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Intravenous Anesthetics Differentially Modulate Ligand-gated Ion Channels

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Background: **Heteromeric neuronal nicotinic acetylcholine receptors (nAChRs) are potently inhibited by volatile anesthetics, but it is not known whether they are affected by intravenous anesthetics. Ketamine potentiates γ-aminobutyric acid type A ($GABA_A$) receptors at high concentrations, but it is unknown whether there is potentiation at clinically relevant concentrations. Information about the effects of intravenous anesthetics with different behavioral profiles on specific ligand-gated ion channels may lead to hypotheses as to which ion channel effect produces a specific anesthetic behavior.**

Methods: **A heteromeric nAChR composed of α4 and β4 subunits was expressed heterologously in *Xenopus laevis* oocytes. Using the two-electrode voltage clamp technique, peak ACh-gated current was measured before and during application of ketamine, etomidate, or thiopental. The response to GABA of α1β2γ2s $GABA_A$ receptors expressed in human embryonic kidney cells and *Xenopus* oocytes was compared with and without coapplication of ketamine from 1 μM to 10 mM.**

Results: **Ketamine caused potent, concentration-dependent inhibition of the α4β4 nAChR current with an IC_{50} of 0.24 μM. The inhibition by ketamine was use-dependent; the antagonist was more effective when the channel had been opened by agonist. Ketamine did not modulate the α1β2γ2s $GABA_A$ receptor response in the clinically relevant concentration range. Thiopental caused 27% inhibition of ACh response at its clinical EC_{50}. Etomidate did not modulate the α4β4 nAChR response in the clinically relevant concentration range, although there was inhibition at very high concentrations.**

Conclusions: **The α4β4 nAChR, which is predominantly found in the central nervous system (CNS), is differentially affected by clinically relevant concentrations of intravenous anesthetics. Ketamine, commonly known to be an inhibitor at the *N*-methyl-D-aspartate receptor, is also a potent inhibitor at a central nAChR. It has little effect on a common CNS $GABA_A$ receptor in a clinically relevant concentration range. Interaction between ketamine and specific subtypes of nAChRs in the CNS may result in anesthetic behaviors such as inattention to surgical stimulus and in analgesia. Thiopental causes minor inhibition at the α4β4 nAChR. Modulation of the α4β4 nAChR by etomidate is unlikely to be important in anesthesia practice based on the insensitivity of this receptor to clinically used concentrations. (Key words: Anesthetic mechanism; barbiturates; general anesthetic; ketamine.)**

SEVERAL members of the ligand-gated ion channel family have been identified as molecular targets of general anesthetics in the clinically relevant concentration range. Heteromeric neuronal nicotinic acetylcholine receptors (nAChRs) found in the central nervous system (CNS) are potential targets of volatile anesthetic drugs as they are inhibited at minimum alveolar concentration.[1,2] The intravenous anesthetic propofol does not modulate the central-type nAChRs in the clinically relevant concentration range.[1,2]

We hypothesize that the intravenous anesthetics ketamine, thiopental, and etomidate may have in common with volatile anesthetics modulation of nAChRs expressed in the CNS. We studied a central-type nAChR composed of α4 and β4 subunits. The α4 and β4 nAChR subunits are expressed in anatomic locations within the CNS, which make it a potential target for mediating anesthetic behavioral responses. Although RNA for the α4 subunit partner in the α4β4 nAChR is distributed diffusely throughout the CNS,[3] the β4 subunit has more specific CNS distribution. RNA message for β4 subunits is detected in the hippocampal formation, in layer I-IV of the cerebral cortex, in the cerebellum, in the medial habenula, in the interpeduncular and pontine and trigeminal nuclei, and in the locus coeruleus. The combination of α4 and β4 nAChR subunits is therefore anatomically located in an appropriate manner to subserve anesthetic behaviors such as sedation, amnesia, and cen-

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tral modification of autonomic reflexes.[4] The study of neuronal nAChR modulation by anesthetics is particularly pertinent because they have been implicated in memory, arousal, analgesia, and autonomic control.[5]

Although ketamine potentiates γ-aminobutyric acid type A ($GABA_A$) receptor response at higher-than-clinical concentrations, concentration dependence has not been determined, and ketamine effect at clinical concentrations is unknown.[6] We hypothesize that ketamine is unusual among anesthetics in not potentiating the response to GABA at clinical concentrations.

