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Subunit-dependent Inhibition of Human Neuronal Nicotinic Acetylcholine Receptors and Other Ligand-gated Ion Channels by Dissociative Anesthetics Ketamine and Dizocilpine

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Background: **The neuronal mechanisms responsible for dissociative anesthesia remain controversial. *N*-methyl-D-aspartate (NMDA) receptors are inhibited by ketamine and related drugs at concentrations lower than those required for anesthetic effects. Thus, the authors studied whether ligand-gated ion channels other than NMDA receptors might display a sensitivity to ketamine and dizocilpine that is consistent with concentrations required for anesthesia.**

Methods: **Heteromeric human neuronal nicotinic acetylcholine receptors (hnAChR channels $\alpha_2\beta_2$, $\alpha_2\beta_4$, $\alpha_3\beta_2$, $\alpha_3\beta_4$, $\alpha_4\beta_2$ and $\alpha_4\beta_4$), 5-hydroxytryptamine$_3$ (5-HT$_3$), $\alpha_1\beta_2\gamma_{2S}$ γ-aminobutyric acid type A (GABA$_A$) and α_1 glycine receptors were expressed in *Xenopus* oocytes, and effects of ketamine and dizocilpine were studied using the two-electrode voltage-clamp technique.**

Results: **Both ketamine and dizocilpine inhibited hnAChRs in a noncompetitive and voltage-dependent manner. Receptors containing β_4 subunits were more sensitive to ketamine and dizocilpine than those containing β_2 subunits. The inhibitor concentration for half-maximal response (IC_{50}) values for ketamine of hnAChRs composed of β_4 subunits were 9.5–29 μM, whereas those of β_2 subunits were 50–92 μM. Conversely, 5-HT$_3$ receptors were inhibited only by concentrations of ketamine and dizocilpine higher than the anesthetic concentrations. This inhibition was mixed (competitive/noncompetitive). GABA$_A$ and glycine receptors were very resistant to dissociative anesthetics.**

Conclusions: **Human nAChRs are inhibited by ketamine and dizocilpine at concentrations possibly achieved *in vivo* during anesthesia in a subunit-dependent manner, with β subunits being more critical than α subunits. Conversely, 5-HT$_3$, GABA$_A$, and glycine receptors were relatively insensitive to dissociative anesthetics. (Key words: Cholinergic; serotonin; electrophysiology.)**

KETAMINE is a dissociative anesthetic widely used in both clinical practice and animal research. It is known that ketamine blocks *N*-methyl-D-aspartate (NMDA) receptors at concentrations (about 1 μM)[1] lower than plasma concentrations required for anesthetic effects (≈ 10 μM).[2] A more potent channel blocker of NMDA receptors, dizocilpine ((+)MK-801), can also produce ketamine-like anesthetic effects at high doses.[3,4] Because inhibition of NMDA receptors by these dissociative anesthetics occurs at subanesthetic concentrations, other target sites with a lower affinity for these anesthetics than NMDA receptors may be involved in anesthetic effects (see Discussion). During the past few years, a consensus has emerged that general anesthetics act on one or more superfamilies of ligand-gated ion channels that include γ-aminobutyric acid type A (GABA$_A$), glycine, nicotinic acetylcholine, and 5-hydroxytryptamine$_3$ (5-HT$_3$) receptors.[5]

Nicotinic acetylcholine receptors (nAChRs) of skeletal muscle and fish electric organ are composed of the α_1, β_1, δ, and γ or ε subunits. Conversely, for neuronal nAChRs, 11 subunits (α_{2-9}, β_{2-4}) are identified, and they provide physiologic and pharmacologic heterogeneity distinct from muscle nAChRs.[6,7] Although a predominant codistribution of the α_4 and β_2 subunits in the central nervous system and the α_3 and β_4 subunits in the peripheral nervous system has been reported,[6,8] more recent studies have shown that there might be a greater variety of subunits throughout different regions of the brain.[7,9] Furthermore, it is evident that there are a number

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of differences in physiology and pharmacology between neuronal nAChRs in humans and those in other species.[7,10]

Muscle and electric organ nAChRs are known to be affected by dissociative anesthetics.[11-14] Neuronal nAChRs in various neuronal preparations are also reported to be inhibited by ketamine and dizocilpine at concentrations of 3 μM and 1-25 μM, respectively.[13,15,16] However, only a few studies showed the effects on the recombinant neuronal nAChRs (*i.e.*, data on phencyclidine effects on rat $\alpha_{2-4}\beta_2$ channels and dizocilpine effects on human α_7 channels).[17,18] We report the effects of ketamine and dizocilpine on various heteromeric human neuronal nAChRs (hnAChRs) composed of α (α_2, α_3, α_4) and β (β_2, β_4) subunits. We also show the effects of ketamine and dizocilpine on the recombinant 5-HT_3, $GABA_A$, and glycine receptors. The sensitivities to ketamine and dizocilpine of these ligand-gated ion channels expressed in *Xenopus* oocytes were directly compared with the previously reported sensitivities of NMDA receptors measured in a comparable oocyte system.[1]

Materials and Methods

mRNA and cDNA Preparation

The hnAChR subunit cDNAs were provided by SIBIA Neurosciences, Inc., (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.[19] *In vitro* transcripts were prepared using the mRNA capping kit (Stratagene, La Jolla, CA). The cDNA encoding the NCB-20 5-HT_3 receptor[20] in pBK-CMV N/B-200 vector; cDNAs of the human α_1, β_2, and γ_{2S} $GABA_A$ receptor subunits[21] in pBK-CMV N/B-200, pCDM8, and pCIS2 vectors, respectively; and human α_1 glycine receptor subunit cDNA[22] in pBK-CMV N/B-200 vector were used for the nuclear injection.

