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Human Neuronal Nicotinic Acetylcholine Receptors Expressed in Xenopus *Oocytes Predict Efficacy of Halogenated Compounds That Disobey the Meyer-Overton Rule*

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Background: **According to the Meyer-Overton rule, anesthetic potency of a substance can be predicted by its lipid solubility, but a group of halogenated volatile compounds predicted to induce anesthesia does not obey this rule. Thus, these compounds are useful tools for studies of molecular targets of anesthetics. Human neuronal nicotinic acetylcholine receptor (hnAChR) subunits have been recently cloned, which allowed the authors to assess whether these receptors could differentiate among volatile anesthetic and nonimmobilizer compounds. This study provides the first data regarding anesthetic sensitivity of hnAChRs.**

Methods: **$\alpha_2\beta_4$, $\alpha_3\beta_4$, and $\alpha_4\beta_2$ hnAChRs were expressed in *Xenopus* oocytes, and effects of volatile anesthetics isoflurane and F3 (1-chloro-1,2,2-triflurocyclobutane, 1A) and nonimmobilizers F6 (1,2-dichlorohexafluorocyclobutane, 2N) and F8 (2,3-dichlorooctafluorobutane) on the peak acetylcholine-gated currents were studied using the two-electrode voltage-clamp technique.**

Results: **Isoflurane and F3 inhibited all the hnAChRs tested in a concentration-dependent manner. Isoflurane at a concentration corresponding to 1 minimum alveolar concentration (MAC) inhibited 83, 69, and 71% of ACh-induced currents in $\alpha_2\beta_4$, $\alpha_3\beta_4$, and $\alpha_4\beta_2$ hnAChRs, respectively, and 1 MAC of F3 inhibited 64, 44, and 61% of currents gated in those receptors. F6 (8–34 μM) did not cause any changes in currents gated by any of the receptors tested. F8 (4–18 μM) did not alter the currents gated in either $\alpha_3\beta_4$ or $\alpha_4\beta_2$ receptors, but caused a small potentiation of $\alpha_2\beta_4$ hnAChRs without a concentration–response relation.**

Conclusion: **The *in vivo* potency and effectiveness of volatile anesthetic and nonimmobilizer compounds were consistent with their actions on hnAChRs expressed in a recombinant expression system, suggesting a potential participation of these receptors in the mechanisms of anesthesia. (Key words: Electrophysiology; ligand-gated ion channel; nonimmobilizer; recombinant receptor.)**

AT the end of the nineteenth century, Meyer and Overton[1] described a correlation between anesthetic potency and lipid solubility that led to the view that anesthetics exert their effects by disrupting the structure of lipids in nerve membranes. This theory was attractive because it represented a simple and unifying explanation for the molecular mechanisms of such a heterogeneous group of compounds, but its limitations have been noted. For example, there are findings that strongly support the concept that anesthetic sites of action are proteins rather than lipids.[2-3] In their pioneer work, Franks and Lieb[4] demonstrated that anesthetics inhibit a lipid-free preparation of an enzyme (firefly luciferase), and, subsequently, they showed that anesthetics act in a stereospecific manner on ion channels.[5] Recently, it was shown that specific amino acids in glycine and γ-aminobutyric type A ($GABA_A$) receptors and a glutamate receptor (GluR6) are necessary for the modulation of these receptors by anesthetics.[6-7]

F3, (1-chloro-1,2,2-triflurocyclobutane; also termed 1A), F6, (1,2-dichlorohexafluorocyclobutane; also termed 2N) and F8 (2,3-dichlorooctafluorobutane) are polyhalogenated compounds that are soluble in lipids that, according to the Meyer-Overton hypothesis, would have anesthetic proper-

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ties. However, only F3 produced anesthesia *in vivo*,[8] which makes these compounds useful pharmacologic tools for anesthesia research. Therefore, proteins that constitute targets for anesthetic action *in vivo* should be sensitive to F3 but not to F6 or F8, and this prediction was valid for $GABA_A$, glycine, and glutamate receptors.[9-11] However, the picture is not so clear for nicotinic acetylcholine receptors (nAChRs). Raines[12] showed that nAChRs from *Torpedo nobiliana* are differentially affected by volatile anesthetic and nonimmobilizer compounds, being sensitive to isoflurane and enflurane but not to F6 or F8. Conversely, Forman and Raines[13] found that enflurane and the nonimmobilizer compounds F6 and F8 inhibit mouse muscle nAChRs. The distinct sensitivity of nAChRs from *Torpedo* and from rodent muscle to nonimmobilizers might be caused by the evolutionary distance between those receptors[14]; therefore, studies using the neuronal type of nAChRs are an undeniable necessity for a better understanding of effects of anesthetic in the nervous system.

