schild), and setting \(X = 1/K_i\),

\[
\frac{3r^2}{1 + 2r} = 1 + \frac{[\text{iso}]}{K_i}
\]

This equation is used in figure 5b.

When a similar analysis is undertaken for the propofol data, a mechanistic scheme where two molecules of propofol bind to each receptor molecule is required.

\[
\begin{align*}
2k_1 & \quad k_2 & \quad \beta \\
A+R & \rightarrow AR+\alpha & \rightarrow ARA \rightarrow O^*
\end{align*}
\]

\[
\begin{align*}
k_0 \quad k_0 & \quad k_1 & \quad k_0 \quad k_1 & \quad 2k_{-2} & \quad \alpha \\
R-P & \rightarrow AR-P & \rightarrow AR-P
\end{align*}
\]

\[
\begin{align*}
k_{-1} & \quad k_{-1} & \quad k_{-1} & \quad k_{-1} & \quad R-2P & \rightarrow AR-2P
\end{align*}
\]

In this case we obtain

\[
\frac{3r^2}{1 + 2r} = 1 + \frac{[P]}{K_{p1}^{-1}} + \frac{[P]^2}{K_{p1}^{-1}K_{p2}^{-1}}
\]

used in figure 5b.

Note that the equations derived and used in figures 3b and 5b, are simplified to the original Schild equation as \(r^{-1}\).