Isoflurane Hyperalgesia Is Modulated by Nicotinic Inhibition

Pamela Flood, M.D.,* James M. Sonner, M.D.,† Diane Gong, B.S.,‡ Kristen M. Coates, B.S.§

Background: The inhaled anesthetic isoflurane inhibits neuronal nicotinic acetylcholine receptors (nAChRs) at concentrations lower than those used for anesthesia. Isoflurane produces biphasic nociceptive responses, with both hyperalgesia and analgesia within this concentration range. Because nicotinic agonists act as analgesics, the authors hypothesized that inhibition of nicotinic transmission by isoflurane causes hyperalgesia.

Methods: The authors studied female mice at 6–8 weeks of age. They measured hind paw withdrawal latency at isoflurane concentrations from 0 to 0.98 vol% after the animals had received a nicotinic agonist (nicotine), a nicotinic antagonist (mecamylamine or chlorisondamine), or saline intraperitoneally. In addition, the authors tested the interactions between mecamylamine and isoflurane and nicotine and isoflurane in heterologously expressed α4β2 nAChRs.

Results: Female mice had significant hyperalgesia from isoflurane. Nicotine administration prevented isoflurane-induced hyperalgesia without altering the antinociception produced by higher isoflurane concentrations. Mecamylamine treatment caused a biphasic nociceptive response similar to that caused by isoflurane. Mecamylamine and isoflurane had an additive effect, both at heterologously expressed α4β2 nAChRs and on the production of hyperalgesia in vivo. Mecamylamine thus potentiated hyperalgesia but did not affect analgesia.

Conclusions: Since hyperalgesia occurs in vivo at isoflurane doses that antagonize nAChRs in vitro, isoflurane is prevented by a nicotinic agonist, and is mimicked and potentiated by nicotinic antagonists, the authors conclude that isoflurane inhibition of nAChRs activation is involved in the pathway that causes hyperalgesia. At subanesthetic doses, isoflurane can either enhance pain responses (produce hyperalgesia) or be analgesic (antinociceptive). In rats, low volatile anesthetic concentrations (0.1–0.2 minimum alveolar concentration [MAC]) elicit hyperalgesia, while 0.4–0.6 MAC elicits antinociception.

The mechanisms by which isoflurane acts as an analgesic and anesthetic are unknown. However, a current hypothesis suggests that isoflurane acts by inhibiting synaptic transmission. This may result from the modulation of the function of ligand-gated ion channels.1,2 Isoflurane modulates GABAergic, glycine, glutamate, and nicotinic acetylcholine receptors (nAChRs) at clinically relevant concentrations.3–9 Nonetheless, despite ample evidence for modulation of the above ion channels at appropriate anesthetic concentrations, a link between modulation of specific ion channels and anesthetic-induced behavior has not been established.

Nicotinic acetylcholine receptors are the most potently modulated target of inhaled anesthetics (i.e., they are blocked at the lowest multiple of minimum alveolar concentration [MAC]).2 Their inhibition occurs at concentrations well below MAC. The IC50 values for the inhibition of heteromeric nAChRs by isoflurane are between 0.2 and 0.3 MAC.10,11 Nicotinic agonists are known to be potent analgesic agents. Epibatidine, a nicotinic agonist, is approximately 200 times as potent as morphine for analgesia.12,13 Because isoflurane inhibits heteromeric nAChRs in the same concentration range that produces hyperalgesia and because nicotinic agonists are potent analgesic agents, we hypothesized that isoflurane exerted its hyperalgesic effects by antagonism of nAChRs. To test this hypothesis, we studied the effect of isoflurane, a nicotinic agonist, and two nicotinic antagonists on nicotinic responses in vitro and pain behavior in vivo.