Oocyte Expression

The use of experimental animals (frogs) was approved by the Animal Care and Use Committees of University of Texas. Isolation of *Xenopus laevis* oocytes and microinjection of the mRNA and cDNA was performed as described previously.[23] Isolated oocytes were placed in modified Barth's saline (MBS) containing : NaCl 88 mM, KCl 1 mM, HEPES 10 mM, $MgSO_4$ 0.82 mM, $NaHCO_3$ 2.4 mM, $CaCl_2$ 0.91 mM, and $Ca(NO_3)_2$ 0.33 mM adjusted to pH 7.5. Oocytes were injected with 40 nl diethyl pyrocarbonate-treated water containing 10-50 ng of $\alpha_x\beta_y$ hnAChR subunit combinations of mRNA in a 1:1 molar ratio. The 5-HT_3 and α_1 glycine receptor cDNAs (1.5 and 0.5 ng/30 nl, respectively) and α_1, β_2, and γ_{2S} $GABA_A$ receptor subunit cDNAs (2.0 ng/30 nl in a 1:1:2 molar ratio) were injected into the animal poles of oocytes by the blind method. The injected oocytes were singly placed in Corning cell wells (Corning Glass Works, Corning, NY) containing incubation medium (sterile MBS supplemented with 10 mg/l streptomycin, 10,000 U/l penicillin, 50 mg/l gentamicin, 90 mg/l theophylline, and 220 mg/l pyruvate) and incubated at 15-19°C. On 2-5 days after injection, oocytes were used in electrophysiologic recording.

Table 1. The Acetylcholine EC_{50} and Hill Coefficient Values of Heteromeric hnAChRs Expressed in *Xenopus* Oocytes

hnAChRs	EC_{50} (μM)	Hill Coefficient	n
$\alpha_2\beta_2$	3.7 ± 0.7	1.1 ± 0.06	6
$\alpha_2\beta_4$	76 ± 7	1.7 ± 0.12	8
$\alpha_3\beta_2$	7.7 ± 0.7	1.4 ± 0.11	7
$\alpha_3\beta_4$	138 ± 10	2.0 ± 0.09	7
$\alpha_4\beta_2$	2.2 ± 0.1	1.2 ± 0.02	6
$\alpha_4\beta_4$	46 ± 4	1.9 ± 0.13	8

Values are mean ± SEM.

n = number of oocytes included in each group.

hnAChR = human neuronal nicotinic acetylcholine receptors.

Electrophysiologic Recording

Oocytes expressing hnAChRs were placed in a rectangular chamber (≈ 100 μl volume) and perfused (2 ml/min) with Ba^{2+} Ringer's solution to minimize the effects of secondarily activated Ca^{2+}-dependent Cl^- currents (115 mM NaCl, 2.5 mM KCl, 1.8 mM $BaCl_2$, and 10 mM HEPES, pH 7.4) containing 1 μM atropine sulfate. For the 5-HT_3 receptors, oocytes were perfused with low Ca^{2+} Ringer's solution (115 mM NaCl, 2.5 mM KCl, 0.18 mM $CaCl_2$, and 10 mM HEPES, pH 7.4) to reduce Ca^{2+} inhibition of currents; for the $GABA_A$ and glycine receptors, MBS was perfused. The animal poles of oocytes were impaled with two glass electrodes (0.5-10 MOhm) filled with 3 M KCl and voltage clamped at −70 mV using a Warner Instruments model OC-725A oocyte clamp (Hamden, CT). Acetylcholine (ACh), γ-aminobutyric acid (GABA), and glycine were dissolved in Ringer's solution and applied for 20 s; 5-hydroxytryptamine (5-HT) was applied for 30 s to reach equilibrium state. Anesthetics were tested against EC_{30-60} concentrations of ACh, (*i.e.*, concentrations of agonists giving 30-60% of the maximal response calculated based on ACh dose-response curves [3-10 μM for $\alpha_x\beta_2$ and 30-100 μM for $\alpha_x\beta_4$]; table 1). The dissociative anesthetics were preapplied for 30 s before being coapplied with ACh. Preapplication of dis-

sociative anesthetics alone did not produce any current responses of receptors tested. A 5–10 min washout period was allowed between drug applications. Effects of dissociative anesthetics were expressed as the fraction of control responses that were measured before and after anesthetic applications to take into account possible shifts in the control current throughout the experiment. Data were obtained from 4 to 12 oocytes taken from at least two different frogs. All experiments were performed at room temperature.

Compounds

Xenopus laevis female frogs were purchased from Xenopus I (Ann Arbor, MI). Acetylcholine chloride, ketamine hydrochloride, 5-hydroxytryptamine (serotonin) hydrochloride, glycine, and other reagents were purchased from Sigma Co. (St. Louis, MO). Dizocilpine ((+)MK-801) maleate and γ-aminobutyric acid (GABA) were purchased from RBI (Natick, MA). 2,6-Diisopropylphenol (propofol) was purchased from Aldrich (Milwaukee, WI). Propofol was dissolved in dimethyl sulfoxide at a concentration of 1 M. The dimethyl sulfoxide stocks were diluted to appropriate concentrations in Ringer's solution. Because 60% of propofol was lost from vial to bath,[24] the concentrations used represent the final bath concentrations. Perfusion of the final dimethyl sulfoxide concentrations of 0.01% for propofol used in this investigation did not affect hnAChRs.