Neuronal nAChRs are ligand-gated ion channels and are part of the superfamily that includes $GABA_A$, glycine, and 5-hydroxytryptamine$_3$ ($5\text{-}HT_3$) receptors. A family of genes that encode 11 different neuronal subunits has been identified (α_{2-9} and β_{2-4}), and a predominant distribution of $\alpha_4\beta_2$ in the central nervous system and $\alpha_3\beta_4$ in the peripheral nervous system has been reported[15]; more recent studies have shown that there might be a greater variety of subunits throughout different regions of the brain.[15,16] Neuronal nAChRs have been found both in pre- and postsynaptic sites. To date, there are few reports showing neuronal nAChRs mediating postsynaptic events, and there is extensive literature showing that neuronal nAChRs modulate a variety of neurotransmitter release throughout activation of presynaptic sites.[17] Recent studies have found that volatile anesthetics inhibit the function of neuronal nAChRs from rats and chickens.[18,19] Only recently, human neuronal nAChRs have been cloned and characterized, [20,21] and striking differences in the pharmacologic properties between receptors from different species were found.[16,21] The aim of this study was to evaluate the effects of halogenated volatile anesthetics and nonimmobilizers on clones of human neuronal nAChRs (hnAChRs) expressed in *Xenopus laevis* oocytes.

Materials and Methods

Materials

Xenopus laevis female frogs were purchased from Xenopus I (Ann Arbor, MI) or Nasco (Fort Atkinson, WI). The use of experimental animals (frogs) was approved by the Animal Care and Use Committees of the Universities of Texas (Austin, Texas) and Colorado (Health Sciences Center, Denver, Colorado); all studies were performed following the appropriate guidelines. Acetylcholine chloride, atropine sulfate, collagenase type 1A, streptomycin/penicillin, gentamicin, and other reagents were purchased from Sigma Co. (St. Louis, MO). Isoflurane was obtained from Ohmeda Pharmaceutical Products (Liberty Corner, NJ). 1-chloro-1,2,2-trifluorocyclobutane (F3, 97%), 1,2-dicholorohexafluorocyclobutane (F6, 97%), and 2,3-chlorooctafluorobutane (F8, 97%) were from obtained from PCR Inc. (Gainesville, FL). XL-1 Blue cells were obtained from Stratagene (La Jolla, CA). The QIAFilter Maxi Kit was obtained from Qiagen (Chatworth, CA) and the mCAP mRNA capping kit was obtained from Stratagene (La Jolla, CA).

cDNA and cRNA Preparation

The hnAChR subunit cDNAs were provided by SIBIA Neurosciences (La Jolla, CA) in different expression vectors, α_2 and α_3 in pCMV-T7-3, α_4 and β_4 in pcDNA3 and β_2 in pSP64T.[20] The cDNAs were transformed and amplified in XL-1 Blue cells and purified using the QIAFilter Maxi Kit. *In vitro* transcripts were prepared using the mRNA capping kit.

Oocyte Preparation and cRNA Injection

Oocytes were obtained from *Xenopus laevis* frogs kept in aquarium tanks at 19-21°C, on a 12-h light-dark cycle. Frogs were fed frog brittle (Nasco or Xenopus I) three times per week. Mature females were anesthetized by immersion for approximately 30 min in a 0.12% 3-aminobenzoic acid ethyl ester solution, a small incision was made in the abdominal wall, and a piece of ovary was removed and placed in Modified Barth's solution (88 mM NaCl, 1 mM KCl, 10 mM HEPES, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 0.91 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, pH 7.5). Each frog underwent this procedure at most once a month.

To facilitate manual dissection of oocytes, a section of ovary was transferred from modified Barth's solution to a hypertonic buffer containing 108 mM NaCl, 2 mM KCl, 2 mM EDTA, 10 mM HEPES, pH 7.5, and theca and epithelial layers of mature oocytes (stages V and VI) were removed with surgical forceps. The follicular layer was removed by a 10-min immersion in 0.5 mg/ml collagenase in buffer containing 83 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES.

Oocytes were injected with 40 nl of diethyl pyrocarbonate-treated water containing 20-100 ng $\alpha_x\beta_y$ subunit com-

binations of cRNA in a 1:1 ratio. Injections were made with micropipettes (20 μm tip diameter) connected to a microdispenser (Drummond Scientific Co., Broomwall, PA) attached to a micromanipulator. After injection, oocytes were individually placed in wells of 96-well microtiter plates containing incubation medium (modified Barth's solution supplemented with 10 mg/l streptomycin, 10,000 U/l penicillin G, 50 mg/l gentamicin, 2 mM sodium pyruvate, and 0.5 mM theophylline) that was sterilized by passage through a 0.2-μm filter. Oocytes were incubated at 19°C for 3–5 days after injection.

Eletrophysiologic Recording of Xenopus *Oocytes*

Oocytes were placed in a rectangular chamber (approximately 100 μl) and perfused (2 ml/min) with buffer ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM HEPES, pH 7.4) containing 1 μM atropine sulfate. In some experiments, the role of calcium influx through the nicotinic receptors was assessed by comparing responses obtained in ND96 with those obtained from calcium-free Ba^{2+}-Ringer's (Ba-R) solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $BaCl_2$, and 10 mM HEPES, 1 μM atropine, pH 7.4). Oocytes were impaled with two glass electrodes (0.5–10 MΩ) filled with 3 M KCl and clamped at −70 mV using a Warner Instruments (Hamden, CT) oocyte clamp (model OC-725C).

