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Volatile anesthetics and endogenous cannabinoid anandamide have additive and independent inhibitory effects on α_7 -nicotinic acetylcholine receptor-mediated responses in *Xenopus* oocytes

Shelley N. Jackson², Singhal K. Sachin³, Amina S. Woods², Marisela Morales², Toni Shippenberg¹, Li Zhang³, and Murat Oz¹

¹ National Institute on Drug Abuse, NIH/DHHS, Intramural Research Program, Integrative Neuroscience Section, 5500 Nathan Shock Drive, Baltimore MD, 21224, U.S.A.

² National Institute on Drug Abuse, NIH/DHHS, Intramural Research Program, Cellular Neurobiology Branch, 5500 Nathan Shock Drive, Baltimore MD, 21224, U.S.A.

³ National Institute on Alcohol Abuse & Alcoholism, NIH/DHHS, Laboratory for Integrative Neuroscience, 5625 Fishers Lane, Rockville, MD, 20852, U.S.A

Abstract

In earlier studies, the volatile anesthetics and the endogenous cannabinoid anandamide have been shown to inhibit the function of α_7 -nicotinic acetylcholine receptors. In the present study, interactions between the effects of volatile anesthetics and anandamide on the function of α_7 -nicotinic acetylcholine receptors expressed in *Xenopus* oocytes were investigated using the two-electrode voltage-clamp technique. Anandamide and volatile anesthetics isoflurane and halothane inhibited currents evoked with acetylcholine (100 μ M) in a reversible and concentration-dependent manner. Coapplication of anandamide and volatile anesthetics caused a significantly greater inhibition of α_7 -nicotinic acetylcholine receptor function than anandamide or volatile anesthetics alone. Analyses of oocytes by matrix-assisted laser desorption/ionization mass spectroscopy indicated that volatile anesthetics did not alter the lipid profile of oocytes. Results of studies with chimeric α_7 -nicotinic acetylcholine -5-HT₃ receptors comprised of the N-terminal domain of the α_7 -nicotinic acetylcholine receptor and the transmembrane and carboxyl-terminal domains of 5-HT₃ receptors suggest that while isoflurane inhibition of the α_7 -nicotinic acetylcholine receptor is likely to involve the N-terminal region of the receptor, the site of action for anandamide involves transmembrane and carboxyl-terminal domains of the receptors. These data indicate that endocannabinoids and isoflurane have additive inhibitory effects on α_7 -nicotinic acetylcholine receptor function through allosteric binding sites located on the distinct regions of the receptor.

Keywords

anandamide; volatile anesthetics; nicotinic receptors; *Xenopus* oocytes

Corresponding author: Murat Oz M.D., Ph.D., National Institute on Drug Abuse/IRP, Cellular Neurobiology Branch, 5500 Nathan Shock Drive, Baltimore, MD 21224, Phone: (410) 550-6565; Fax: (410) 550-1621; E-mail: moz@intra.nida.nih.gov.

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1. Introduction

Anandamide (arachidonylethanolamide) is an endogenous cannabinoid composed of an amide of arachidonic acid (Howlett et al., 2002). Anandamide, following its synthesis from lipid precursors in cell membranes via calcium and G-protein dependent processes, binds to cannabinoid receptors and mimics the pharmacological effects of Δ^9 -tetrahydrocannabinol (Δ^9 -THC), psychoactive active ingredient of marijuana (Howlett et al., 2002). However, several reports indicate that some of the effects of anandamide are not mediated by the activation of the cloned cannabinoid CB₁ and CB₂ receptors. For example, it has been demonstrated that anandamide modulates the functions of voltage-dependent-Ca²⁺ channels (Oz et al., 2000, 2004a; Chemin et al., 2001; IC₅₀= 1–10 μ M), Na⁺ channels (Nicholson et al., 2003; IC₅₀= 10 μ M), K⁺ channels (Poling et al., 1996; Maingret et al., 2001; Oliver et al., 2004; IC₅₀= 0.1–3 μ M), TRPV1 receptors (Smart et al., 2000; IC₅₀= 10–20 μ M), serotonin type 3 (5-HT₃) receptors (Barann et al., 2002; Oz et al., 2002a, IC₅₀= 0.2–4 μ M), and glycine (Gly) receptors (Lozovaya et al., 2005; Hejazi et al., 2006; IC₅₀= 0.2–1 μ M). These studies suggest that additional molecular targets for endocannabinoids exist in the peripheral and central nervous system (for a recent review Oz, 2006).

Similar to anandamide, volatile general anesthetics such as halothane and isoflurane also interact directly with voltage-dependent Ca²⁺ channels (Oz et al., 2001; 2002b), Na⁺ channels (Ouyang et al., 2003; Shiraishi and Harris, 2004), K⁺ channels (Patel et al., 1999; Yamakura et al., 2001a), 5-HT₃ receptors (Machu and Harris, 1994; Jenkins et al., 1996), and Gly receptors (Mihic et al., 1997). These studies suggest that anandamide and volatile anesthetics share common molecular targets in their pharmacological actions.

In recent years, changes in the levels of anandamide during a broad range of physiological and pathological conditions have been reported in mammalian tissues and biological fluids (for a review Cravatt and Lichtman 2004). Furthermore, general anesthetics such as propofol (Patel et al., 2003) and sevoflurane (Schelling et al., 2006) and drugs of abuse such as ethanol and cocaine alter anandamide levels in *in vivo* and *in vitro* studies (for reviews Basavarajappa and Hungund, 2002; Gonzalez et al., 2002). As a result of altered anandamide levels, changes in the density and the function of the cannabinoid receptors have also been shown in earlier studies (Basavarajappa and Hungund, 2002; Gonzalez et al., 2002). However, altered anandamide levels can also affect the actions of volatile anesthetics on the above mentioned common target proteins.

One of the common target proteins for anandamide (Oz et al., 2003b, 2004a; 2004b) and volatile anesthetics (Zhang et al., 1997; Mori et al., 2001; Flood and Coates, 2002) is α_7 -nicotinic acetylcholine receptors, a member of the ligand-gated ion channel super family (Lindstrom et al., 1996). The potential involvement of these receptors in pharmacological actions of volatile anesthetics has been reported in earlier studies (for reviews Tassonyi et al., 2002; Yamakura et al., 2002b). Since, both volatile anesthetics and anandamide are allosteric modulators of α_7 -nicotinic acetylcholine receptor function, the present study was performed to investigate if there is an interaction between the effects of anandamide and volatile anesthetics on the functional properties of α_7 -nicotinic acetylcholine receptors. The results indicate that volatile anesthetics and anandamide have additive inhibitory effects on the function of α_7 -nicotinic acetylcholine receptor by interacting with distinct regions of the receptor.