Statistical Analysis

The inhibitor concentration for half-maximal response (IC_{50}) and the Hill coefficient values for dissociative anesthetics were calculated according to the equation $I/I_{con} = 1/[1 + (D/IC_{50})^n]$, where I represents the current, I_{con} the control current, D the concentration of dissociative anesthetics, and n the Hill coefficient. The agonist concentration for half-maximal response (EC_{50}) value for agonists was calculated according to the equation $I/I_{max} = F/[1 + (EC_{50}/A)^n]$, where I represents the current, I_{max} the maximal current, F the residual fraction by anesthetic inhibition of the maximal current, A the concentration of agonists, and n the Hill coefficient. To calculate the antagonist dissociation constant (K_i) value of ketamine for 5-HT_3 receptors, the 5-HT dose–response curve in the presence of ketamine was analyzed as a shifted version of the control curve using null models; the concentration–response relation in the presence of ketamine is expressed as $nR = 1/[1 + (EC_{50}/(A/S))^n]$, where nR represents the normalized response, A the concentration of agonists, S the dose shift (dose ratio), and n the Hill coefficient. The dose shift (S) for competitive antagonism is $1 + D/K_i$ and that for noncompetitive antagonism is $1/(1 - Y) + Y/(1 - Y) \times (1/EC_{50}) \times A$, where D represents the concentration of dissociative anesthetics, Y the portion of receptors occluded, and A the concentration of agonists.[25] Parameter estimation was carried out by nonlinear regression using SPSS software (SPSS Inc., Chicago, IL) and fitting to models was analyzed using F tests. The other results obtained were statistically analyzed using one-way analysis of variance

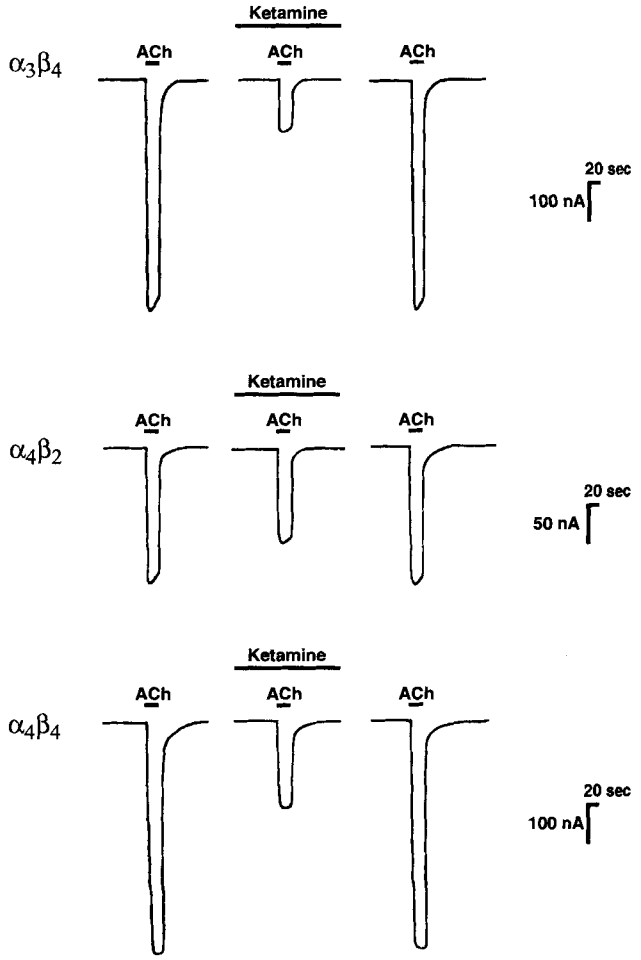

Fig. 1. Representative tracings of current responses of hnAChRs before (*left*), during (*middle*), and after (*right*) perfusion of 30 μM ketamine. The current responses of the $\alpha_3\beta_4$, $\alpha_4\beta_2$, and $\alpha_4\beta_4$ channels were evoked by EC_{30-60} (agonist concentration giving 30–60% of the maximal response) concentrations of acetylcholine (100, 3, and 30 μM for the $\alpha_3\beta_4$, $\alpha_4\beta_2$, and $\alpha_4\beta_4$ channels, respectively). Inward current is downward. The period of treatment with drugs is indicated by bars.

(ANOVA). $P < 0.05$ was considered significant. Data are represented as mean ± SEM.