Acetylcholine was applied for 20 s at 5-min intervals at an EC_{30} concentration in all experiments. Applications of 20 s were sufficient to allow currents to reach an equilibrium state in most oocytes (see Results). The EC_{30} for each subunit combination was calculated based on acetylcholine dose–response curves previously obtained in our laboratory (data not shown). Volatile compounds were preapplied for 2 min to allow complete equilibration in the bath and then immediately coapplied with acetylcholine for 20 s. Acetylcholine and anesthetic solutions were prepared on the day of the experiment. Saturated solutions of F3, F6, and F8 were obtained by 30-min bath sonication of 200 μl volatile anesthetic in 20 ml ND96 buffer. These solutions were equilibrated in a sealed vial protected from light for at least 24 h before use. The concentrations of the saturated solutions were 14.011 mM for F3, 0.225 mM for F6, and 0.035 mM for F8. Final solutions of those compounds were prepared from the respective saturated solution. The concentration in the figures represent bath concentrations that were calculated based on previously published values from our laboratory.[9] All the experiments were performed at room temperature.

Statistical Analysis

Results are expressed as percent of change from control responses, which were measured before and after each anesthetic application to account for possible shifts in the control current throughout the experiment. The "n" values refer to number of oocytes studied. Each experiment was performed with oocytes from at least two different frogs. Effects of anesthetics and nonimmobilizers were analyzed by one-sample Student *t* test (against a theoretical mean of zero) and one-way analysis of variance using GraphPad Prizm software (San Diego, CA). Data are presented as mean ± SEM.

Results

We first compared the time course of acetylcholine action and the concentration–response curves for acetylcholine using ND96 and calcium-free (Ba-R) solutions. The maximum effect of acetylcholine was achieved by 20 s (fig. 1), and this application time was used in all studies. The acetylcholine concentration–response curves were similar for ND96 and for Ba-R, indicating that calcium flux through the receptors did not influence acetylcholine potency (fig. 2).

We tested the effects of volatile anesthetic (isoflurane and F3) and nonimmobilizer (F6 and F8) compounds on the currents gated by acetylcholine in three different combinations of hnAChRs, $\alpha_2\beta_4$, $\alpha_3\beta_4$, and $\alpha_4\beta_2$, expressed in *Xenopus* oocytes. Because working at high concentrations of agonist often causes problems, including desensitization of the receptors, an EC_{30} concentration of acetylcholine was used for all hnAChRs studied: 23 μM for $\alpha_2\beta_4$, 127 μM for $\alpha_3\beta_4$, and 0.5 μM for $\alpha_4\beta_2$.

For all subunit combinations tested, isoflurane inhibited acetylcholine-gated currents, and this inhibition was similar when experiments were performed using ND96 or Ba-R, indicating that calcium did not play a critical role in the actions of isoflurane (fig. 3). In Ba-R buffer, isoflurane markedly inhibited the maximal response to acetylcholine, with only small changes in the EC_{50} (fig. 4). Isoflurane, at a concentration corresponding to the minimum alveolar concentration (MAC, 320 μM), inhibited 83, 69, and 71% of acetylcholine-induced currents in the hnAChR subunit combinations $\alpha_2\beta_4$, $\alpha_3\beta_4$, and $\alpha_4\beta_2$, respectively, and the isoflurane IC_{50} values were 25, 56, and 82 μM, respectively (fig. 5). These receptors showed high sensitivity to isoflurane, with significant inhibition occurring at concentrations as low as 3.5 μM for $\alpha_2\beta_4$ and $\alpha_3\beta_4$ and 14 μM for $\alpha_4\beta_2$ nAChRs (Student *t* test, $P < 0.05$).