2. Methods

2.1. Recordings from oocytes

Mature female *Xenopus laevis* frogs were purchased from Xenopus laevis I (Ann Arbor, MI) and were housed in dechlorinated tap water at 19–21° C with 12/12 hour light/dark cycling,

and fed with beef liver twice a week. Clusters of oocytes were removed surgically under tricaine (Sigma, St. Louis, MO) local anesthesia (0.15 % w/v) and individual oocytes were dissected away manually in a solution containing (in mM): NaCl, 88; KCl, 1; NaHCO₃, 2.4; MgSO₄, 0.8; HEPES, 10 (pH 7.5). Later, dissected oocytes were stored 2–7 days in modified Barth's solution (MBS) containing (in mM): NaCl, 88; KCl, 1; NaHCO₃, 2.4; Ca(NO₃)₂, 0.3; CaCl₂, 0.9; MgSO₄, 0.8; HEPES, 10 (pH 7.5), supplemented with sodium pyruvate 2 mM, penicillin 10,000 IU/L, streptomycin 10 mg/L, gentamicin 50 mg/L, and theophylline 0.5 mM. The oocytes were placed in a 0.2 ml recording chamber and superfused at a constant rate of 4–5 ml/min. The bathing solution consisted of (in mM): NaCl, 95; KCl, 2; CaCl₂, 2; and HEPES 5 (pH 7.5). The cells were impaled at the animal pole with two standard glass microelectrodes filled with a 3 M KCl (1–10 MΩ). The oocytes were voltage-clamped routinely at a holding potential of –70 mV using GeneClamp-500 amplifier (Axon Instruments Inc., Burlingame, CA), and current responses were recorded directly on a Gould 2400 rectilinear pen recorder (Gould Inc., Cleveland, OH). Current-voltage curves were generated by holding each membrane potential in a series for 30 s, followed by a return to –70 mV for 10 min. Oocyte capacitance was measured by a paired ramp method described earlier (Oz et al., 2004c). Briefly, voltage ramps were employed to elicit constant capacitive current, I_{cap} , and the charge associated with this current was calculated by the integration of I_{cap} . Ramps had slopes of 2 V/s and durations of 20 ms, and started at a holding potential of –90 mV. A series of 10 paired-ramps was delivered at 1 s intervals, and averaged traces were used for charge calculations. In each oocyte, the averages of 5–7 measurements were used to obtain values for membrane capacitance (C_m). Currents for I_{cap} recordings were filtered at 20 kHz and sampled at 50 kHz.

All chemicals used in preparing the solutions were from Sigma-Aldrich (St. Louis, MO). Anandamide, (–)-nicotine, and α -bungarotoxin were from Sigma-RBI (St. Louis, MO). Acetylcholine was applied by gravity flow via micropipette positioned 3 mm from the oocyte. Anandamide and/or volatile anesthetics were applied externally by addition to the superfusate and to acetylcholine containing solution in the micropipette. Procedures for the injections of BAPTA (50–100 nl, 100 mM) were performed as described previously (Oz et al., 1998). BAPTA was prepared in Cs₄-BAPTA. Injections were performed 1 h prior to recordings using an oil-driven ultra microsyringe pump (Micro4, WPI, Inc. Sarasota, FL). Stock solutions of anandamide were prepared in dimethylsulfoxide (DMSO) at a concentration of 100 mM. DMSO alone did not affect nicotinic receptors when added at concentrations up to 0.3 % (v/v) in MBS solutions, a concentration twice as high as that resulting from the most concentrated application of the agents used. The acetylcholine (3–4 s)-induced inward currents were elicited at 10 min intervals to avoid receptor desensitization.

The isoflurane (Abbott Laboratories, Chicago, IL) and halothane (Wyeth-Ayerst Laboratories, Philadelphia, PA) solutions were prepared immediately before use and applied with a Hamilton gas-tight syringe connected to Teflon tubing. The concentrations of the anesthetics given in the text refer to bath concentration and they were confirmed by gas chromatography.

2.2. Synthesis of cRNA and chimeric construct

The cDNA clones of the chick nicotinic acetylcholine α_7 subunit and mouse 5-HT_{3A} subunit were provided by Dr. Jon Lindstrom (University of Pennsylvania, PA) and Dr. David Julies (University of California, San Francisco, CA), respectively. Capped cRNA transcripts were synthesized *in vitro* using a mMESSAGE mMACHINE kit from Ambion (Austin, TX) and analyzed on 1.2 % formaldehyde agarose gel to check the size and the quality of the transcripts. The chimeric α_7 -nicotinic acetylcholine -5-HT_{3A} receptor was constructed as described previously (Eisele et al., 1993, Yu et al., 1996).

2.3. Mass Spectrometry

A voyager De-Pro matrix-assisted laser desorption/ionization (MALDI) time-of-flight instrument (PE-Biosystem, Framingham, MA) was used for MALDI analysis of *Xenopus* oocytes. All mass spectra were acquired in positive ion mode and are the sum of 100 laser shots. Samples were prepared from eight to nine oocytes in MBS (control solution) or in 2 mM of isoflurane or halothane containing MBS solutions. The individual oocytes were then fractured and spread across the target surface. 2, 5-Dihydroxybenzoic acid (DHB) was used as the matrix. It was deposited directly onto the sample and left to dry at room temperature before insertion into the mass spectrometer. DHB was prepared in a 50:50 ethanol:water solution.

2.4. Data analysis

Average values were calculated as mean $\pm$ standard error mean (S.E.M.). Statistical significance was analyzed using Student's *t* test or ANOVA as indicated. Concentration-response curves were obtained by fitting the data to the logistic equation,

$$y = \{(I_{\max} - I_{\min}) / (1 + [IC_{50}/x]^n)\} + I_{\min},$$

where *x* and *y* are concentration and response, respectively, $I_{\max}$ is the maximal inhibition, $I_{\min}$ is the minimal inhibition, IC_{50} is the half-maximal inhibition, and *n* is the slope factor (apparent Hill coefficient).

3. Results

In the present study, we investigated the interaction between the inhibitory effects of anandamide and isoflurane in oocytes expressing α_7 -nicotinic acetylcholine receptors. In line with earlier studies (Oz et al., 2003a, Zhang et al., 1997; Flood and Coates, 2002), application of isoflurane (0.5 mM) or anandamide (100 nM) for 20 min inhibited acetylcholine (100 μ M)-induced ion currents mediated by α_7 -nicotinic acetylcholine receptors (Fig. 1A). In the majority of experiments, test concentration of 100 μ M acetylcholine was used, since this concentration of acetylcholine is close to the EC_{50} value of 100–120 μ M for acetylcholine in oocyte expression system (Oz et al., 2004). Perfusion of oocytes for 20 min with extracellular solution containing 0.5 mM isoflurane and 100 nM anandamide caused a further inhibition of acetylcholine -induced currents (Fig. 1B). Results of experiments demonstrating the time courses of the effects of anandamide, isoflurane and anandamide + isoflurane applications on the mean amplitudes of the acetylcholine-induced currents (normalized to current amplitudes induced by 100 μ M acetylcholine) from 5–7 oocytes are presented in figure 1C. Results summarizing the effects of isoflurane, halothane, anandamide, anandamide + isoflurane, and anandamide + halothane on acetylcholine-induced responses are demonstrated in figure 1D.