Results

Effects of Dissociative Anesthetics on hnAChRs

Heteromeric hnAChRs composed of α subunits (α_2, α_3, α_4); β subunits (β_2, β_4); $\alpha_2\beta_2$, $\alpha_2\beta_4$, $\alpha_3\beta_2$, $\alpha_3\beta_4$, $\alpha_4\beta_2$, and $\alpha_4\beta_4$ subunit combinations were expressed in *Xenopus* oocytes by the injection of respective subunit-specific mRNAs synthesized *in vitro* from cloned cDNAs. The effects of dissociative anesthetics ketamine and dizocilpine on these hnAChRs were examined by measuring current responses to EC_{30-60} concentrations of ACh during incubation of anesthetics. We used Ba^{2+} Ringer's solution to prevent Ca^{2+} flux through hnAChRs. Ketamine inhibited hnAChRs in a fully reversible manner (fig. 1). The $\alpha_3\beta_4$ channel was inhibited more effectively than the $\alpha_4\beta_2$ channel by 30 μM ketamine. The dose-inhibition relations for ketamine and dizocilpine of heteromeric hnAChRs were examined. Ketamine inhibited hnAChRs in a dose-dependent manner, and receptors composed of β_4 subunits were more sensitive to ketamine than those containing β_2 subunits (fig. 2A). The sensitivities were also different with α subunits, especially for channels composed of β_4 subunits. The α_3 subunit-containing hnAChRs were most sensitive to ketamine, and the α_2 subunit-containing channels were least sensitive. The IC_{50} value for ketamine of the most sensitive channel ($\alpha_3\beta_4$) was 9.5 μM, whereas that of the most resistant channel ($\alpha_2\beta_2$) was 92 μM. The rank order of sensitivity to ketamine was $\alpha_3\beta_4 > \alpha_4\beta_4 > \alpha_2\beta_4 > \alpha_3\beta_2 > \alpha_4\beta_2 > \alpha_2\beta_2$. Dizocilpine also inhibited hnAChRs in a fully reversible manner. Inhibition by dizocilpine was dependent on hnAChR subunit combinations, and the sensitivity of each channel to dizocilpine was two to four times higher than that to ketamine (fig. 2B). The rank order of sensitivities of various heteromeric channels to dizocilpine was similar to that of ketamine, with β_4 subunit-containing channels being more sensitive to dizocilpine than β_2 subunit-containing channels. For β_4 subunit-containing channels, sensitivities to dizocilpine were also different among α subunits. The IC_{50} value for dizocilpine of the most sensitive channel ($\alpha_3\beta_4$) was 2.7 μM, whereas that of the most resistant channel ($\alpha_2\beta_2$) was 36 μM.

Effects of Ketamine on Acetylcholine Sensitivity of hnAChRs

To characterize the ketamine inhibition of hnAChRs, we examined the effects of ketamine on the ACh dose-response relations of hnAChRs (fig. 3). Ketamine 10 μM markedly inhibited the maximal responses to ACh of the$\alpha_3\beta_4$ and $\alpha_4\beta_4$ channels. The EC_{50} value for ACh of the $\alpha_3\beta_4$ channel during treatment with ketamine was not significantly different from that before treatment (138 ± 33 and 132 ± 26 μM [n = 6], respectively). The EC_{50} values for ACh of the $\alpha_4\beta_4$ channel before and during treatment with ketamine were 42 ± 4 and 43 ± 4 μM (n = 4), respectively. Similarly, dizocilpine inhibited the maximal responses of the $\alpha_3\beta_4$ and $\alpha_4\beta_2$ chan-

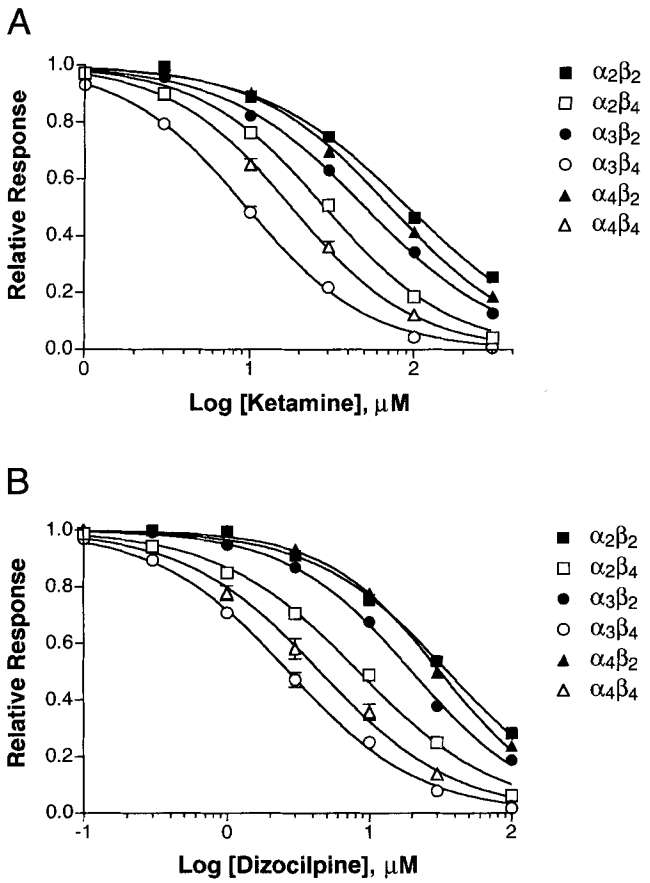

Fig. 2. Dissociative anesthetics inhibit various heteromeric human neuronal nicotinic acetylcholine receptors. (*A*) The dose–inhibition relations for ketamine. Each point represents the mean ± SEM of measurement on seven to eleven oocytes; SEM are indicated by bars when larger than the symbols. Inhibitor concentration for half-maximal response (IC_{50}) values of the $\alpha_2\beta_2$, $\alpha_2\beta_4$, $\alpha_3\beta_2$, $\alpha_3\beta_4$, $\alpha_4\beta_2$ and $\alpha_4\beta_4$ channels for ketamine were 92, 29, 50, 9.5, 72, and 18 μM, respectively, and the Hill coefficient values of those were 1.0, 1.1, 1.0, 1.2, 1.1, and 1.2, respectively. (*B*) The dose–inhibition relations for dizocilpine. IC_{50} values of the $\alpha_2\beta_2$, $\alpha_2\beta_4$, $\alpha_3\beta_2$, $\alpha_3\beta_4$, $\alpha_4\beta_2$ and $\alpha_4\beta_4$ channels for dizocilpine were 36, 8.5, 20, 2.7, 32, and 4.5 μM, respectively, and the Hill coefficient values of those were 0.9, 0.9, 1.0, 0.9, 1.1, and 0.9, respectively (n = 8–12).