Materials and Methods

Behavior

With approval of the UCSF Committee on Animal Research (San Francisco, California), we studied female, 129J strain mice at 6–8 weeks of age that weighed 15–20 g and were obtained from the Jackson Laboratories (Bar Harbor, ME). We measured hind paw withdrawal latency (HPWL) with a modification of the automatic device (Plantar Tes; Ugo Basile Biologic Research Apparatus, Comerio, Italy) described by Hargreaves et al.14 in up to five unrestrained mice (per study) housed individually in clear plastic chambers. The chambers rested on a clear glass plate. Over the chambers, we placed a clear Plexiglas enclosure that rested on a silicone rubber gasket that produced a seal to the glass plate. Gas-tight fittings at either end permitted delivery and scavenging of isoflurane. Isoflurane in oxygen was delivered from a variable-bypass vaporizer. Concentrations of isoflurane were monitored with an infrared analyzer (RGM; Datex-Ohmeda, Madison, WI) and were analyzed at the end of each concentration step with gas chromatography. The chromatograph reading was accepted as the value for the exposure concentration. Heating strips warmed the glass plate to minimize body heat loss. To diminish exploratory activity, the mice were acclimatized to this environment for at least 30 min before commencing the study. After acclimation, a mov-
ISOFLURANE INHIBITS TONIC NICOTINIC ANALGESIA

able source of radiant heat was applied from a projector lamp (Radium tungsten halogen lamp, model EJY, 19 V, 80 W; General Electric, Glen Allen, VA) through a 7-mm aperture under the glass plate to the hind paw of the resting mouse. A photocell within the housing that surrounds the lamp sensed the light reflecting from the hind paw of the mouse (i.e., whether the paw remained in place). The device automatically measured the time from the onset of application of the light (heat) to the time the mouse moved the hind limb (as determined by the moment the light no longer reflected from the paw to the photocell).

Animals were allocated into four study groups: saline, mecamylamine (Sigma, Milwaukee, WI), chlorisondamine (Tocris, Ballwin, MO), or nicotine (Sigma). Each drug was administered by intraperitoneal injection. Five to 28 mice were studied per group. Some mice were used for more than one study, and at least 2 days separated such studies. In all experiments, an HPWL measurement was made for each hind paw 5 times (total of 10 measurements). Measurements on each paw were made at approximately 5-min intervals. The 10 readings were averaged to produce the value for each control or anesthetic level. After obtaining control measurements, isoﬂurane (Abbot Laboratories, North Chicago, IL) was delivered in a stepwise manner at inspired concentrations of 0.14, 0.28, 0.56, 0.84, and, in some cases, 0.98% inspired concentration of isoﬂurane (i.e., 0.1, 0.2, 0.4, 0.6, and 0.7 MAC; MAC for isoﬂurane equals 1.4% in these mice [data not shown]). At the end of equilibration, we determined HPWL. After the final equilibration, anesthetic delivery was discontinued, and after 1 h, we again measured HPWL to demonstrate recovery. All animals returned to control HPWL within 1 h.

In all experiments with mecamylamine, chlorisondamine, and their saline controls, animals were injected intraperitoneally with mecamylamine in a saline solution or saline (control) at a volume of 10 ml/kg, at least 30 min before HPWL testing. The duration of mecamylamine’s action was tested with two control experiments. First, five mice were injected with 5 mg/kg intraperitoneal mecamylamine or saline. These mice were then tested at 1-h intervals with 1 mg/kg intraperitoneal nicotine. The mice that were previously treated with mecamylamine did not show prostration from the nicotine for up to 4 h, indicating continued blockade by mecamylamine during this time period. Untreated mice lay prone within 5 min. Second, to determine whether the antinociceptive effects of mecamylamine were stable over the testing period, five mice were tested immediately with 0.84% isoﬂurane, which is normally the anesthetic concentration tested 3 h after mecamylamine treatment. There was no signiﬁcant difference in the response to 0.84% isoﬂurane whether the mice were tested at 1 or 3 h after treatment with mecamylamine. Mecamylamine plasma concentration was measured in ﬁve female mice, 1 h after intraperitoneal injection using a combination of gas chromatography and mass spectroscopy described by Jacob et al.15