Several traditional pharmacologic criteria have been used to select potentially relevant molecular targets of general anesthetics.[7,8] Among these criteria is the requirement that the target be modulated in the clinically relevant concentration range. The range of concentrations that is clinically relevant is controversial, and many drugs affect ligand-gated ion channels at high concentrations. However, if there is no effect on a molecular target at the clinical EC_{50} for a drug, the target is unlikely to be responsible for drug-induced changes in behavior. The concentrations of the intravenous anesthetics that modulate $\alpha4\beta4$ nAChRs may be compared with the clinical EC_{50}s for those anesthetics, derived from the literature. The reported EC_{50}s for anesthetics in humans vary considerably. One reason for the variability is that for many anesthetics, there is a great degree of protein binding, which has not always been taken into account. The concentration of ketamine measured in blood on awakening is reported to be between 2.7 and 4.7 μM.[9,10] Analgesia has been reported at 0.17 and 0.63 μM.[11] Ketamine is approximately 50% bound by protein in human plasma[12]; therefore, the EC_{50} concentration for free ketamine to produce anesthesia is between 1.3 and 2.4 μM. Analgesia is caused by 0.08–0.32 μM free ketamine. Fifty percent of patients fail to respond to a painful stimulus at 178 μM thiopental. When 85% protein binding is accounted for, the EC_{50} for free thiopental is approximately 25 μM.[8] The EC_{50} value for etomidate has been measured at 8.7 μM. Protein binding is 78%; thus, the EC_{50} for free etomidate is approximately 2 μM.[13]

We have found that the intravenous anesthetics ketamine, thiopental, and etomidate differentially modulate both nAChRs and $GABA_A$ receptors. Anesthetic drugs can perturb many systems; the crucial next step is in distinguishing targets that result in desirable anesthetic effects and undesirable side effects from those that are epiphenomena.

Materials and Methods

nAChRs and GABA Receptors in Xenopus *Oocytes*

The chick α4 and β4 nAChR subunit cRNAs were prepared from appropriate cDNAs in a modified PGH19 vector using standard techniques.[14] The rat α1, β1, γ2s $GABA_A$ subunit cRNAs were prepared from the appropriate cDNAs in a modified PGEM vector. The vector was linearized and used as a template for run-off transcription from the T7 (α4 nAChR and α1, β1, and γ2s $GABA_A$ receptors) or SP6 (β4 nAChR) promoter. *Xenopus laevis* oocytes were surgically removed from female frogs and defolliculated with collagenase. After incubation overnight in L-15 oocyte medium (Specialty Media, Phillipsburg, NJ), approximately 5 ng each subunit cRNA was injected per oocyte using a Nanoject Variable automatic injector (Drummond Scientific Co., 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, Inc., 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 $MgCl_2$, 10 mM HEPES, 1 mM $CaCl_2$, pH = 7.2. Calcium was omitted in experiments with $GABA_A$ receptors in oocytes. Experiments were performed at room temperature (20–24°C). Ketamine (Parke-Davis, Morris Plains, NJ) was prepared as a 1-mM stock solution in external recording solution. Thiopental (Sigma, St.Louis, MO) was prepared as a 10-mM solution on the day the experiments were conducted. Etomidate (Abbott Laboratories, Chicago, IL) was prepared as a 1-mM solution. Test solutions were prepared by serial dilution. All anesthetic drugs were racemic mixtures.