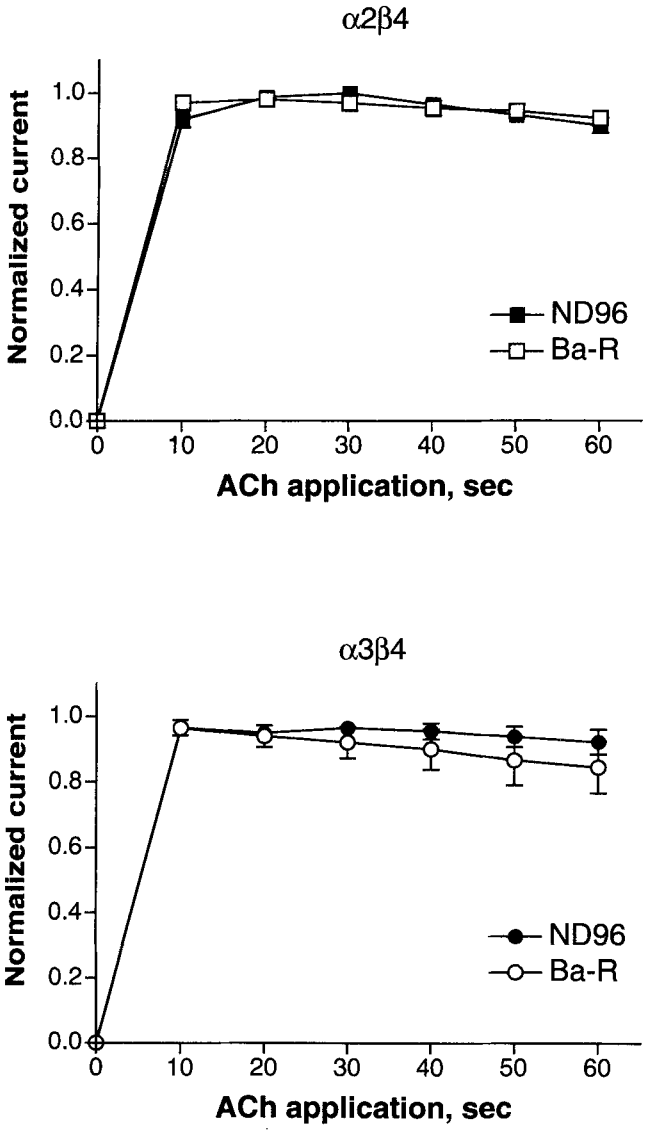

Fig. 1. Comparisons of the time courses of acetylcholine current responses of human neuronal nicotinic acetylcholine receptor (hnAChR) in ND96 and calcium-free Ba^{2+}–Ringer's solution (Ba-R). Each current response was normalized to the peak current response during application of EC_{30} concentrations of acetylcholine for 60 s. Error bars are smaller than the symbols for some values; n = 4–6.

F3 also inhibited all subunit combinations tested in a concentration-dependent manner (fig. 6; analysis of variance, $P < 0.0001$). 1 MAC F3 (0.8 mM) inhibited 64, 44, and 61% of currents gated in $\alpha_2\beta_4$, $\alpha_3\beta_4$, and $\alpha_4\beta_2$ hnAChRs, respectively. IC_{50} values for these receptors were 0.44, 1.17, and 0.33 mM, and all were significantly inhibited by the lowest concentration tested (100 μM, Student *t* test, $P < 0.05$). Isoflurane (3.5–448 μM) and F3 (0.1–3.2 mM) did not activate any currents during the preapplication period in any of the hnAChRs tested.

F6, at concentrations corresponding to 0.5, 1.0, and 2.0 predicted MAC (8, 17, and 34 μM, respectively), did not induce any significant change in acetylcholine-gated currents in $\alpha_2\beta_4$, $\alpha_3\beta_4$ and $\alpha_4\beta_2$ hnAChRs (fig. 7A; Student *t* test, $P < 0.05$). F8, at concentrations ranging from 0.5 predicted MAC to 2.0 predicted MAC (4.4–18 μM), did not change the currents gated by $\alpha_3\beta_4$ and $\alpha_4\beta_2$ hnAChRs. F8 (4.4 μM and 18 μM) induced statistically significant potentiation of acetylcholine-gated currents in $\alpha_2\beta_4$ subunit combination (fig. 7B; 4.4 μM: 10 ± 4%; 18 μM: 17 ± 3%; Student *t* test, $P < 0.05$). Despite the potentiation observed at 4.4 and 18 μM F8, 8.8 μM of this compound did not cause any changes in acetylcholine-gated currents in $\alpha_2\beta_4$ receptors (1.2 ± 2.7%, $P > 0.05$). Although statistically significant, the potentiation induced by F8 was very small and was not concentration-dependent.

Discussion

Many proteins are affected by volatile anesthetics but are not necessarily important for anesthesia, *e.g.*, luciferase.[4] One criterion for a role of a protein in the immobility component of anesthesia is that it should be sensitive to volatile anesthetics such as F3, but resistant to the structurally related nonimmobilizers F6 and F8.[8,9] Previous studies asked whether non-neuronal nAChRs could differentiate among volatile anesthetics and non-

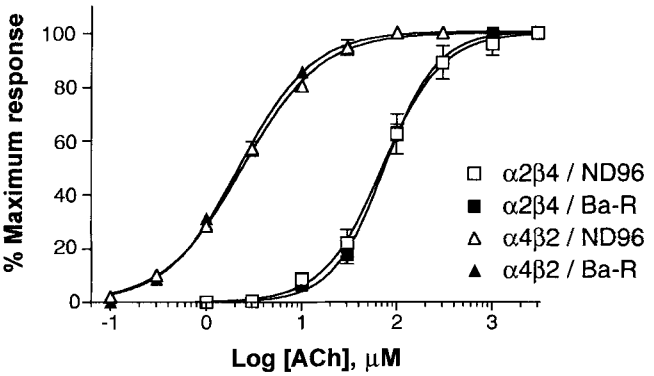

Fig. 2. Comparison of acetylcholine concentration–response relations of human neuronal nicotinic acetylcholine receptors (hnAChRs) in ND96 and Ba^{2+}–Ringer's (Ba-R) solution. The EC_{50} values (μM) of the $\alpha_2\beta_4$ hnAChR for acetylcholine in ND96 and Ba-R solution were 72 ± 15 and 76 ± 7, and the Hill coefficient values of those were 1.5 ± 0.1 and 1.7 ± 0.1, respectively (n = 6 or 7). The EC_{50} values (μM) of the $\alpha_4\beta_2$ hnAChR for acetylcholine in ND96 and Ba-R solution were 2.5 ± 0.2 and 2.2 ± 0.1, and the Hill coefficient values of those were 1.11 ± 0.05 and 1.15 ± 0.02, respectively (n = 6 or 7).