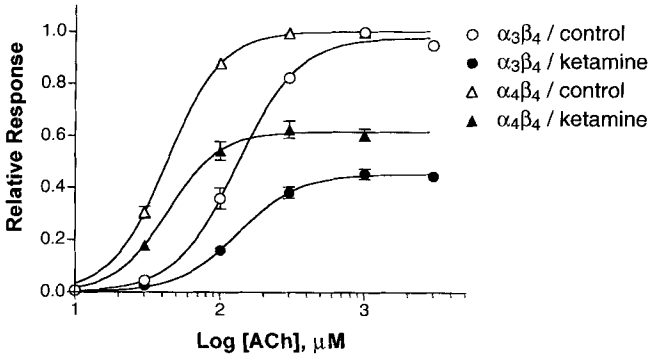

Fig. 3. Effects of ketamine on the acetylcholine concentration–response curves of human neuronal nicotinic acetylcholine receptors. The concentration–response relations of the $\alpha_3\beta_4$ and $\alpha_4\beta_4$ channels for acetylcholine before and during perfusion of 10 μM ketamine were examined. The agonist concentration for half-maximal response (EC_{50}) values of the $\alpha_3\beta_4$ channel for acetylcholine before and during treatment with ketamine were 132 ± 26 and 138 ± 33 μM, and Hill coefficient values of those were 2.2 ± 0.3 and 2.6 ± 1.2, respectively (n = 6). The EC_{50} values of the $\alpha_4\beta_4$ channel for acetylcholine before and during treatment with ketamine were 42 ± 4 and 43 ± 4 μM, and Hill coefficient values of those were 2.3 ± 0.3 and 2.6 ± 0.7, respectively (n = 4).

nels to ACh without changing the EC_{50} values (data not shown). These results suggest a noncompetitive mechanism of inhibition of hnAChRs by dissociative anesthetics.

Effects of Agonist Application Times on Anesthetic Inhibition

To determine if the inhibition of hnAChRs by dissociative anesthetics progresses due to the application times of agonists (use-dependent block), we measured current responses evoked by repeated applications of ACh during continuous perfusion of dissociative anesthetics (fig. 4). Ketamine 10 μM effectively inhibited the first current response of the $\alpha_3\beta_4$ channel, and the extent of inhibition of the second and third currents was not significantly different from that of the first current (ANOVA, $P > 0.68$). Similarly, inhibition of the $\alpha_4\beta_2$ channel by 100 μM ketamine and of the $\alpha_2\beta_4$ channel by 10 μM dizocilpine was not dependent on the application times of ACh (ANOVA, $P > 0.54$ and $P > 0.70$, respectively).

Effects of Membrane Potential on Anesthetic Inhibition

To test whether the inhibition of hnAChRs by dissociative anesthetics is dependent on the membrane potential, the effects of dissociative anesthetics on hnAChRs were measured at different holding potentials (fig. 5). Ketamine 10 μM markedly inhibited the $\alpha_3\beta_4$ channel by 72 ± 1% (n = 8) at a membrane potential of −110 mV, whereas it inhibited by only 23 ± 2% at −10 mV. The extent of inhibition was significantly different depending on membrane potentials (ANOVA, $P < 0.001$). Similarly, 3 μM dizocilpine inhibited the $\alpha_3\beta_4$ channel in a voltage-dependent manner (ANOVA, $P < 0.001$). We examined the voltage dependency of another intravenous anesthetic, propofol, which is reported to inhibit the nAChRs.[26] In contrast to dissociative anesthetics, propofol inhibition of the $\alpha_3\beta_4$ channel was not dependent on membrane potentials (ANOVA, $P > 0.89$), suggesting that mechanisms of inhibition are different between dissociative anesthetics and propofol. A propofol concentration of 40 μM produced about 50% inhibition; however, this is much higher than the concentration (≈ 1 μM) required for anesthesia.

Effects of Dissociative Anesthetics on 5-HT_3 Receptors

We next examined the effects of dissociative anesthetics on other ligand-gated ion channels. Ketamine inhibited 5-HT_3 receptors in a dose-dependent manner (fig. 6A). The extent of inhibition was dependent on the 5-HT concentrations; more effective inhibition was observed at lower concentrations of 5-HT. Similarly, dizocilpine inhibited 5-HT_3 receptors in a manner dependent on the 5-HT concentrations (fig. 6B). Effects of ketamine on the

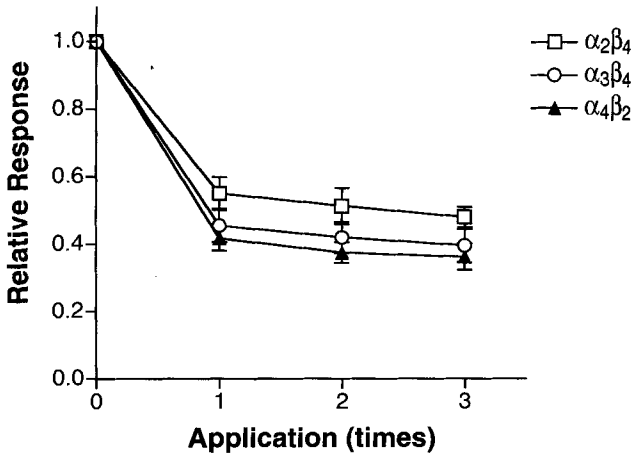

Fig. 4. Effects of acetylcholine application times during perfusion of dissociative anesthetics on currents of human neuronal nicotinic acetylcholine receptors. The current responses of the $\alpha_2\beta_4$, $\alpha_3\beta_4$, and $\alpha_4\beta_2$ channels were evoked by repeated applications of acetylcholine during continuous perfusion of 10 μM dizocilpine, 10 μM ketamine, and 100 μM ketamine, respectively (n = 4–5).