In the next series of experiments, the concentration-dependent inhibition of nicotinic receptors by anandamide was investigated in the absence and presence of a constant isoflurane concentration (0.5 mM). The IC_{50} values of 246 ± 38 nM for anandamide alone and 124 ± 32 nM in the presence of anandamide + isoflurane were significantly different (Fig. 2A; $n = 4-6$, ANOVA, $P < 0.05$). However, after subtracting isoflurane-induced tonic inhibition (approximately 40% for 0.5 mM isoflurane) and normalizing to maximal inhibition in each group (*inset* to Fig. 2A), reanalysis of the data indicated that IC_{50} values for anandamide ($IC_{50} = 215 \pm 34$ nM) and anandamide + isoflurane ($IC_{50} = 192 \pm 26$ nM) were not altered ($n = 4-5$; ANOVA, $P > 0.05$); i.e., coapplication of anandamide and isoflurane did not alter the potency of anandamide on nicotinic receptors (*inset* to Fig. 2A).

The effect of increasing concentrations of isoflurane in the presence of a constant (100 nM) anandamide concentration was also tested (Fig. 2B). The IC_{50} value of 0.87 ± 0.24 mM for

isoflurane alone decreased significantly to 0.51 ± 0.16 mM during coapplication of isoflurane and anandamide (Fig. 2B; $n=4-5$, ANOVA, $P<0.05$). Reanalysis of data by subtracting the tonic anandamide inhibition and normalizing to maximal effect in each group indicated that the IC_{50} value for isoflurane remained unaltered in the presence of anandamide (*inset* to Fig. 2B). The IC_{50} values for normalized concentration-inhibition curves were 0.68 ± 0.26 mM and of 0.72 ± 0.28 mM for isoflurane alone and isoflurane + anandamide applications, respectively.

The data from Figure 2A was further re-analyzed to give Figure 3A. Figure 3A (filled circles) plots the difference between the two inhibition curves of Figure 2A, to give the difference between the mean inhibition obtained at increasing concentrations of AEA, in the presence and absence of 0.5 mM isoflurane. This difference was then normalized to the mean inhibition produced by 100 nM anandamide alone. A similar re-analysis of data from Figure 2B gives the normalized relative inhibition obtained by increasing concentrations of isoflurane with and without 100 nM anandamide.

In our earlier studies, we have demonstrated that the inhibitory effect of anandamide is not affected by the inhibition of fatty acid amide hydrolase (FAAH), an enzyme that hydrolyses anandamide (Oz et al., 2003). However, a possible modulation of FAAH activity by isoflurane can alter anandamide levels in bathing solution. To evaluate a potential role of anandamide hydrolysis by FAAH in our system, we compared the extent of anandamide + isoflurane-induced inhibition of nicotinic acetylcholine responses in the presence and absence of phenylmethylsulfonyl fluoride (PMSF; 0.2 mM), an inhibitor for FAAH. The amount of inhibition by anandamide + isoflurane on α_7 -nicotinic acetylcholine receptor-mediated currents was not altered significantly in the presence of PMSF (Fig 4A). In addition, R-methanandamide (100 nM), a metabolically stable chiral analogue of anandamide that is resistant to hydrolytic inactivation by fatty acid amide hydrolase, and isoflurane (0.5 mM) inhibited α_7 -nicotinic acetylcholine receptor-mediated currents to 26 ± 5 % of controls ($n=4$), suggesting that hydrolytic degradation products of anandamide are not involved in inhibition of α_7 -nicotinic acetylcholine receptor.

In *Xenopus* oocytes, activation of α_7 -nicotinic acetylcholine receptors permits sufficient Ca^{2+} entry to activate endogenous Ca^{2+} -dependent Cl^- channels and the outward Cl^- currents carries significant portion of total acetylcholine-induced current (Sands et al., 1993). Since anandamide and volatile anesthetics have been shown to enhance intracellular Ca^{2+} concentrations (Howlett and Mukhopadhyay, 2000; Gomez and Guatimosim, 2003), we investigated whether or not coapplication of anandamide + isoflurane interact directly with endogenous Ca^{2+} -dependent Cl^- channels or secondarily, on other currents induced by Ca^{2+} entry. Thus, extracellular Ca^{2+} was replaced with Ba^{2+} , since Ba^{2+} can pass through α_7 -nicotinic acetylcholine receptors (Sands et al., 1993) but causes little, if any, activation of Ca^{2+} -dependent Cl^- channels. In addition, because a small Ca^{2+} -dependent Cl^- current remains, even in Ba^{2+} , we injected oocytes with the Ca^{2+} chelator BAPTA (Sands et al., 1993). Under these conditions, coapplication of anandamide (100 nM) + isoflurane (0.5 mM) produced the same level of inhibition (71 ± 5 % in controls versus 74 ± 4 % in BAPTA injected group) of acetylcholine-induced currents when compared to control oocytes (Fig. 4B). We also examined the effects of isoflurane and anandamide coapplication on membrane resistance (R_m), membrane capacitance (C_m) and resting membrane potential (V_m) in oocytes expressing α_7 -nicotinic acetylcholine receptor. In controls and in the presence of anandamide (300 nM) together with isoflurane (1 mM), the means of R_m , C_m , and V_m values were 1.2 ± 0.2 and 1.1 ± 0.2 M Ω , 192 ± 14 and 197 ± 16 nF, and -32 ± 3 and -35 ± 4 mV, respectively ($n=6-8$, ANOVA, $P>0.05$), indicating that coapplication of anandamide and isoflurane did not cause a significant effect on passive membrane properties of oocytes.

Examination of the voltage-dependence of the inhibition by anandamide + isoflurane coapplication indicated that the extent of inhibition did not vary with membrane potential (Fig. 4C). There was no change on the reversal potential of the acetylcholine -activated ion currents (2 ± 3 mV in controls versus 4 ± 4 mV in anandamide + isoflurane; $n=4-5$, ANOVA, $P>0.05$).

To study possible effects of isoflurane on endogenous anandamide levels, we looked for quantitative detection of anandamide in control and 2 mM isoflurane-treated oocytes by matrix-assisted laser desorption/ionization (MALDI) analysis. In our earlier study, we demonstrated that anandamide was easily detectable down to 150 fmol by using this technique (Oz et al., 2005). In the present study, we compared mass spectra profiles of oocytes before and after 30 min treatment with 1 mM isoflurane. Anandamide was not observed in mass spectra of either control (Fig. 5A) or 1 mM isoflurane-treated oocytes (Fig. 5B). Moreover, lipid distribution was not altered by isoflurane treatment (Figs 5B). Similar results were obtained after 30 min treatment with 2 mM halothane ($n=7-8$, data not shown).