Oocytes were held at a membrane potential of −60 mV (unless specified otherwise), and peak current was measured in response to ACh or GABA, with and without anesthetic. The drug solutions were placed in a closed syringe at the time of application and injected into a closed loop of tubing with a volume of 1 ml. When activated by a manual switch, the drug containing solution joined the column of solution that bathes a specially prepared recording dish consisting of a 125-μl cylindrical channel. Perfusate was run continually at approximately 4 ml/min. Drugs were thus applied for an approximately 15-s pulse of known volume and concentration. The cells were perfused with the test concentration of

anesthetic for 5 min before agonist/anesthetic coapplication. Five minutes was allowed to elapse between repeated agonist application in all experiments to minimize the contribution of nAChR desensitization. This waiting time was adequate for recovery from desensitization in control experiments. A baseline control response to ACh or GABA was measured before each agonist–anesthetic coapplication. Response to agonist was measured after each anesthetic application. Responses that did not return to within 80% of baseline values were rejected for analysis. Each anesthetic response is expressed relative to the preceding control current amplitude. Unless otherwise indicated, test ACh concentrations were 1 mM, which is saturating for the $\alpha4\beta4$ receptor (data not shown). Test GABA concentration was 0.2 μM, EC_{20} derived in control experiments (data not shown). $N \geq 3$ for each data point.

Statistics and Curve Fitting

Concentration–response curves were prepared by plotting the fraction of current remaining after the coapplication of ACh and varying concentrations of intravenous anesthetic relative to the current response elicited by ACh alone. These data were fit to a Hill equation,

$$y = 100/(1 + (x/IC_{50})^n)$$

where y is the percentage of current remaining with antagonist application, and x is the concentration of antagonist. IC_{50} is the dose at which 50% of available receptors are modulated, and n is the Hill coefficient. All fitting algorithms and graphs were produced with Microcal Origins 5.0 software (Microcal Software Inc., Northamton, MA). Responses were acquired on-line using Pclamp7 (Axon Instruments), low pass filtered at 5 kHz, and digitized (Digidata 1200 interface; Axon Instruments) using Plcamp7 software. Data are fit using a nonlinear regression method within Microcal Origin. Data are expressed as mean ± SE.

$GABA_A$ Receptors in Human Embryonic Kidney Cells

cDNAs for human GABA $\alpha1$, $\gamma2s$ and rat $\beta2$ subunits were expressed in human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Rockville, MD) *via* the pCIS2 vector, which contains the strong promotor from cytomegalovirus and a polyadenylation sequence from SV40. HEK cells were cultured in Eagle's minimal essential media (Sigma) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), L-glutamine (0.292 μg/ml; Gibco BRL, Grand Island, NY), penicillin G sodium (100 U/ml; Gibco BRL), and streptomycin sulfate (100 μg/ml; Gibco BRL). For electrophysiology, cells were plated on glass coverslips coated with poly-D-lysine (Sigma). The cells were transfected using the calcium phosphate precipitation technique.[15]

Electrophysiologic recordings were performed at 22°C using whole-cell patch-clamp technique as previously described.[15] The coverslips were transferred 24–72 h after removal of the cDNA to a 70-ml chamber that was continuously perfused (2–3 ml/min) with extracellular medium containing 145 mM NaCl, 3 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM D-glucose, and 10 mM HEPES, pH 7.4. The intracellular solution contained 145 m[scap[m *N*-methyl-D-glucamine hydrocloride, 5 mM K_2ATP, 5 mM HEPES/KOH, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 1.1 mM EGTA, pH 7.2. Pipette-to-bath resistance was 4–6 MΩ. Cells were voltage clamped at −60 mV. The test GABA concentration was always EC_{20}. GABA and ketamine were applied with rapid solution changes (< 50 ms exchange time) to the cell by local perfusion using a motor-driven solution exchange device (Bio Logic Rapid Solution Changer RSC-100; Molecular Kinetics, Pullman, WA).[16] The solution changer was driven by protocols within the acquisition program Pclamp5 (Axon Instruments). Each data point represents five current measurements. Responses were digitized (TL-1-125 interface; Axon Instruments) using Pclamp5 and stored for off-line analysis. All concentration–response curves are plotted and analyzed using Microcal Origin 5.0 (Microcal Software Inc.). Data are expressed as mean ± SE. Data are fit using a nonlinear regression method within Microcal Origin.