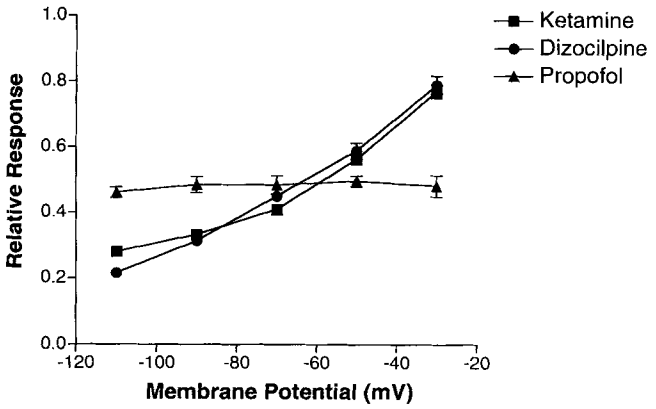

Fig. 5. The extent of inhibition of human neuronal nicotinic acetylcholine receptors by anesthetics as a function of membrane potential. Inhibition of the $\alpha_3\beta_4$ channel by 10 μM ketamine, 3 μM dizocilpine and 40 μM propofol was examined at different membrane potentials (n = 6–8).

5-HT dose-response curve were examined, and we found that ketamine 300 μM inhibited the maximal responses to 5-HT and also shifted the curve to the right (fig. 6C). This suggests both competitive and noncompetitive antagonism by ketamine. To calculate the K_i value for competitive antagonistic effects of ketamine, the 5-HT dose-response curve in the presence of ketamine was analyzed as a shifted version of the control curve (see Materials and Methods).[25] This analysis shows that K_i value for competitive antagonistic effects of ketamine is 420 ± 60 μM (n = 4). The IC_{50} values for noncompetitive antagonistic effects of ketamine was calculated to be 910 ± 30 μM (n = 6) from the effects against maximal currents to 100 μM 5-HT (fig. 6A).

Effects of Dissociative Anesthetics on $GABA_A$ and Glycine Receptors

We also examined effects of dissociative anesthetics on $GABA_A$ and glycine receptors. Both $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ and α_1 glycine receptors were very resistant to ketamine (fig. 7A). Only slight potentiation of $GABA_A$ receptors and inhibition of glycine receptors were observed with 1 mM ketamine. Similarly, dizocilpine did not affect $GABA_A$ and glycine receptors, even at 300 μM (fig. 7B).

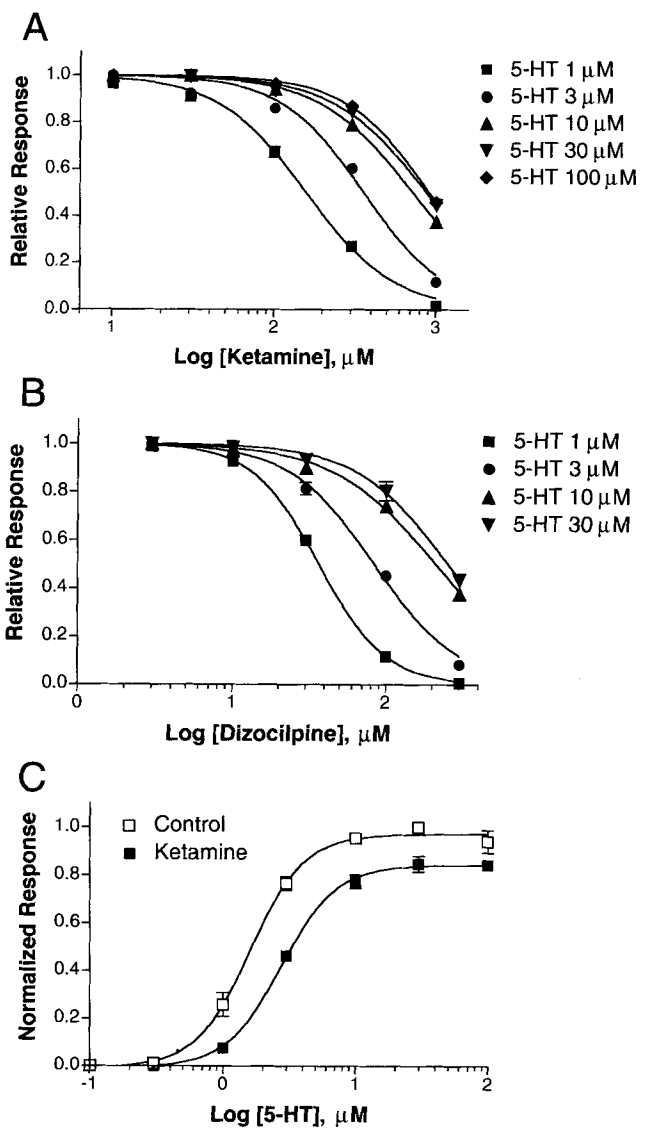

Fig. 6. Effects of dissociative anesthetics on 5-hydroxytryptamine$_3$ (5-HT$_3$) receptors. (*A*) The ketamine dose–inhibition relations for the currents evoked by different concentrations of 5-HT (n = 6–8). (*B*) The dizocilpine dose–inhibition relations for the currents evoked by different concentrations of 5-HT (n = 4–6). (*C*) Effects of ketamine on the 5-HT concentration–response curve. The concentration–response relations for 5-HT before and during perfusion of 300 μM ketamine were examined. The agonist concentration for half-maximal response (EC_{50}) values of the 5-HT$_3$ receptors before and during treatment with ketamine were 1.6 ± 0.4 and 2.8 ± 0.2 μM, and Hill coefficient values of those were 2.3 ± 0.3 and 2.1 ± 0.3, respectively (n = 4).