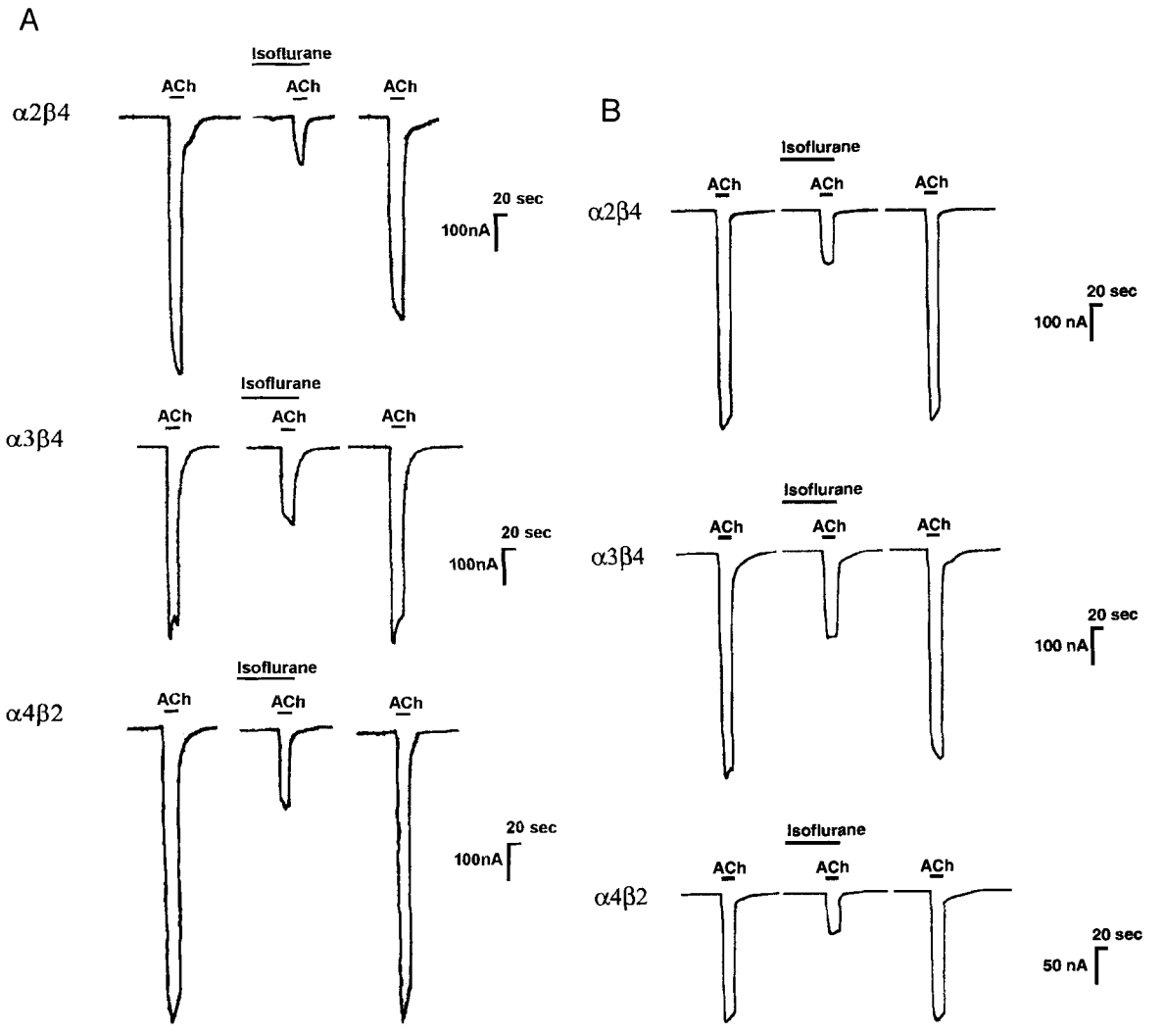

Fig. 3. (*A*) Representative tracings of inhibition induced by 100 μM isoflurane in different subunit combinations of human neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes and measured in ND96 buffer. Oocytes were stimulated with EC_{30} concentrations of acetylcholine for 20 s (23 μM for $\alpha_2\beta_4$, 127 μM for $\alpha_3\beta_4$, and 0.5 μM for $\alpha_4\beta_2$), followed by 5 min of wash, then 2 min of preincubation of isoflurane. Immediately after isoflurane preincubation, acetylcholine was coapplied with isoflurane for 20 s, and then washed once more for 5 min and reexposed to acetylcholine. Note that the effect of isoflurane in all receptors was reversible. Bars represent period of treatment with drugs. (*B*) Similar to (*A*), except Ba^{2+}–Ringer's buffer was used.