Because nicotine’s analgesic effect is known to be short lived,16 mice were injected intraperitoneally with 1 mg/kg Sc(-)-nicotine (Sigma) or saline in a total volume of 10 ml/kg, 5 min before HPWL testing. In mice, nicotine at 1 mg/kg reaches a peak concentration of approximately 2 μM at 5 min and is undetectable by HPLC at 40 min.17 In studies with nicotine, mice breathed oxygen or the desired anesthetic concentration for 25 min, were injected with nicotine or saline, and then were reequilibrated for 5 min prior to HPWL testing. Injections of nicotine were separated by at least 1 h. As each testing period lasted approximately 25 min, we determined the peak effects of nicotine with this methodology. The first HPWL measurements for each paw were not signiﬁcantly different than the last HPWL measurements in these experiments. The control animals studied with the multiple injection protocol had a slightly different baseline than control animals that did not receive multiple injections; thus, animals tested with nicotine were compared to their own controls.

Electrophysiology

The human α4 and β2 type nAChRs were in a pSP64 expression vector. Standard techniques were used to linearize the vectors and use them as templates to make cRNA using SP6 as the polymerase. The human nicotinic clones were a gift from Dr. Jon Lindstrom, Ph.D. (Department of Neuroscience, University of Pennsylvania, Philadelphia, Pennsylvania). Xenopus laevis oocytes were removed from the females and defolliculated with collagenase. After the oocytes rested for 24 h in L-15 oocyte medium, about 10 ng of a 1:1 ratio of α4 to β2 cRNA were injected into individual oocytes. A manual injector was used for this process (Nanoject; Drummond Scientific, Broomall, PA). The oocytes were incubated for 2–5 days in ND-96 medium (96 mm NaCl, 2 mm KCl, 1 mm MgCl2, 1.8 mm CaCl2, H2O, 5 mm HEPES, 2.5 mm Na-pyruvate, 0.5 mm theophylline, and 10 mg/L gentamicin, adjusted to pH 7.5).

Whole oocytes were used to record currents using a Gene-Clamp 500 two-microelectrode voltage-clamp amplifier with an active ground (Axon Instruments, Inc., Foster, CA). The recording electrodes were pulled from glass capillary tubing (Drummond) to obtain a resistance between 1 and 5 MΩ and were filled with 3 m KCl. Ba2+ Ringer’s solution was used as the extracellular solution to avoid current amplification by calcium-activated chloride currents (115 mm NaCl, 25 mm KCl, 1.8 mm BaCl2, 10 mm HEPES, 1 μM atropine, pH 7.4). Atropine was included to avoid activation of intrinsic muscarinic receptors. Experiments were performed at room temperature. Isoflurane was prepared from a saturated solution.
by serial dilution. Concentrations were verified by gas chromatography.

Oocytes were tested at a membrane potential of $-60$ mV. Bolus application of the agonist $\pm$ indicated antagonist(s) was applied at a rate of $4$ ml/min for a $2$-s application. Antagonists were preapplied for $2$ min prior to activation. Concentration-response curves were made from the percent change in peak current from acetylcholine (ACh) activation in the presence of antagonist(s), compared to ACh alone. As the ACh concentration at central neuronal nAChRs is unknown, 1 mM ACh (saturating) was used to detect inhibition by isoflurane and mecamylamine experiments with mecamylamine. ACh, $2 \mu$m, was used to detect potentiation by nicotine. Currents were measured in five to eight cells for each data point. Clampex 7 (Axon Instruments) was used for data acquisition.