In earlier studies we found that the IC_{50} values for anandamide were 229 nM and 3.7 μ M at α_7 -nicotinic acetylcholine and 5-HT₃ receptors, respectively (Oz et al., 2002a, 2003a). The development of chimeric α_7 -nicotinic acetylcholine-5HT₃ receptors (Eisele et al., 1993, Yu et al., 1996; Zhang et al., 1997) and the differences in binding sites for anandamide and isoflurane at α_7 -nicotinic acetylcholine and 5-HT₃ receptors provided an opportunity to evaluate further the effect of dissociating these binding sites on the interaction between isoflurane and anandamide. Therefore, we utilized a functional chimeric receptor-ion channel constructed with the N-terminal domain of the α_7 -nicotinic acetylcholine receptor and the C-terminal and transmembrane domains of the 5-HT₃ receptors (Eisele et al., 1993, Yu et al., 1996; Zhang et al., 1997). As described previously (Eisele et al., 1993), the properties of these chimeric receptors are largely consistent to the native α_7 -nicotinic acetylcholine receptor with regard to the potency and efficacy of acetylcholine except that the chimeric receptors display slower activation and inactivation kinetics than that of the native α_7 -nicotinic acetylcholine receptors. In agreement with our earlier results (Oz et al., 2003a), applications of 100 nM anandamide and 0.5 mM isoflurane inhibited the acetylcholine -induced currents mediated by α_7 -nicotinic acetylcholine receptors to 66 ± 5 % and 69 ± 6 % of controls, respectively (Fig. 6A). Coapplication of anandamide and isoflurane further inhibited acetylcholine -induced currents to 36 ± 4 % of controls. Consistent with our previous observations (Oz et al., 2002a), this same concentration of anandamide (100 nM) had no significant effect on 5-HT₃ receptors (Fig. 6B) and coapplication of anandamide and isoflurane did not cause an alteration on the maximal amplitudes of 5-HT₃ receptor-mediated currents. Similarly, the abilities of anandamide and anandamide + isoflurane to inhibit acetylcholine-induced currents mediated by the α_7 -nicotinic acetylcholine-5-HT₃ chimeric receptors were examined (Fig. 6C). In line with our earlier results (Oz et al., 2004b, Zhang et al., 1996), although anandamide (100 nM) did not cause a significant alteration, isoflurane (0.5 mM) inhibited chimeric receptor-mediated ion currents to 68 ± 7 % of controls ($n=5$). Coapplication of isoflurane and anandamide also resulted in suppression of acetylcholine-induced currents mediated by the α_7 -nicotinic acetylcholine-5-HT₃ chimeric receptors (Fig. 6C). Maximal acetylcholine-induced responses were inhibited to 68 ± 7 % and 71 ± 8 % of controls in the presences of isoflurane and anandamide + isoflurane, respectively. There was no statistically significant difference between these values ($n=4-5$; ANOVA, $P>0.05$).

Arachidonic acid, structurally similar to anandamide, also inhibits the function of α_7 -nicotinic acetylcholine receptors (Vijayaraghavan et al., 1995; Oz et al., 2004). Thus, it was likely that isoflurane also interact with the inhibitory effect of arachidonic acid on nicotinic receptors. In order to test this hypothesis, we have compared the effect of the coapplication of arachidonic acid (1 μ M) and isoflurane (0.5 mM) with the effects of arachidonic acid alone and isoflurane alone on nicotinic responses. The extent of inhibition by the coapplication of arachidonic acid

and isoflurane was significantly greater than those induced by arachidonic acid alone and isoflurane alone (Fig. 6D).

4. Discussion

The results indicate that volatile anesthetics and the endogenous cannabinoid anandamide have additive inhibitory effects on the function of neuronal α_7 -nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. Our results also suggest that sites of actions for volatile anesthetics and anandamide are different on α_7 -nicotinic acetylcholine receptors.

Volatile anesthetics and anandamide have been shown to alter intracellular Ca^{2+} levels in various cell types (for reviews, Howlett and Mukhopadhyay, 2000; Gomez and Guatimosim, 2003). In oocytes, Ca^{2+} -activated Cl^- currents have been employed routinely to monitor intracellular Ca^{2+} levels (for a review, Dascal, 1987). Under voltage-clamp conditions, the coapplication of anandamide and isoflurane to oocytes expressing α_7 -nicotinic acetylcholine receptors did not cause a significant change in baseline holding currents, suggesting that the intracellular concentration of Ca^{2+} was not altered by the coapplication of isoflurane and anandamide. In addition, passive membrane properties of oocytes were not altered significantly by anandamide and isoflurane, suggesting that coapplication of these reagents did not disrupt the integrity of the oocyte membrane.

Anandamide, at the concentration range used in this study, has been shown to activate cannabinoid receptors (for a review, Howlett et al., 2002). However, cannabinoid receptors are not expressed endogenously in *Xenopus* oocytes (Henry and Chavkin, 1995). In addition, the cannabinoid CB_1 receptor antagonist SR141716A, the cannabinoid CB_2 receptor antagonist SR144528, and pertussis toxin do not alter the anandamide-induced inhibition of α_7 -nicotinic acetylcholine receptors expressed in oocytes (Oz et al., 2003a). Thus, the actions of anandamide and isoflurane on α_7 -nicotinic acetylcholine receptors are not likely to be mediated by cannabinoid receptors.

In recent studies, general anesthetics such as propofol (Patel et al., 2003) and sevoflurane (Schelling et al., 2006) have been shown to alter anandamide levels. Isoflurane-induced changes in anandamide metabolism could mediate isoflurane potentiation of anandamide effect on α_7 -nicotinic acetylcholine receptors. However, inhibition of FAAH activity by PMSF did not alter the effect of isoflurane on the anandamide inhibition of α_7 -nicotinic acetylcholine receptors, suggesting that the changes in the anandamide hydrolysis do not mediate the effect of isoflurane in the oocyte expression system. In addition, R-methanandamide (100 nM), a metabolically stable chiral analogue of anandamide that is resistant to hydrolytic inactivation by the fatty acid amide hydrolase, mimicked the effect of anandamide. Furthermore, comparison of the spectra profiles of oocyte preparations under control conditions and after exposure to isoflurane for 30 min indicated that anandamide levels were not detectable in either control or isoflurane-treated oocytes. Thus, in the oocyte expression system, isoflurane-induced alterations in anandamide levels do not account for the enhancement of anandamide inhibition of α_7 -nicotinic acetylcholine receptors by isoflurane.