immobilizers, and conflicting results were found. Neuronal acetylcholine receptors from *Torpedo nobiliana* were affected differentially by those compounds,[12] but muscle nAChRs expressed in *Xenopus* oocytes were inhibited by enflurane and by F6 and F8, although the inhibition by enflurane and the nonimmobilizers happened at distinct kinetic states of the receptor.[13] In the current study, different combinations of neuronal hnAChR subunits were capable of differentiating among volatile anesthetics and nonimmobilizers. Therefore, there may be important differences in the intramolecular sites or mechanisms of anesthetic action between muscle and neuronal nAChRs, emphasizing the need for studies characterizing neuronal nicotinic receptors. Comparison of our results with other recent publications suggests that there are not marked species differences in the sensitivity of neuronal nAChRs to volatile anesthetics. For example, the IC_{50} for isoflurane inhibition of $\alpha_4\beta_2$ nAChRs was similar for receptors formed by subunits from rat, [18] chicken,[19] and human clones (current report). However, isoflurane inhibition of human nAChRs largely resulted from the noncompetitive mech-

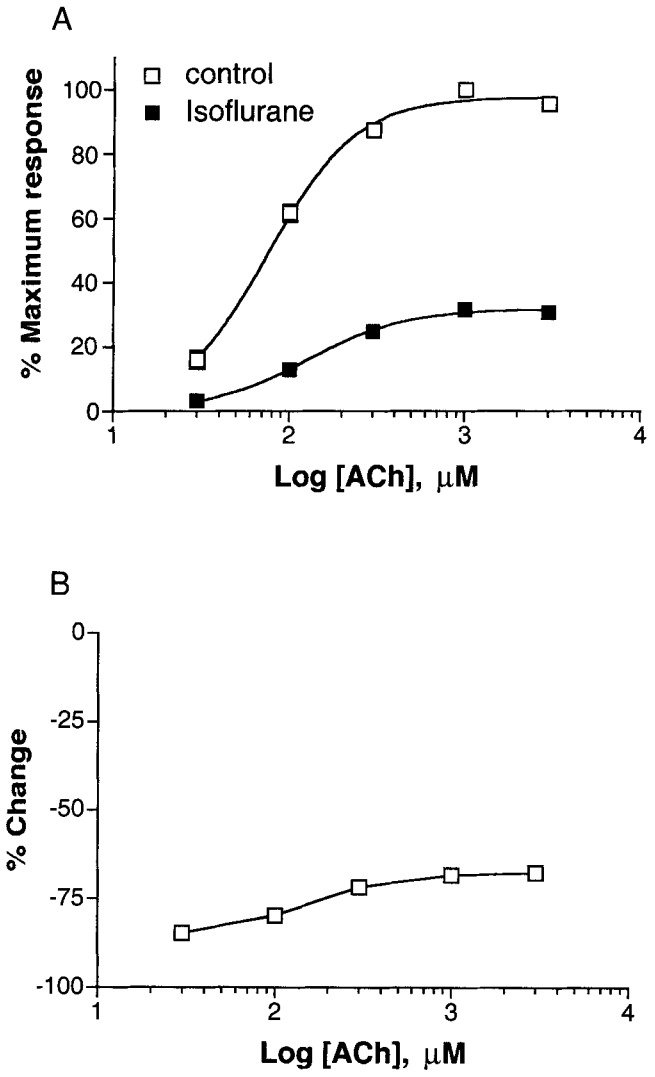

Fig. 4. Effects of isoflurane on the acetylcholine concentration–response relations of human neuronal nicotinic acetylcholine receptors (hnAChRs). (*A*) The acetylcholine concentration–response of the $\alpha_2\beta_4$ hnAChR for acetylcholine before and during perfusion of 200 μM isoflurane in Ba^{2+}–Ringer's solution. The EC_{50} values (μM) of the $\alpha_2\beta_4$ hnAChR for acetylcholine before and during perfusion of isoflurane were 77 ± 6 and 131 ± 9, and the Hill coefficient values of those were 1.8 ± 0.1 and 1.6 ± 0.1, respectively (n = 6 or 7). (*B*) The extent of inhibition of the $\alpha_2\beta_4$ hnAChR by 200 μM isoflurane as a function of acetylcholine concentration.

anism, although acetylcholine dose–response curve was shifted slightly to the right, whereas chicken nAChRs were shown to be inhibited by the competitive antagonism.[19] The human receptors have only recently been cloned,[20] and our study provides the first data regarding anesthetic sensitivity of human nAChRs.