Statistical Analysis

Microcal Origins 5.0 (Microcal, Northampton, MA) was used for statistical calculations and graphical presentation. The in vitro data were fit to a modified Hill equation,

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

where $IC_{50}$ is the concentration of drug at which of 50% of the response is inhibited, and $n$ is the Hill coefficient. Interaction between isoflurane and mecamylamine was interpreted using an isobolographic analysis in which the concentrations that cause 50% inhibition of $\alpha_2\beta_2$ nAChR activation are displayed graphically with a line of additivity and 95% confidence intervals. The concentrations of the combined drugs that cause 50% inhibition are displayed, those that fall within the 95% confidence intervals are considered to interact additively.

Hind paw withdrawal latency data for females in response to isoflurane and mecamylamine had a biphasic response; thus, the extent of maximal hyperalgesia in the presence of isoflurane was compared to baseline with a paired $t$ test, using the Bonferroni correction for multiple comparisons.

Results

Isoflurane Hyperalgesia in Female Mice

The nociceptive response produced by 0.28–0.98% isoflurane was tested by measuring HPWL in female mice (fig. 1). The mice were significantly hyperalgesic while breathing 0.28% isoflurane as compared to the oxygen control (fig. 1; $t$ test, $P < 0.01$). HPWL returned to baseline at 0.56% isoflurane, and higher isoflurane concentrations resulted in progressively increasing analgesia. HPWL returned to baseline by 1 h after isoflurane washout in all mice (data not shown).

Fig. 1. Nociceptive response to isoflurane in female mice. Hind paw withdrawal latency (HPWL) is shown for female mice given isoflurane from 0 to 0.98 vol% (0–256 $\mu$m). Female mice have a biphasic nociceptive response to subanesthetic concentrations of isoflurane. At a low 0.28% isoflurane, the mice have reduced HPWL to 83% of control ($t$ test, $P < 0.01$). HPWL returns to baseline at 0.56% isoflurane, and at higher concentrations, there is concentration-dependent antinociception. Symbols represent mean ± SE; n = 20.

Behavioral Effects of Nicotinic Antagonists

Mecamylamine intraperitoneally administered to female mice caused a biphasic response, with significantly increased nociception at 2 and 4 mg/kg intraperitoneally ($t$ test; $P < 0.001$) and analgesia at doses of 5 mg/kg and greater (fig. 2). Mice assumed a hunched posture, made rapid back-and-forth rocking motions, and aggressively groomed themselves after injection of mecamylamine at 7.5 or 10 mg/kg. The plasma concentration of mecamylamine was measured with gas chromatography–mass spectroscopy 1 h after intraperitoneal injection of 5 mg/kg in female mice and was found to be $203 ± 67$ nm.

Fig. 2. Nociceptive response to mecamylamine in female mice. Female mice have a biphasic nociceptive response to the non-competitive nicotinic inhibitor mecamylamine. In female mice, 2 or 4 mg/kg intraperitoneal mecamylamine reduces hind paw withdrawal latency (HPWL) compared to control ($t$ test, $P < 0.001$). Mecamylamine, 7.5–10 mg/kg, increased HPWL in females. Symbols represent mean ± SE; n = 10.
ISOFLURANE INHIBITS TONIC NICOTINIC ANALGESIA

Interaction of Isoflurane and Mecamylamine on the Activation of α₄β₂ nAChRs Expressed in Xenopus Oocytes

Isoflurane caused hyperalgesia in female mice within the same low concentration range (0.28–0.56% or 63–128 μM) as α₄β₂ nAChRs were inhibited in vitro (10,11,21) (fig. 5). To provide additional evidence for a role for the nAChR in the nociceptive response to isoflurane, we studied the effects of nicotine and mecamylamine on isoflurane inhibition of α₄β₂ nAChRs at concentrations relevant to those used in the behavioral experiments.