Discussion

In the current investigation, we demonstrated that the dissociative anesthetics ketamine and dizocilpine inhibit various heteromeric hnAChRs and that sensitivity of hnAChRs to dissociative anesthetics depends on the subunit combinations. The β subunit was more critical for determining the sensitivity than the α subunit; channels containing the β_4 subunit are more sensitive than those containing the β_2 subunit. The α subunit also contrib-

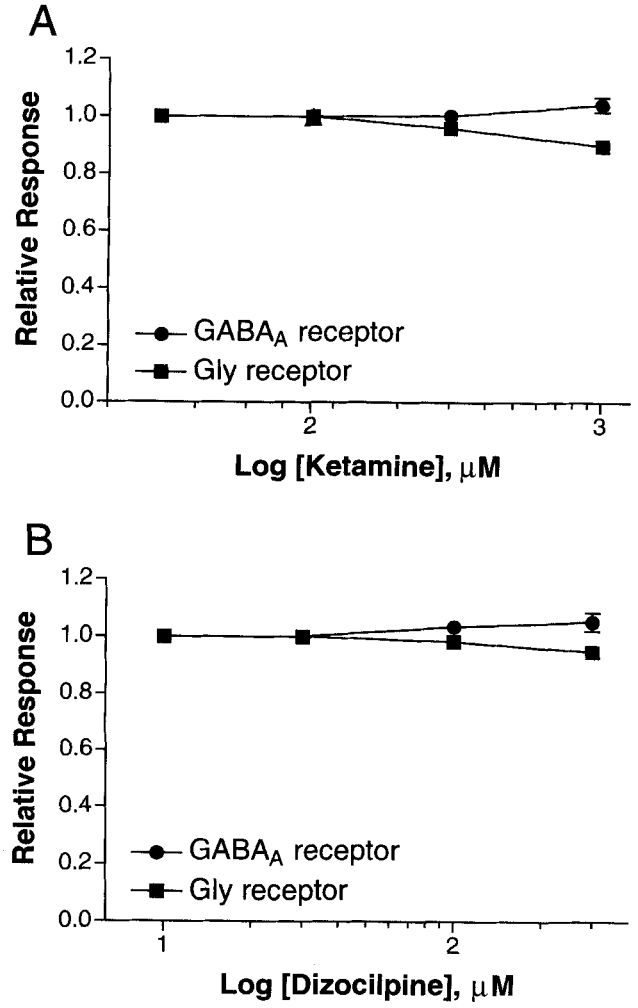

Fig. 7. Effects of dissociative anesthetics on γ-aminobutyric acid$_A$ (GABA$_A$) and glycine receptors. (*A*) Ketamine effects on the current responses of $\alpha_1\beta_2\gamma_{2S}$ GABA$_A$ and α_1 glycine receptors to EC_{50-60} (agonist concentration giving 50–60% of the maximal response) concentrations of GABA and glycine, respectively. (n = 4). (*B*) Dizocilpine effects on the current responses of $\alpha_1\beta_2\gamma_{2S}$ GABA$_A$ and α_1 glycine receptors to EC_{50-60} concentrations of GABA and glycine, respectively (n = 4).

uted to determining the sensitivity, although less prominently; channels composed of α_3 subunits were more sensitive than those containing the other α subunits. Because distribution of hnAChRs subunits in the central and peripheral nervous system is distinct,[6,7] sensitivity of native hnAChRs to dissociative anesthetics could be different depending on regions of the nervous system. For example, previous electrophysiologic and biochemical studies showed that nAChRs of retinal ganglion cells were more sensitive to dizocilpine than those of hippocampal synaptosomes in rats.[13] Neuronal nAChRs of the retinal ganglion cells are closely related to those of autonomic ganglion neurons,[27] where the α_3 and β_4 subunits are expressed.[8] Thus, the high dizocilpine sensitivity of nAChRs of the retinal ganglion cells may be related to higher sensitivity of the $\alpha_3\beta_4$ channel to dizocilpine than the other channels.

We found that IC_{50} values of heteromeric hnAChRs for ketamine were 9.5–29 μM for channels composed of β_4 subunits and 50–92 μM for channels composed of β_2 subunits. The IC_{50} values for dizocilpine were 2.7–8.5 for β_4 subunit-containing channels and 20–36 μM for β_2 subunit-containing channels. These concentrations are almost consistent with those reported for ketamine to inhibit native neuronal nAChRs in rat pheochromocytoma cell line PC12 (3 μM) and those for dizocilpine to block nAChRs in rat hippocampal neurons and bovine adrenomedullary chromaffin cells (1–25 μM).[13,16] Muscle nAChRs are reported to display median inhibitory concentrations of 15–30 μM for ketamine[11,12] and 3–10 μM for dizocilpine.[13,14] Thus, neuronal nAChRs containing the β_4 subunit are suggested to have sensitivity to dissociative anesthetics similar to muscle nAChRs, whereas nAChRs containing the β_2 subunit are slightly less sensitive than muscle nAChRs.