It is possible that the nonimmobilizers have a dual

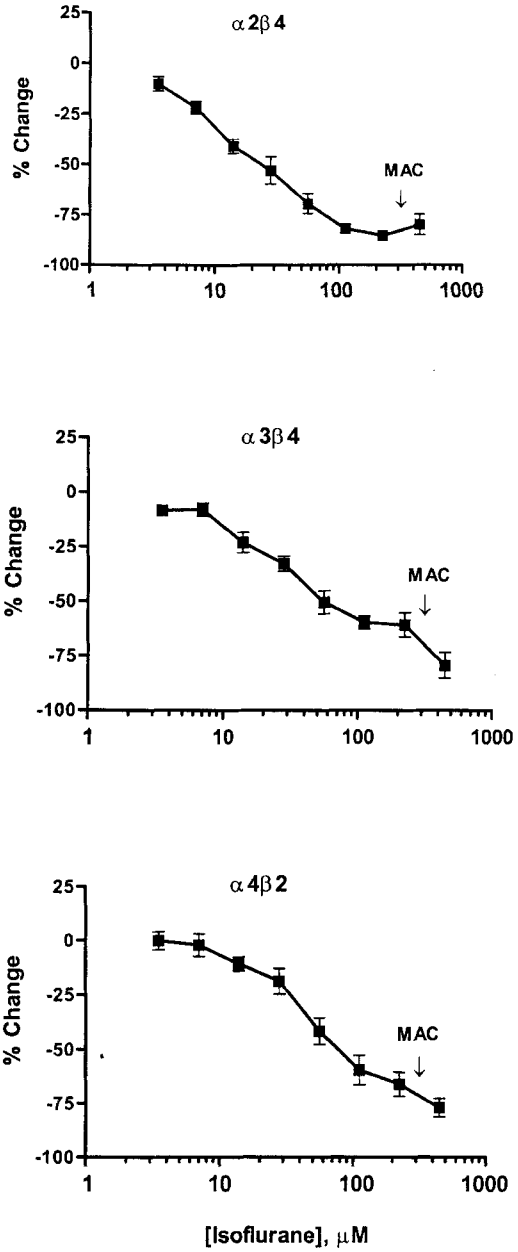

Fig. 5. Effect of isoflurane on acetylcholine-induced currents in oocytes expressing human neuronal nicotinic acetylcholine receptors with the following subunit composition: $\alpha_2\beta_4$ (*top*), $\alpha_3\beta_4$ (*middle*), and $\alpha_4\beta_2$ (*bottom*). Isoflurane was applied for 2 min before coapplication with acetylcholine EC_{30} for 20 s. The concentration corresponding to 1 MAC is indicated in each panel. Values are mean ± SE from 4 oocytes to 10 oocytes. Error bars not visible are smaller than symbols.

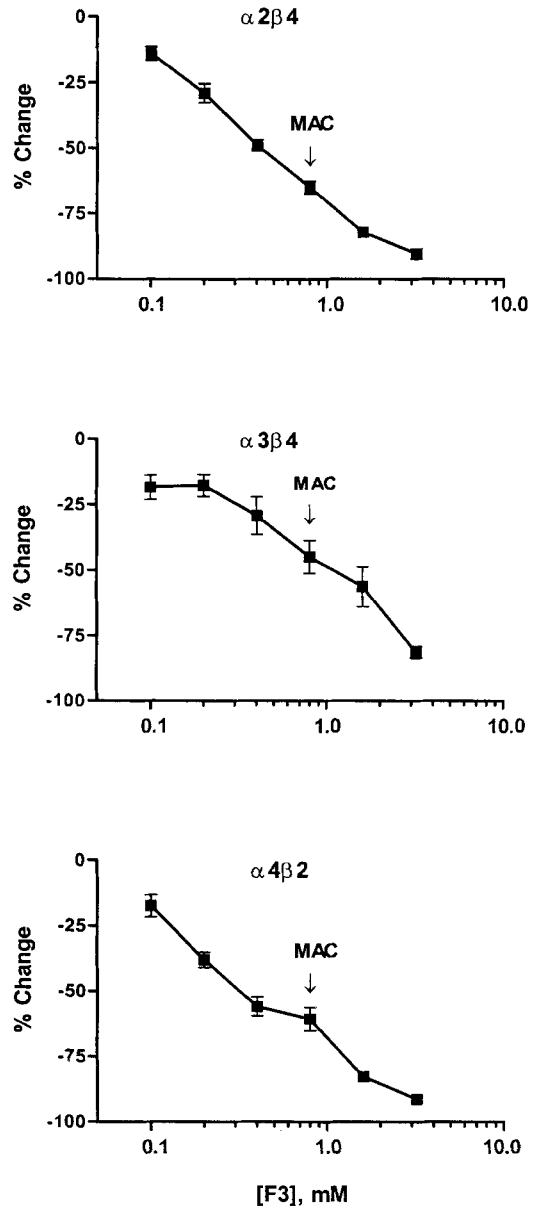

Fig. 6. Effect of the anesthetic F3 on acetylcholine-induced currents in oocytes expressing human neuronal nicotinic acetylcholine receptors: $\alpha_2\beta_4$ (*top*), $\alpha_3\beta_4$ (*middle*), and $\alpha_4\beta_2$ (*bottom*). For experimental details, see legend to figure 5. Values are mean ± SE from 5 oocytes to 8 oocytes. Error bars not visible are smaller than symbols.