Both isoflurane and mecamylamine act as noncompetitive antagonists at heteromeric nAChRs (figs. 5A and B).11,22 To study the role of nicotinic modulation in the isoflurane nociceptive response, we evaluated the interaction between isoflurane and mecamylamine in vitro on heteromeric nAChRs. Figure 4A shows representative current traces from α₄β₂ nAChRs activated by 1 mM ACh alone, in the presence of 44 μM isoflurane or 0.2 μM mecamylamine. The half-maximal inhibitory concentration for isoflurane inhibition of α₄β₂ nAChRs was 44 μM. The concentration of mecamylamine chosen for study was approximately IC₅₀ for inhibition of the α₄β₂ nAChR (0.29 ± 0.05 μM) and was close to the mecamylamine concentration measured in plasma from female mice injected with 5 mg/kg (0.20 ± 0.07 μM). A concentration-response relationship for inhibition of α₄β₂ nAChRs by isoflurane with and without mecamylamine 0.2 μM is shown in figure 5B. Isobolographic analysis in figure 5C indicates that inhibition of α₄β₂ nAChR activation by mecamylamine and isoflurane applied together is within the 95% confidence intervals for additivity.

As expected, the addition of 2 μM nicotine (the approximate concentration measured by HPLC in a mouse injected with 1 mg/kg intraperitoneal nicotine)17 to 2 μM ACh produces a larger current than ACh alone (fig.

Fig. 3. Effect of nicotinic antagonists on isoflurane nociception in female mice. The nicotinic antagonist mecamylamine (2 mg/kg intraperitoneally) causes hyperalgesia at baseline in female mice (t test, P < 0.001; n = 10). Mecamylamine (square) increased the reduction in hind paw withdrawal latency (HPWL) caused by 0.28% isoflurane (t test, P < 0.01; n = 10). Mecamylamine has no significant effect on antinociception caused by higher isoflurane concentrations. Chlorisondamine (triangle) caused hyperalgesia. Points are means ± SE. Symbols represent mean ± SE.

Fig. 4. Effect of nicotine on isoflurane nociception in female mice. Female mice in the presence of nicotine do not have hyperalgesia at low isoflurane concentrations. Antinociception produced by higher concentrations of isoflurane is not affected by nicotine. Note that these experiments were conducted using a different protocol from those in figures 1 and 3; thus, the control values are different. Symbols represent mean ± SE, n = 10.

Anesthesiology, V 97, No 1, Jul 2002
In the presence of a given concentration of isoflurane, currents generated with 2 \( \mu M \) ACh plus 2 \( \mu M \) nicotine are always larger than those generated by 2 \( \mu M \) ACh alone.

Discussion

We present several lines of evidence, summarized in cartoon form in figure 7A, that suggest that the nociceptive action of isoflurane is due to the inhibition of heteromeric nicotinic receptors by isoflurane, while the analgesic phase is mediated by another mechanism.

1. Isoflurane inhibits the activation of the most common nicotinic subunit combination expressed in the central nervous system within the same concentration range in which hyperalgesia occurs in vivo (figs. 1 and 4). Although our in vitro experiments were conducted on nAChRs of human origin, little difference in the effect of isoflurane between species as diverse as chick, rat, and human has been identified.\(^{10,11,21}\)

2. Mecamylamine and isoflurane, both noncompetitive nicotinic inhibitors, cause a similar biphasic nociceptive response in the female, with hyperalgesia at low concentrations that are more specific for nicotinic inhibition.\(^{23–25}\) Mecamylamine potentiates the hyperalgesia caused by isoflurane (fig. 3). Chlorisondamine, another nicotinic antagonist, at 10 mg/kg also causes hyperalgesia (fig. 3), presumably through inhibition of tonic nicotinic activity.

3. Nicotine, an agonist, specifically prevents isoflurane hyperalgesia in females at a concentration that does not cause analgesia alone or effect analgesic concentrations of isoflurane (fig. 4).