Interestingly, increasing the concentrations of either agent diminished the extent of other agent's relative inhibitory effect (Fig 3A). This finding is markedly different from the effect of ethanol on anandamide inhibition of α_7 -nicotinic acetylcholine receptors (Oz et al., 2005), where, even at high anandamide concentrations, the extent of ethanol inhibition was not altered and the effects of anandamide and ethanol were additive (Fig 3B). It appears that although the inhibition caused by the coapplication of isoflurane and anandamide was additive, the extent of this additive inhibition decreased significantly with increasing the concentrations of either agent (Fig 3). It is possible that, at high concentrations, these agents can compete with a

common site for their action; therefore causing a decrease in the extent of their additive inhibitory effects. It is unlikely that a chemical antagonism occurs between anandamide and isoflurane since these compounds are not charged under our experimental conditions.

Volatile anesthetics, fatty acids and anandamide are noncompetitive inhibitors of nicotinic acetylcholine receptors (Arias et al., 2003, 2006; Barrantes, 2004; Oz, 2006). They all share common pharmacological properties: A) they do not interact with the agonist binding site of the receptor (i.e. allosteric modulators), B) they are not open channel blockers C) they are highly lipophilic molecules acting as allosteric modulators of the nicotinic acetylcholine receptors (Arias et al., 2003, 2006; Barrantes, 2004). Several studies indicate that there is a functional interaction between these allosteric modulators. For example, arachidonic acid and volatile anesthetics have been shown to modulate each others effects on the function of proteins (Ueda and Yoshida, 1999), and ion channels (Fletcher et al., 1991; Fletcher and Welter, 1994; Denson et al., 1996). Likewise, allosteric interactions between volatile anesthetics such as halothane and membrane cholesterol on the functions of nicotinic acetylcholine receptors and voltage-gated Na⁺ channels have also been demonstrated (Lechleiter et al., 1986; Rehberg et al., 1995). In another study, cholesterol treatment of excised patches containing Ca²⁺-activated K⁺ channels antagonized the effect of halothane on channel activity, indicating a negative allosteric interaction between halothane and cholesterol (Denson et al., 1996).

Recently the interaction between inhibitory effects of steroids and fatty acids has been studied on *Torpedo californica* nicotinic acetylcholine receptor (Nievas et al., 2007). Results of this study indicated that fatty acids and steroids share a common binding side on the receptor and they occlude each others inhibitory effect (Nievas et al., 2007). In our study, when isoflurane and anandamide are coapplied at low concentrations, additive inhibition of the α_7 -nicotinic acetylcholine receptors was observed. However, the extent of this additive effect was attenuated with increasing concentrations of either compound. Most of the hydrophobic allosteric agents including fatty acids, steroids and volatile anesthetics have multiple binding sites on nicotinic acetylcholine receptors (Arias et al., 2003, 2006; Gaspar et al., 2007). It is likely that anandamide have also multiple binding sites with high and low affinities to these agents. We hypothesize that the additive effects of anandamide and isoflurane observed at low concentrations of these agents is mediated by different high affinity binding sites for these agents. However, at high concentrations, anandamide and isoflurane competes for a common low affinity binding site(s), causing attenuation of their additive effects when these agents are coapplied at higher concentrations range.

The functional chimeric α_7 -nicotinic acetylcholine-5-HT₃ receptor consisted of an N-terminal domain of the α_7 -nicotinic acetylcholine receptor and the transmembrane and C-terminal domains of the 5-HT₃ receptor (Eisele et al., 1993; Zhang et al., 1997). Thus, we have examined if the chimeric α_7 -nicotinic acetylcholine-5-HT₃ receptor also uncouples a possible linkage between the allosteric modulatory sites for isoflurane and anandamide. Earlier studies have shown that anandamide inhibits the function of α_7 -nicotinic acetylcholine receptors with a potency that was an order of magnitude higher than at 5-HT₃ receptors in *Xenopus* oocytes (Oz et al., 2002a, 2003a). In the present study, we found that anandamide did not inhibit the chimeric α_7 -nicotinic acetylcholine-5-HT₃ receptor; however, coapplication of anandamide and isoflurane caused a significant inhibition of the chimeric α_7 -nicotinic acetylcholine-5-HT₃ receptor, suggesting that the effects of anandamide and isoflurane are mediated through topographically distinct allosteric binding sites on the nicotinic acetylcholine receptor.

Although the modulation of α_7 -nicotinic acetylcholine receptors by volatile anesthetics is probably not the main mechanism by which these compounds produce anesthesia, the role played by nicotinic receptors in anesthetic-induced side effects such as postoperative hyperalgesia may be substantial (Flood et al., 2002; Flood and Daniel, 2004). Thus, because

of allosteric interaction between anandamide and volatile anesthetics, the alterations in endocannabinoid levels would modulate the actions of volatile anesthetics on α_7 -nicotinic acetylcholine receptors. The potencies of these compounds on nicotinic acetylcholine receptors were not altered by their allosteric interaction when they are calculated after subtraction of tonic inhibitory influences of either compound. However, in *in vivo* conditions, tissue anandamide levels can exert a tonic influence on the activity of α_7 -nicotinic acetylcholine receptors and the potency of volatile anesthetics would be altered significantly. Several physiological and pathological conditions have been shown to alter tissue anandamide levels (Cravatt and Lichtman, 2002; Basavarajappa and Hungund, 2002; Gonzalez et al., 2002). It is likely that the effects of volatile anesthetics on the function of α_7 -nicotinic acetylcholine receptors are altered under these conditions. Due to the presence of α_7 -nicotinic acetylcholine receptors in critical locations such as presynaptic terminals, endocannabinoids released from postsynaptic sites *in situ* may further modulate the effects of volatile anesthetics on α_7 -nicotinic acetylcholine receptors.

In conclusion, our results indicate that the changes in endocannabinoid concentrations modulate the inhibitory effects of volatile anesthetics on α_7 -nicotinic acetylcholine receptor-mediated currents in *Xenopus* oocytes. The results of studies with chimeric α_7 -nicotinic acetylcholine-5-HT₃ receptor suggest that there are multiple allosteric modulatory sites for volatile anesthetics and anandamide. Collectively, these data suggest that α_7 -nicotinic acetylcholine receptors may represent a novel link between volatile anesthetics and endocannabinoids.