Results

The three intravenous anesthetics studied—ketamine, thiopental, and etomidate—inhibit the ACh activation of the $\alpha4\beta4$ nAChR; however, the effects of only ketamine and possibly thiopental occur in a clinically relevant concentration range (figs. 1A–F).

Ketamine

Ketamine at its clinical EC_{50} almost completely inhibits ACh-gated current from the $\alpha4\beta4$ nAChR (fig. 1A). The inhibition by ketamine was readily reversible on washout of the drug. The application of ketamine alone at concentrations up to 1 mM had no direct effect on baseline membrane current. The inhibition by ketamine of the ACh response of the $\alpha4\beta4$ nAChR is concentration-dependent (fig. 1B). When the concentration–re-

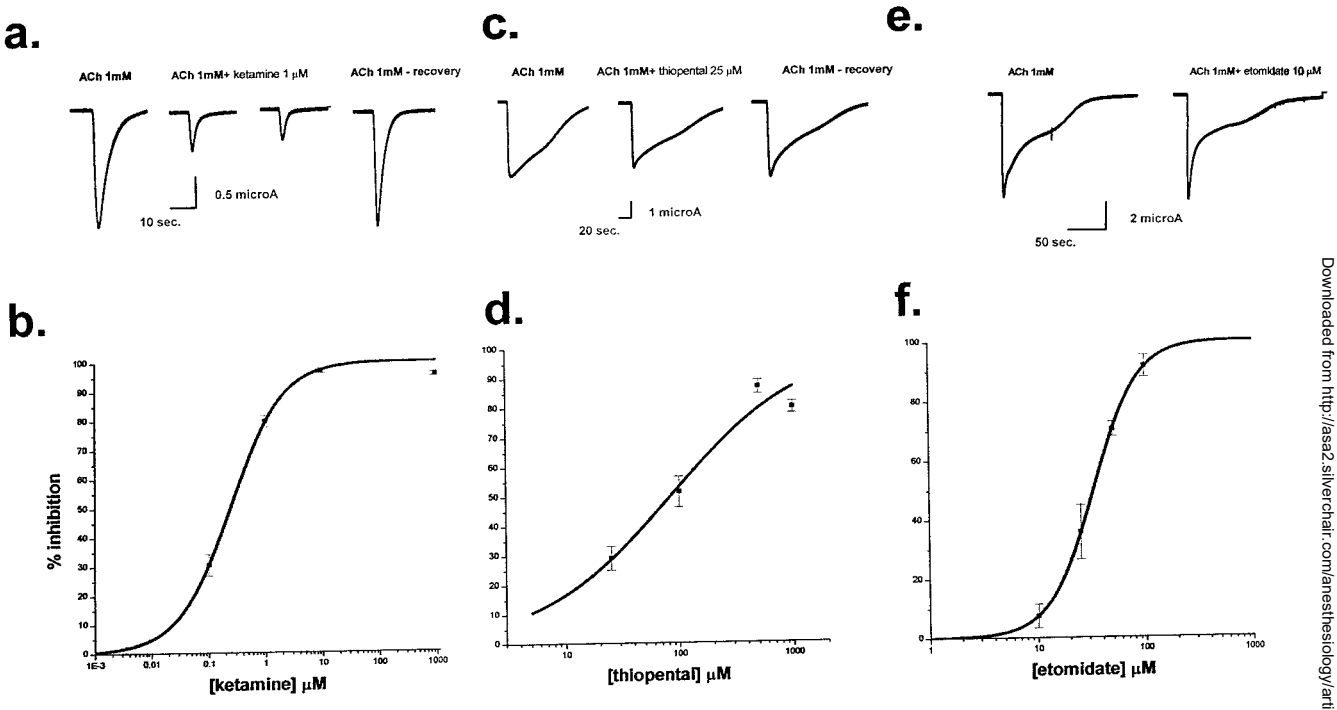


Fig. 1. Intravenous anesthetics, near clinical EC_{50}, differentially modulate nAChRs expressed in *Xenopus* oocytes. The modulation is concentration-dependent. (*A*) Ketamine 1 μM reversibly inhibits the maximal α4β4 nAChR response to 1 mM ACh by 80%. (*B*) Ketamine inhibition is concentration-responsive with an IC_{50} of 0.24 (± 0.3) μM and a Hill coefficient of 0.95 (± 0.1; n = 12). (*C*) Thiopental 25 μm reversibly inhibits the α4β4 nAChR response to ACh by 20%. (*D*) Thiopental at high clinical concentrations causes concentration-dependent inhibition with an IC_{50} of 84 (± 22) μM and a Hill coefficient of 0.75 (± 0.15; n = 12). (*E*) Etomidate 10 μM does not significantly inhibit the α4β4 nAChR response to ACh. (*F*) Etomidate at greater than clinical concentrations causes concentration-dependent inhibition with an IC_{50} of 33 (± 0.08) μM and a Hill coefficient of 2.1 (± 0.01; n = 12). Anesthetics were applied for 5 min before agonist–anesthetic coapplication.