action on hnAChRs; that is, inhibition at the anesthetic site and enhancement at another site, and these effects could cancel each other, resulting in an apparent noneffect. However, it seems unlikely because a dual effect of those compounds has not been observed in other studies of ligand-gated ion channels.[3,10,22]

Neuronal acetylcholine receptors are part of the superfamily of ligand-gated ion channels and share with other members of the family differential responses to volatile anesthetics compared to nonimmobilizers. Conversely, one major distinction between nAChRs and the other members of this family is that $GABA_A$, glycine, and $5\text{-}HT_3$ receptors are potentiated by anesthetics,[9,10,22] but acetylcholine-induced currents in neuronal nAChRs are inhibited, as shown in the current study and other reports.[18,19] It is important to emphasize that not all receptors differentiate among volatile anesthetics and nonimmobilizer compounds. Indeed, in a recent series of publications, Minami *et al.*[23-25] demonstrated that metabotropic glutamate receptors (mGluR1, mGluR5), muscarinic m_1, and $5\text{-}HT_{2A}$ receptors,

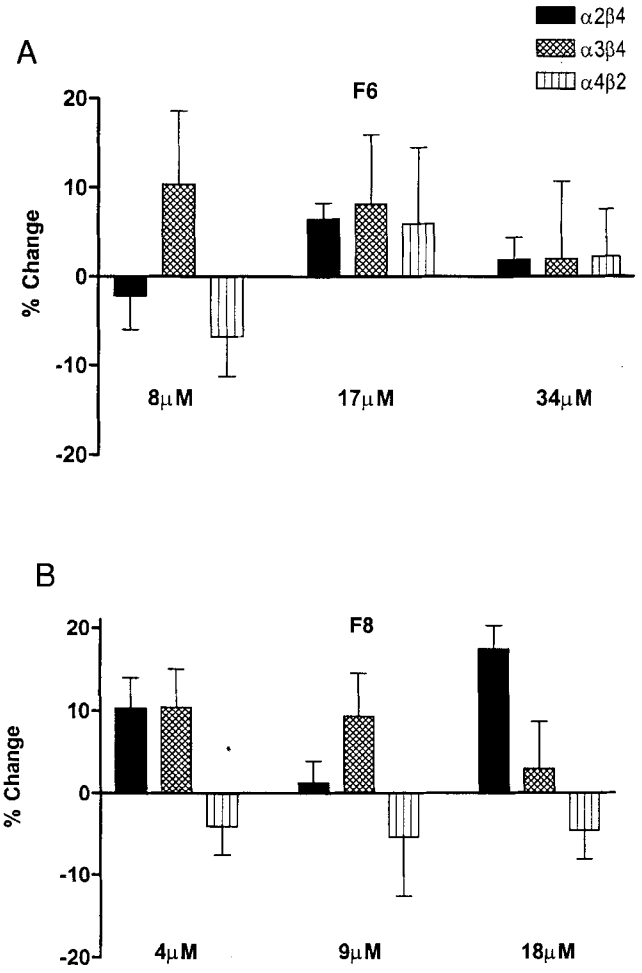

Fig. 7. Effect of the nonimmobilizers F6 and F8 on acetylcholine-induced currents in oocytes expressing human neuronal nicotinic acetylcholine receptors. F6 (*A*) or F8 (*B*) was applied for 2 min before coapplication with acetylcholine for 20 s. Acetylcholine concentrations were the EC_{30} for each receptor: 23 μM for $\alpha_2\beta_4$, 127 μM for $\alpha_3\beta_4$, and 0.5 μM for $\alpha_4\beta_2$. Values are mean ± SE from 6 oocytes to 11 oocytes.

which are all G-protein coupled, are inhibited by F3 and by F6 (albeit by distinct mechanisms), but not by F8. Considering that F6 suppresses learning in rodents,[26] the authors suggested that G-protein-coupled receptors may be responsible for the amnesia caused by anesthetics.

Recently, it was shown that halothane and isoflurane both inhibit nicotine-induced release of dopamine by acting on presynaptic nAChRs in the rat striatum.[27] Presynaptic neuronal nAChRs not only modulate release of dopamine, but also modulate the release of norepinephrine, glycine, GABA, 5-HT, acetylcholine, and glutamate.[17] Therefore, inhibition of nAChRs by anesthetics may cause changes in the balance of neurotransmitter release at central levels, which could contribute to the mechanism of anesthesia.

In summary, heteromeric human neuronal nAChRs expressed in *Xenopus laevis* oocytes were strongly inhibited by volatile anesthetics and were capable of differentiating between anesthetics and structurally related nonimmobilizers, suggesting the participation of these receptors in anesthesia.

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