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References

- Arias HR, Kem WR, Trudell JR, Blanton MP. Unique general anesthetic binding sites within distinct conformational states of the nicotinic acetylcholine receptor. *Int Rev Neurobiol* 2003;54:1–50. [PubMed: 12785284]
- Arias HR, Bhumireddy P, Bouzat C. Molecular mechanisms and binding site locations for noncompetitive antagonists of nicotinic acetylcholine receptors. *Int J Biochem Cell Biol* 2006;38:1254–1276. [PubMed: 16520081]
- Azad SC, Rammes G. Cannabinoids in anaesthesia and pain therapy. *Curr Opin Anaesthesiol* 2005;18:424–427. [PubMed: 16534269]
- Barann M, Molderings G, Bruss M, Bonisch H, Urban BW, Gothert M. Direct inhibition by cannabinoids of human 5-HT_{3A} receptors: probable involvement of an allosteric modulatory site. *Br J Pharmacol* 2002;137:589–596. [PubMed: 12381672]
- Barrantes FJ. Structural basis for lipid modulation of nicotinic acetylcholine receptor function. *Brain Res Brain Res Rev* 2004;47:71–95. [PubMed: 15572164]
- Basavarajappa BS, Hungund BL. Neuromodulatory role of the endocannabinoid signaling system in alcoholism: an overview. *Prostaglandins Leukot Essent Fatty Acids* 2002;66:287–299. [PubMed: 12052043]
- Chemin J, Monteil A, Perez-Reyes E, Nargeot J, Lory P. Direct inhibition of T-type calcium channels by the endogenous cannabinoid anandamide. *EMBO J* 2001;20:7033–7040. [PubMed: 11742980]
- Cravatt BF, Lichtman AH. The enzymatic inactivation of the fatty acid amide class of signaling lipids. *Chem Phys Lipids* 2002;121:135–148. [PubMed: 12505696]
- Dascal N. The use of *Xenopus* oocytes for the study of ion channels. *CRC Critical Reviews in Biochemistry* 1987;22:317–387. [PubMed: 2449311]
- Denson DD, Worrell RT, Eaton DC. A possible role for phospholipase A₂ in the action of general anesthetics. *Am J Physiol* 1996;270:C636–644. [PubMed: 8779929]

- Downie DL, Vicente-Agullo F, Campos-Caro A, Bushell TJ, Lieb WR, Franks NP. Determinants of the anesthetic sensitivity of neuronal nicotinic acetylcholine receptors. *J Biol Chem* 2002;277:10367–10373. [PubMed: 11741933]
- Eisele JL, Bertrand S, Galzi JL, Devillers-Thiery A, Changeux JP, Bertrand D. Chimaeric nicotinic-serotonergic receptor combines distinct ligand binding and channel specificities. *Nature* 1993;366:479–483. [PubMed: 8247158]
- Fletcher JE, Mayerberger S, Tripolitis L, Yudkowsky M, Rosenberg H. Fatty acids markedly lower the threshold for halothane-induced calcium release from the terminal cisternae in human and porcine normal and malignant hyperthermia susceptible skeletal muscle. *Life Sci* 1991;49:1651–1657. [PubMed: 1943469]
- Fletcher JE, Welter VE. Enhancement of halothane action at the ryanodine receptor by unsaturated fatty acids. *Adv Pharmacol* 1994;31:323–331. [PubMed: 7873421]
- Flood P, Coates KM. Sensitivity of the $\alpha 7$ nicotinic acetylcholine receptor to isoflurane may depend on receptor inactivation. *Anesth Analg* 2002;95:83–77. [PubMed: 12088948]
- Flood P, Sonner JM, Gong D, Coates KM. Isoflurane hyperalgesia is modulated by nicotinic inhibition. *Anesthesiology* 2002;97:192–198. [PubMed: 12131122]
- Flood P, Daniel D. Intranasal nicotine for postoperative pain treatment. *Anesthesiology* 2004;101:1417–21. [PubMed: 15564950]
- Gomez RS, Guatimosim C. Mechanism of action of volatile anesthetics: involvement of intracellular calcium signaling. *Curr Drug Targets CNS Neurol Disord* 2003;2:123–129. [PubMed: 12769804]
- Gonzalez S, Cascio MG, Fernandez-Ruiz J, Fezza F, Di Marzo V, Ramos JA. Changes in endocannabinoid contents in the brain of rats chronically exposed to nicotine, ethanol or cocaine. *Brain Res* 2002;954:73–81. [PubMed: 12393235]
- Henry DJ, Chavkin C. Activation of inwardly rectifying potassium channels (GIRK1) by co-expressed rat brain cannabinoid receptors in *Xenopus* oocytes. *Neuroscience Lett* 1995;186:91–94.
- Hejazi N, Zhou C, Oz M, Sun H, Ye JH, Zhang L. $\Delta 9$ -tetrahydrocannabinol and endogenous cannabinoid anandamide directly potentiate the function of glycine receptors. *Mol Pharmacol* 2006;69:991–997. [PubMed: 16332990]
- Howlett AC, Mukhopadhyay S. Cellular signal transduction by anandamide and 2-arachidonoylglycerol. *Chem Phys Lipids* 2000;108:53–70. [PubMed: 11106782]
- Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, Mechoulam R, Pertwee RG. International Union of Pharmacology. XXVII Classification of cannabinoid receptors. *Pharmacol Rev* 2002;54:161–202. [PubMed: 12037135]
- Jenkins A, Franks NP, Lieb WR. Actions of general anaesthetics on 5-HT₃ receptors in N1E-115 neuroblastoma cells. *Br J Pharmacol* 1996;117:1507–1515. [PubMed: 8730747]
- Krasowski MD, Harrison NL. General anaesthetic actions on ligand-gated ion channels. *Cell Mol Life Sci* 1999;55:1278–1303. [PubMed: 10487207]
- Lechleiter J, Wells M, Gruener R. Halothane-induced changes in acetylcholine receptor channel kinetics are attenuated by cholesterol. *Biochim Biophys Acta* 1986;856:640–645. [PubMed: 2421772]
- Lindstrom J, Anand R, Gerzanich V, Peng X, Wang F, Wells G. Structure and function of neuronal nicotinic acetylcholine receptors. *Prog Brain Res* 1996;109:125–137. [PubMed: 9009699]
- Lozovaya N, Yatsenko N, Beketov A, Tsintsadze T, Burnashev N. Glycine receptors in CNS neurons as a target for nonretrograde action of cannabinoids. *J Neurosci* 2005;25:7499–7506. [PubMed: 16107637]
- Machu TK, Harris RA. Alcohols and anesthetics enhance the function of 5-hydroxytryptamine₃ receptors expressed in *Xenopus laevis* oocytes. *J Pharmacol Exp Ther* 1994;271:898–905. [PubMed: 7965811]
- Maingret F, Patel AJ, Lazdunski M, Honore E. The endocannabinoid anandamide is a direct and selective blocker of the background K(+) channel TASK-1. *EMBO J* 2001;20:47–54. [PubMed: 11226154]
- Mihic SJ, Ye Q, Wick MJ, Koltchine VV, Krasowski MD, Finn SE, Mascia MP, Valenzuela CF, Hanson KK, Greenblatt EP, Harris RA, Harrison NL. Sites of alcohol and volatile anaesthetic action on GABA(A) and glycine receptors. *Nature* 1997;389:385–389. [PubMed: 9311780]
- Mori T, Zhao X, Zuo Y, Aistrup GL, Nishikawa K, Marszalec W, Yeh JZ, Narahashi T. Modulation of neuronal nicotinic acetylcholine receptors by halothane in rat cortical neurons. *Mol Pharmacol* 2001;59:732–743. [PubMed: 11259617]