sponse curve for ketamine inhibition of current response to 1 mM ACh is fit by the Hill equation, the IC_{50} is determined to be 0.24 μM (± 0.03), and the Hill coefficient is 0.95 (± 0.01). After 5 min of ketamine preapplication, the first response to the application of agonist with ketamine is always larger than the response to subsequent applications (fig. 2A). Inhibition of the α4β4 nAChR response by any ketamine concentration is more potent when activated by a higher agonist concentration (fig. 2B). The inhibition of ACh response is not dependent on membrane holding potential from −30 to −60 mV (fig. 3).

In marked contrast to the potent modulation by ketamine at nAChRs, ketamine does not modulate the function of the GABA α1β2γ2s receptor at clinically relevant concentrations. A clinically relevant concentration of 1 μM ketamine does not alter the response to GABA in this receptor when expressed in HEK cells (fig. 4) or *Xenopus* oocytes (3 ± 6% potentiation, n = 3 oocytes). Ketamine did produce GABA potentiation in HEK cells but only at very high ketamine concentrations (> 500 μM), with an EC_{50} of 1.2 mM (± 0.6 mM) and a Hill slope of 2.7 (± 0.3; n = 60).

Thiopental

Thiopental produces weak inhibition of central nAChRs within the clinically relevant range (fig. 1C). Thiopental inhibition of the α4β4 nAChR is also concentration-dependent (fig. 1D). A concentration–response curve with thiopental inhibition of the response to 1 mM ACh, fit by the Hill equation as above, yields an IC_{50} of 84 μM (± 22) thiopental and a Hill coefficient of 0.75 (± 0.15; n = 12).

Etomidate

Etomidate does not alter the current response to ACh in a clinically relevant concentration range (fig. 1E). Etomidate inhibition of the α4β4 nAChR is concentration-dependent but occurs at well above a clinically relevant concentration range (fig. 1F). Etomidate inhibits