Taken together, these findings suggest that nicotinic blockade mediates isoflurane’s hyperalgesic effect, while other mechanisms may contribute to isoflurane’s analgesic actions. It is unlikely that isoflurane analgesia is caused by heteromeric nicotinic inhibition as it is unaffected by nicotine and mecamylamine. At high concentrations, both isoflurane and mecamylamine are known
to have activity other than nicotinic targets. Isoflurane modulates the activation of receptors for GABA, 5,5,6 glycine, S,8 and glutamate 7,9 at concentrations higher than those relevant for nAChR inhibition. Mecamylamine has NMDA antagonist properties at concentrations in the 100–1000 μM range. 23,26 Mecamylamine inhibits seizures induced by NMDA with an ED50 of 12 ± 3.2 mg/kg. 24 The analgesic properties of high concentrations of isoflurane and mecamylamine are more likely to be mediated through one or more of the above or other mechanisms. 27,28 The analgesic activity of nicotinic agonists is mediated, in part through modulation of the descending 5HT3 projections from the raphe magnus. 27,28 Cordero-Erausquin and Changeux 29 have recently proposed a model for nicotinic modulation of 5HT3 transmission based on pharmacologic modulation of 5HT3 release in the mouse spinal cord. They propose the existence of three pharmacologically distinct populations of nAChRs, including a tonically active presynaptic nAChR represented in figure 7B. While our experiments were not designed to differentiate between brain and spinal action of isoflurane or to detect interaction with other neurotransmitters, their model may suggest one potential mechanistic explanation for our findings. Inhibition of a tonically activated excitatory presynaptic receptor by isoflurane or mecamylamine at a low concentration would be expected to reduce the release of serotonin and on this basis cause hyperalgesia. The antinociceptive properties of systemically administered nAChR agents are also mediated by descending noradrenergic and muscarinic inhibitory pathways in addition to serotonergic pathways, and we cannot rule out the involvement of these systems. 28

The nicotinic analgesic system is particularly important, and tonically active in the female. All volatile anesthetics tested by Zhang et al. 30 produced hyperalgesia at low concentrations. The concentrations of volatile anesthetics that cause hyperalgesia in animals (0.1–0.38% isoflurane) are commonly present in patients on emergence from general anesthesia. The significant incidence of emergence agitation when volatile anesthetics are used may be in part due to hyperalgesia from residual anesthetic.

References


Fig. 7. Cartoon data summary. (A) The activation of nicotinic receptors provides analgesia. Exogenous nicotine can increase that analgesia. Low concentrations of isoflurane inhibit tonic nicotinic activity that is present in the female and cause hyperalgesia. Higher concentrations of isoflurane act on other targets to cause analgesia. Higher concentrations of mecamylamine act on other targets receptors to cause analgesia. (B) A possible molecular explanation. Cordero-Erausquin and Changeux 29 have provided evidence for three different nicotinic receptor subtypes that regulate descending serotonergic inhibition. One tonically active, heteromeric nicotinic acetylcholine receptor (nAChR) is located on the serotonergic terminals. This receptor would also be inhibited by low concentrations of isoflurane and mecamylamine, thus reducing the release of serotonin and causing hyperalgesia in females. As the analgesic action of isoflurane was not affected by mecamylamine or nicotine, it is also possible that the analgesic action of isoflurane and mecamylamine at high concentration is through interaction with other targets. Nicotinic acetylcholine receptors play a presynaptic role in inhibitory systems that utilize muscarinic and adrenergic receptors as well, and we have not ruled out the involvement of these systems.
modulation of human gamma-aminobutyric acid type A and glycine receptors by the inhalation anesthetic isoflurane. Mol Pharmacol 1993; 44:628–32
10. Flood P, Ramirez-Latorre J, Role L. αβ Neuronal nicotinic acetylcholine receptors in the central nervous system are inhibited by isoflurane and propofol, but α-type nicotinic acetylcholine receptors are unaffected. Anesthesiology 1997; 86:859–65
23. O’Dell TJ, Christensen BN: Mecamylamine is a selective non-competitive antagonist of N-methyl-D-aspartate- and aspartate-induced currents in horizontal cells dissociated from the catfish retina. Neurosci Lett 1988; 94:93–8

Anesthesiology, V 97, No 1, Jul 2002