- Nicholson RA, Liao C, Zheng J, David LS, Coyne L, Errington AC, Singh G, Lees G. Sodium channel inhibition by anandamide and synthetic cannabimimetics in brain. *Brain Res* 2003;978:194–204. [PubMed: 12834914]
- Nievas GA, Barrantes FJ, Antollini SS. Conformation-sensitive steroid and fatty acid sites in the transmembrane domain of the nicotinic acetylcholine receptor. *Biochemistry* 2007;46:3503–3512. [PubMed: 17319650]
- Oliver D, Lien CC, Soom M, Baukrowitz T, Jonas P, Fakler B. Functional conversion between A-type and delayed rectifier K⁺ channels by membrane lipids. *Science* 2004;304:265–270. [PubMed: 15031437]
- Ouyang W, Wang G, Hemmings HC Jr. Isoflurane and propofol inhibit voltage-gated sodium channels in isolated rat neurohypophysial nerve terminals. *Mol Pharmacol* 2003;64:373–381. [PubMed: 12869642]
- Oz M, Soldatov NM, Melia MT, Abernethy DR, Morad M. Functional coupling of human L-type Ca²⁺ channel and angiotensin AT_{1A} receptor co-expressed in *Xenopus* oocytes. *Mol Pharmacol* 1998;54:1106–1112. [PubMed: 9855640]
- Oz M, Tchugunova Y, Dunn SMJ. Endogenous cannabinoid anandamide directly inhibits voltage-dependent calcium fluxes in rabbit T-tubule membrane preparations. *Eur J Pharmacol* 2000;404:13–20. [PubMed: 10980258]
- Oz M, Zhang L, Morales M. Endogenous cannabinoid, anandamide acts as a non-competitive inhibitor on 5-HT₃ receptor-mediated responses in *Xenopus* oocytes. *Synapse* 2002a;46:150–156. [PubMed: 12325042]
- Oz M, Tchugunova Y, Dunn SMJ. The effects of isoflurane on voltage-dependent calcium fluxes in rabbit T-tubule membranes; comparison with alcohols. *Arch Biochem Biophys* 2002b;398:275–283. [PubMed: 11831860]
- Oz M, Dinc M, Tchugunova Y, Dunn SMJ. Comparison of the effects of xenon and halothane on voltage-dependent Ca(2+) fluxes in rabbit T-tubule membranes. *Naunyn Schmiedeberg's Arch Pharmacol* 2002c;365:413–417. [PubMed: 12012028]
- Oz M, Ravindran A, Diaz-Ruiz O, Zhang L, Morales M. The endogenous cannabinoid anandamide inhibits $\alpha 7$ nicotinic acetylcholine receptor-mediated responses in *Xenopus* oocytes. *J Pharmacol Exp Ther* 2003;306:1003–1010. [PubMed: 12766252]
- Oz M, Tchugunova Y, Dinc M. Differential effects of endocannabinoids and synthetic cannabinoids on voltage-dependent calcium fluxes in rabbit T-tubule membranes; Comparison with fatty acids. *Eur J Pharmacol* 2004a;502:47–58. [PubMed: 15464089]
- Oz M, Zhang L, Ravindran R, Morales M, Lupica CR. Direct and differential effects of cannabinoid receptor ligands on $\alpha 7$ -nicotinic receptor-mediated currents in *Xenopus* oocytes. *J Pharmacol Exp Ther* 2004b;310:1152–1160. [PubMed: 15102930]
- Oz M, Spivak CE, Lupica CR. Tween 80 is a Potent Inhibitor of $\alpha 7$ -nicotinic Acetylcholine Receptor-Mediated Currents in *Xenopus* Oocytes. *J Neurosci Meth* 2004c;137:167–173.
- Oz M, Jackson SN, Woods AS, Morales M, Zhang L. Additive Effects of Endogenous Cannabinoid Anandamide and Ethanol on $\alpha 7$ -nicotinic Acetylcholine Receptor-Mediated Responses in *Xenopus* Oocytes. *J Pharmacol Exp Ther* 2005;313:1272–1280. [PubMed: 15687372]
- Oz M. Direct Effects of Endocannabinoids on Ion Channels. *Curr Pharm Des* 2006;12:227–239. [PubMed: 16454739]
- Patel AJ, Honore E, Lesage F, Fink M, Romey G, Lazdunski M. Inhalational anesthetics activate two-pore-domain background K⁺ channels. *Nat Neurosci* 1999;2:422–426. [PubMed: 10321245]
- Patel S, Wohlfeil ER, Rademacher DJ, Carrier EJ, Perry LJ, Kundu A, Falck JR, Nithipatikom K, Campbell WB, Hillard CJ. The general anesthetic propofol increases brain N-arachidonyl ethanolamine (anandamide) content and inhibits fatty acid amide hydrolase. *Br J Pharmacol* 2003;139:1005–1013. [PubMed: 12839875]
- Poling JS, Rogawski MA, Salem N, Vicini S. Anandamide, an endogenous cannabinoid inhibits Shaker-related voltage-gated K⁺ channels. *Neuropharmacology* 1996;35:983–991. [PubMed: 8938728]
- Rehberg B, Urban BW, Duch DS. The membrane lipid cholesterol modulates anesthetic actions on a human brain ion channel. *Anesthesiology* 1995;82:749–758. [PubMed: 7879943]

- Sands SB, Costa ACS, Patrick JW. Barium permeability of neuronal nicotinic receptor α_7 expressed in *Xenopus* oocytes. *Biophys J* 1993;65:2614–2621. [PubMed: 8312496]
- Schelling G, Hauer D, Azad SC, Schmoelz M, Chouker A, Schmidt M, Hornuss C, Rippberger M, Briegel J, Thiel M, Vogeser M. Effects of General Anesthesia on Anandamide Blood Levels in Humans. *Anesthesiology* 2006;104:273–277. [PubMed: 16436846]
- Shiraishi M, Harris RA. Effects of alcohols and anesthetics on recombinant voltage-gated Na⁺ channels. *J Pharmacol Exp Ther* 2004;309:987–994. [PubMed: 14978193]
- Smart D, Gunthorpe MJ, Jerman JC, Nasir S, Gray J, Muir AI, Chambers JK, Randall AD, Davis JB. The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1). *Br J Pharmacol* 2000;129:227–230. [PubMed: 10694225]
- Tassonyi E, Charpantier E, Muller D, Dumont L, Bertrand D. The role of nicotinic acetylcholine receptors in the mechanisms of anesthesia. *Brain Res Bull* 2002;57:133–150. [PubMed: 11849819]
- Ueda I, Yoshida T. Hydration of lipid membranes and the action mechanisms of anesthetics and alcohols. *Chem Phys Lipids* 1999;101:65–79. [PubMed: 10810926]
- Yamakura T, Lewohl JM, Harris RA. Differential effects of general anesthetics on G protein-coupled inwardly rectifying and other potassium channels. *Anesthesiology* 2001a;95:144–153. [PubMed: 11465552]
- Yamakura T, Bertaccini E, Trudell JR, Harris RA. Anesthetics and ion channels: molecular models and sites of action. *Annu Rev Pharmacol Toxicol* 2001b;41:23–51. [PubMed: 11264449]
- Vijayaraghavan S, Huang B, Blumenthal EM, Berg DK. Arachidonic acid as a possible negative feedback inhibitor of nicotinic acetylcholine receptors on neurons. *J Neurosci* 1995;15:3679–3687. [PubMed: 7751938]
- Zhang L, Oz M, Stewart RR, Peoples RW, Weight FF. Volatile general anesthetic actions on nACh α_7 , 5-HT $_3$ and chimeric nACh α_7 -5-HT $_3$ receptors expressed in *Xenopus* Oocytes. *Brit J Pharmacol* 1997;120:353–355. [PubMed: 9031735]