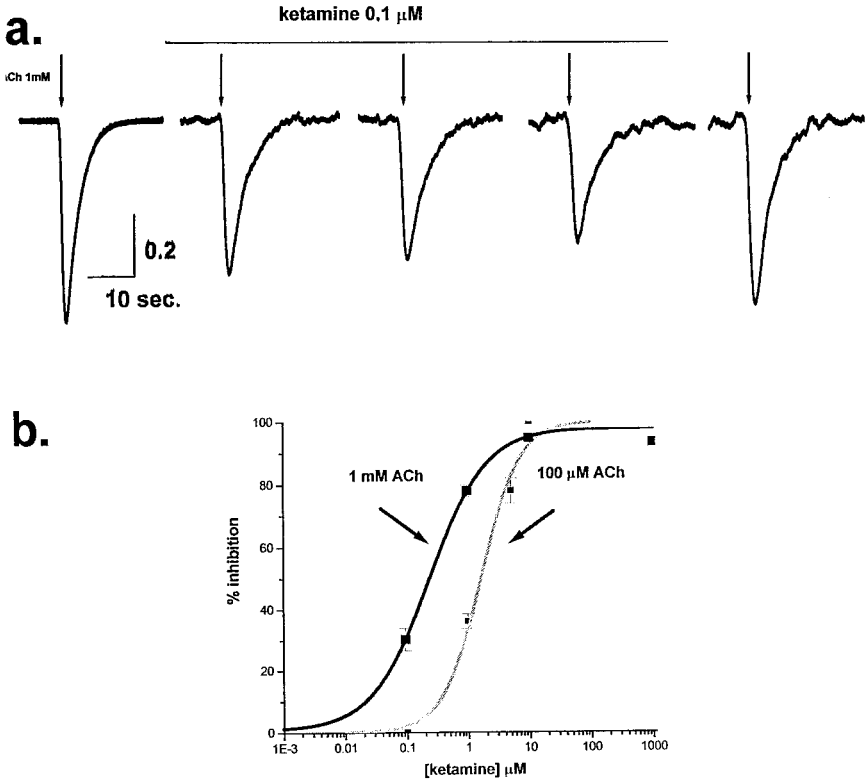


Fig. 2. Evidence for use dependence of ketamine inhibition of the $\alpha4\beta4$ nAChR. (*A*) After 5-min pretreatment with ketamine 0.1 μM, repeated applications of ketamine plus agonist result in increased inhibition. Inhibition with the first coapplication is 20%, with the second is 30%, and with the third is 38%. (*B*) Ketamine inhibition of the $\alpha4\beta4$ nAChR response to 1 mM ACh is more potent than ketamine inhibition of the response to 100 μM ACh. When ACh 100 μM is the agonist, the IC_{50} for ketamine is 1.63 (± 0.28) μM, and the Hill coefficient is 1.38 (± 0.27).

the $\alpha4\beta4$ nAChR response to ACh at high concentrations, with an IC_{50} of approximately 33 μM (± 0.1) etomidate and a Hill number of 2.1 (± 0.0; n = 12).

Discussion

The $\alpha4\beta4$ nAChR is differentially inhibited by intravenous general anesthetics. Ketamine, a dissociative anesthetic, is best known as a potent inhibitor of the *N*-methyl-D-aspartate (NMDA) receptor.[17,18] Ketamine inhibits the NMDA receptor at concentrations between 2 and 50 μM.[17] These data demonstrate that ketamine is a more potent inhibitor of a central nAChR. Ketamine inhibition of the $\alpha4\beta4$ nAChR response to 1 mM ACh is more potent than inhibition of the response to 100 μM ACh (fig. 2B). Thus, when a greater proportion of the nAChRs are in the open state, ketamine is a more potent inhibitor. These data suggest that the inhibition by ketamine of the neuronal nAChR is use-dependent, as it is in the NMDA receptor and muscle nAChR.[18,19] As further evidence for use dependence, the first response to the coapplication of ketamine and agonist is larger than subsequent responses, regardless of preincubation time