It is well known that dissociative anesthetics are potent NMDA receptor channel blockers. Ketamine inhibits NMDA receptors expressed in *Xenopus* oocytes with IC_{50} values of about 1 μM, and dizocilpine exhibits IC_{50} values of 0.03–0.1 μM.[1] Thus, under similar experimental conditions, ketamine has about one order of magnitude higher sensitivity for NMDA receptors than for hnAChRs, whereas dizocilpine shows about two orders of magnitude higher sensitivity for NMDA receptors.

Inhibition of NMDA receptors by ketamine and dizocilpine, as well as recovery from the block, is voltage and use dependent, which is consistent with open channel block mechanisms.[28,29] We showed that hnAChRs are inhibited by ketamine and dizocilpine in a voltage-dependent manner, but use dependency was not found for hnAChRs. Furthermore, hnAChRs exhibited fast and complete recovery from block by both ketamine and dizocilpine. The block and recovery of α_7 homomeric hnAChR by dizocilpine is also shown to be voltage dependent but not use dependent.[18] Thus, the mechanism of block of hnAChRs by dissociative anesthetics may be somewhat different from that of NMDA receptors. Consistent with our observation, inhibition of muscle nAChRs by ketamine and dizocilpine has been proposed to be due to both open and closed channel block mechanisms.[14,30]

Reports on the effects of dissociative anesthetics on 5-HT_3 receptors are controversial. Some studies show that ketamine and dizocilpine potentiate 5-HT_3 receptor-mediated currents or depolarization,[31,32] whereas others report that 5-HT_3 receptors are inhibited by ketamine and dizocilpine.[16,33] The current study shows that recombinant 5-HT_3 receptors are directly inhibited by ketamine and dizocilpine by both competitive and noncompetitive mechanisms. The K_i value for competitive antagonistic effects of ketamine on 5-HT_3 receptors was 420 μM, which is higher than ketamine concentrations for anesthesia. Conversely, ketamine is reported to inhibit 5-HT transporters in a competitive manner (K_i = 162 μM).[34] Thus, reported results of potentiation of 5-HT_3 receptors may be related to inhibition of 5-HT uptake.

The total plasma concentrations of ketamine in humans during anesthesia are approximately 10 μM,[2] whereas subanesthetic effects such as analgesia occur at considerably lower plasma levels than anesthesia ($\approx$ 0.5 μM).[35] The brain:plasma ratio of ketamine is reported to be 6.5:1, and total brain ketamine concentrations required for anesthesia (loss of righting reflex) in rats are $>$ 100 μM.[36] The ketamine-like anesthetic effects of dizocilpine are obtained after high-dose administration.[3] The brain:plasma ratio of dizocilpine is also as high as 13:1,[37] and brain concentrations of dizocilpine in rats for anesthesia could be extrapolated to exceed 60 μM.[4] However, it is likely that free aqueous concentrations of ketamine and dizocilpine in brain would be the most relevant biophase concentrations that should be correlated with *in vitro* effects on ion channels.[5] In the absence of adequate pharmacokinetic data concerning free ketamine and dizocilpine concentrations in brain, it seems plausible that free plasma concentrations would correspond to the biophase concentrations. Because about 50% of ketamine and dizocilpine is bound to plasma proteins,[38,39] free plasma concentrations of ketamine and dizocilpine during anesthesia would be extrapolated to be approximately 5 and 2 μM, respectively.

At these free plasma concentrations, ketamine and dizocilpine markedly inhibit NMDA receptors because IC_{50} values are about 1.0 and 0.03-0.1 μM for ketamine and dizocilpine, respectively,[1] whereas they inhibited hnAChRs containing β_4 subunits by 20-40% and only marginally affected hnAChRs containing β_2 subunits. Conversely, the concentration ratio of ketamine and dizocilpine during anesthesia ($\approx$ 2.5) correlates with the potency ratio of ketamine and dizocilpine for hnAChRs (2-4) rather than that for NMDA receptors (10-30). Thus, it is possible that not only NMDA receptors but also hnAChRs, especially those containing β_4 subunits, are potential targets for anesthetic effects of dissociative anesthetics. In this context, it is of interest to note the effects of other anesthetics on neuronal nAChRs. Clinical concentrations of volatile anesthetics, isoflurane and 1-chloro-1,2,2-trifluorocyclobutane (F3), but not the nonimmobilizer 1,2-dichlorohexafluorocyclobutane (F6), are reported to potently inhibit neuronal nAChRs.[23,26] Intravenous anesthetics such as thiopental and alphaxalone also inhibit neuronal nAChRs at clinically relevant concentrations.[40] Furthermore, fluorinated alcohols, which have *in vivo* anesthetic effects,[41] inhibited hnAChRs.[42] Although the $GABA_A$ receptor is considered a prime target of general anesthetics,[5] dissociative anesthetics and fluorinated alcohols fail to potentiate the $GABA_A$ receptor.[16,43] Inhibition of neuronal nAChRs is generally observed for these anesthetics. In contrast, short-chain alcohols (*e.g.*, ethanol) have anesthetic effects and enhance $GABA_A$ receptor function[44] but do not inhibit hnAChRs; rather, they enhance ACh action.[45] It seems unlikely, therefore, that a single mechanistic effect underlies general anesthesia, but neuronal nAChRs are at least one plausible anesthetic target.

The dissociative anesthetics ketamine and dizocilpine inhibited heteromeric hnAChRs, and the sensitivities were more critically determined by β subunits than α subunits. Conversely, other ligand-gated ion channels (5-HT_3, $GABA_A$, and glycine receptors) were relatively insensitive to dissociative anesthetics. Because hnAChRs were inhibited by ketamine and dizocilpine at concentrations possibly achieved *in vivo* during anesthesia, hnAChRs are likely to be one of potential targets of dissociative anesthetics.

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