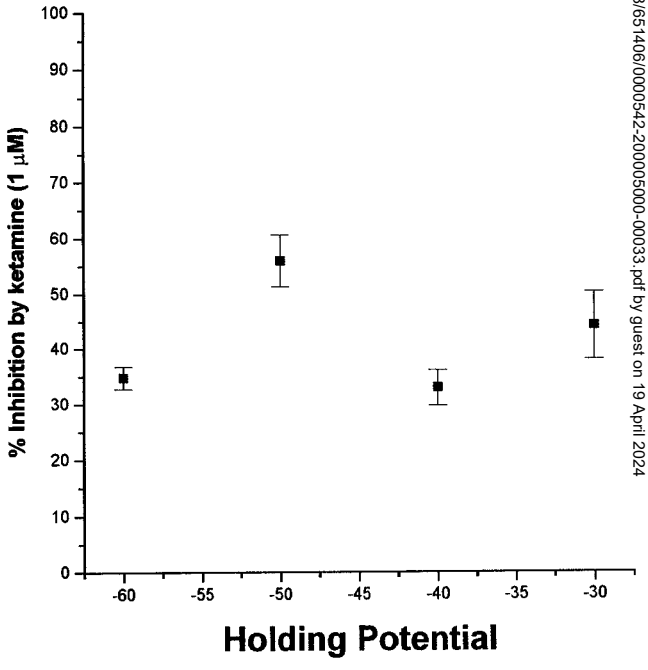
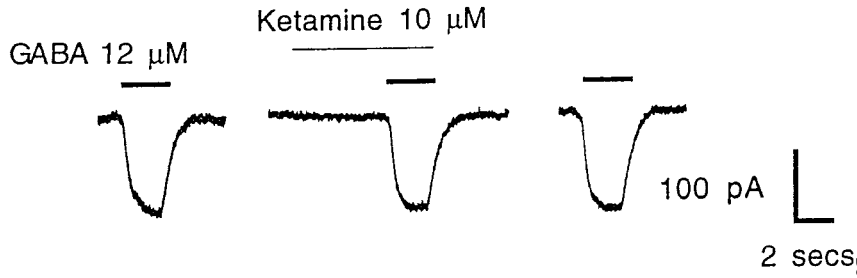


Fig. 3. Lack of voltage dependence of ketamine inhibition. Inhibition of $\alpha4\beta4$ nAChR response to 1 mM ACh is not dependent on membrane holding potential between −30 and −60 mV (n = 16).



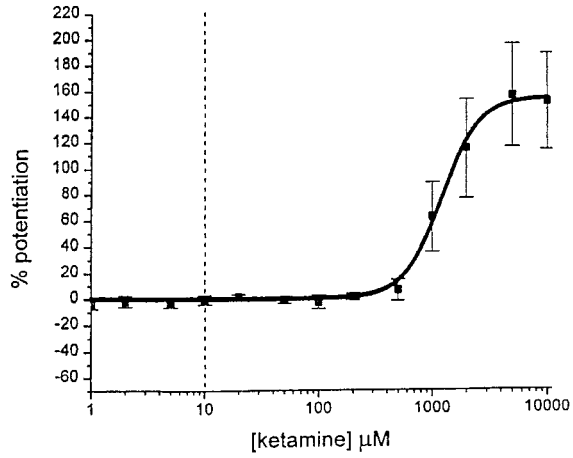


Fig. 4. Ketamine does not modulate $GABA_A$ receptor function in HEK 293 cells at clinically relevant concentrations. (*A*) Ketamine 10 μM does not modulate the $\alpha1\beta2\gamma2s$ $GABA_A$ receptor. (*B*) Ketamine at higher-than-clinical concentrations causes concentration potentiation of GABA response with an EC_{50} of 1.2 mM (± 0.06 mM) and a Hill slope of 2.7 (± 0.3). Maximal efficacy of potentiation is 154% (± 4; n = 60).

with ketamine (fig. 2A). These data suggest that ketamine requires access to the nAChR in the open state for inhibition, although they do not prove that ketamine acts as an open channel blocker as at the NMDA receptor.[18] Inhibition by ketamine is insensitive to the membrane holding potential (fig. 3). The insensitivity of inhibition by this positively charged drug to static voltage changes makes a binding site near the surface of the channel likely. Further mechanistic information will come from experiments in small cells or excised patches that are more suited to mechanistic analysis.

Unlike many other anesthetics, ketamine does not affect $GABA_A$ or glycine receptors at clinically relevant concentrations (fig. 4).[20] This is the first complete concentration–response curve for ketamine potentiation at a recombinant $GABA_A$ receptor, although potentiation at high concentrations has been shown previously.[6] The anesthetic effects of ketamine might be explained by inhibition of the nACh and NMDA receptors. *Xenopus* oocytes also contain an endogenous calcium-gated chloride channel. Heteromeric nAChRs have a relatively small fractional calcium conductance compared with homomeric $\alpha7$ nAChRs,[21] and if the general anesthetics were acting on this current, this would be expected to be a small effect. Effects at other undescribed targets may complement these effects.

Patients anesthetized with anesthetics that inhibit nAChRs (ketamine and volatile anesthetics) have analgesia at subanesthetic concentrations.[22,23] Analgesia with ketamine is present at blood ketamine concentrations of 0.08–0.32 μM.[11] The anesthetics, which do not inhibit nAChRs in the clinical range, such as etomidate and propofol, are not analgesic.[22] Inhibition of nAChRs is thus a candidate mechanism for general anesthetic analgesia. Nicotinic agonists are well known to have analgesic properties. Epibatidine, a potent, specific nicotinic agonist, has analgesic potency 200 times that of morphine.[24] Analgesia by agonist and antagonist need not be paradoxical. It is possible that agonist-induced analgesia occurs because of prolonged desensitization of nicotinic receptors.

Ketamine induces a state of consciousness that is different from that induced by other anesthetics. "Ketamine as a sole anesthetic produces a cataleptic state with nystagmus and intact corneal and light reflexes."[25] Ketamine is also the only anesthetic that does not potentiate $GABA_A$ receptors in a clinically relevant range, with the exception of nitrous oxide and xenon, which, although less extensively studied, may also inhibit the NMDA receptor (fig. 4).[26–28] Although it is currently not possible to explain the mechanism of consciousness, it may be that anesthetics other than ketamine produce their particular state of unconsciousness *via* potentiation of the $GABA_A$ response. Both the native transmitter GABA and the benzodiazepines that act specifically at $GABA_A$ receptors cause unconsciousness.

The anesthetic state achieved with ketamine is better termed "inattentiveness to surroundings" than "unconsciousness." With volatile anesthetics, that inattentiveness is perhaps overshadowed by unconsciousness during the anesthetic but can be appreciated on emergence from anesthesia. Patients typically awaken from a general anesthetic with, for example, 0.2% end-tidal isoflurane, measured by a gas analyzer (approximately 56 μM in solution). At this concentration, there remains approximately 20% central nicotinic inhibition, while $GABA_A$ potentiation is at threshold.[1,15] Drugs that do not inhibit nAChRs in a clinically relevant range, particularly propofol, are notable for the lack of inattentiveness and dysphoria on emergence from anesthesia. Prolonged inattentiveness, dysphoria, and perhaps other side effects of ketamine and volatile anesthetics may be the result of lingering nicotinic blockade. In particular, elderly patients and those suffering from Alzheimer's or Parkinson's disease, who have impaired central nicotinic systems, often emerge from anesthesia inattentive, disoriented, and dysphoric.[22,29] A common treatment for this "emergence delirium" is physostigmine, an acetylcholinesterase inhibitor that would increase the concentration of acetylcholine in the brain. These patients may represent a subgroup, which, because of preexisting abnormalities in their central cholinergic system, is particularly sensitive to nicotinic blockade by general anesthetics. It may be that this is a group of patients in which it is best to avoid general anesthetics that inhibit nAChRs. This area warrants further study and consideration.

Comparison of patterns of ligand-gated ion channel modulation with patterns of anesthetic behavior results in several hypotheses. The inhibition of nAChRs by general anesthetics may mediate analgesia, as well as inattentiveness and delirium. $GABA_A$ augmentation may lead to a particular state of unconsciousness. Validation of these hypotheses and the definition of the central circuitry in which they occur will require further experiments. If these hypotheses prove valid, the designers of future anesthetic drugs will be able to test agents on heterologously expressed ion channels to design *in* the desirable and *out* the undesirable behavioral responses in humans.

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