Figure 1.

The effects of anandamide, isoflurane and anandamide + isoflurane on α_7 -nicotinic acetylcholine receptor-mediated ion currents. **(A)** Currents activated by acetylcholine (100 μ M) and recorded from the same oocyte in controls (*first trace from the left*), at 20 min bath application of 0.5 mM isoflurane (*second trace from the left*), and following 20 min of recovery (*third trace from the left*), after 20 min application of 100 nM anandamide alone (*fourth trace from the left*). **(B)** Currents activated by acetylcholine (100 μ M) and recorded from the same oocyte in controls (*first trace from the left*), at 20 min bath application of 0.5 mM isoflurane (*second trace from the left*), and 20 min after coapplication of 0.5 mM isoflurane and 100 nM anandamide (*third trace from the left*), and following 30 min of recovery (*fourth trace from the left*). **(C)** Time-courses of the effects of anandamide, isoflurane and anandamide + isoflurane on the peak acetylcholine-induced currents. Each data point represents the normalized means $\pm$ s.e.m. of 4 to 5 experiments. The duration of the anandamide or anandamide + isoflurane application is indicated by the horizontal bar. **(D)** Bar graph comparing the inhibitory effects of anandamide (100 nM), isoflurane (0.5 mM), halothane (0.5 mM), anandamide + isoflurane or halothane on the maximal amplitudes of acetylcholine-induced currents. AEA, ISO, and HAL indicate anandamide, isoflurane, and halothane, respectively. Statistical significance at the level of $P < 0.05$ was presented with * (ANOVA).

Figure 2.

The effects of isoflurane and anandamide on concentration-response curves for anandamide and isoflurane-induced inhibition of α_7 -nicotinic acetylcholine receptor-mediated ion currents. **(A)** Concentration-response curve for anandamide (AEA) in the absence and in the presence of 0.5 mM isoflurane. Inset shows analysis of data after the subtraction of tonic inhibition caused by isoflurane and normalizing to maximal inhibition for each data set. Data points represent the mean $\pm$ S.E.M. of 4–5 oocytes. **(B)** Concentration-response curve for isoflurane (ISO) in the absence and in the presence of 100 nM anandamide. Inset shows analysis of data after subtraction of tonic inhibition caused by 100 nM anandamide and normalizing to maximal inhibition for each data set. Data points represent the mean $\pm$ S.E.M. of 4–5 oocytes. The curves

are the best fit of the data to the logistic equation described in the methods. Currents were activated by applying acetylcholine (100 μ M).

Figure 3.

The extent of anandamide and isoflurane inhibitions of α_7 -nicotinic acetylcholine receptor-mediated ion currents in the increasing concentration of either agent. **(A)** The extent of inhibition caused by 100 nM anandamide or 0.5 mM isoflurane was normalized to the inhibition caused by anandamide (AEA) or isoflurane (ISO) alone and plotted as a function of increasing concentration of either agent. Data points were calculated directly from the results shown in Figure 2. Each data points represent the mean of 4–5 experiments and error bars are not shown. **(B)** The extent of inhibition caused by 100 nM anandamide or 30 mM ethanol was normalized to the inhibition caused by anandamide (AEA) or ethanol (EtOH) alone and plotted as a function of increasing concentration of either agent. Data points demonstrated in panel B were calculated

from our earlier studies (Oz et al., 2005). Each data points represent the mean of 4–6 experiments and error bars are not shown.

Figure 4.

Effect of anandamide and isoflurane on acetylcholine-induced currents is not due to alterations in anandamide hydrolysis, anandamide membrane transport, endogenous Ca²⁺-activated Cl⁻ channels and membrane-potential. **(A)** Comparison of the effects of anandamide + isoflurane on the amplitudes of acetylcholine (100 μ M)-induced currents in the absence (*dark gray bar*) or presence of (*gray bar*) PMSF. **(B)** Comparison of the effects of anandamide + isoflurane on the amplitudes of acetylcholine-induced currents in the absence or presence of factors contributing to the activation of Ca²⁺-dependent Cl⁻ channels. Oocytes were either injected with 50 nl distilled water and recorded in a 2 mM Ca²⁺-containing MBS solution (*dark gray bar*) or injected with 50 nl of 100 mM BAPTA (*gray bar*) and recorded in a 2 mM Ba²⁺-

containing MBS solution. Each data point represents the normalized mean $\pm$ s.e.m. of 5 to 6 experiments. (C) Current-voltage relationships of acetylcholine-activated currents in controls, isoflurane (0.5 mM), anandamide (100 nM), and anandamide +isoflurane. Each data point represents the normalized means and S.E.M. of 4 to 5 experiments.

Figure 5.

Lack of anandamide in *Xenopus* oocytes and the effect of isoflurane on mass spectra of *Xenopus* oocytes measured with matrix-assisted laser desorption/ionization. Mass spectra of oocytes in control (A) and in the presence of 2 mM isoflurane (B). Cholesterol and phosphatidylcholine species were detectable in both control and isoflurane treated oocytes.

Figure 6.

Differential effect of anandamide, isoflurane and anandamide + isoflurane on responses mediated by α_7 -nicotinic acetylcholine receptors, 5-hydroxytryptamine type 3 receptors (5-HT₃), and chimeric α_7 -nicotinic acetylcholine-5-HT₃ receptors. The inhibitory effects of anandamide (100 nM) and isoflurane (0.5 mM) on the maximal amplitudes of currents mediated by α_7 -nicotinic acetylcholine receptors (**A**), 5-HT₃ receptors (**B**), the chimeric receptors (**C**). Each bar represents the average of 4 to 5 cells (mean $\pm$ S.E.M.). (**D**) The extent of inhibition in the presence of isoflurane (0.5 mM), arachidonic acid (1 μ M), and isoflurane + arachidonic acid were presented as percent inhibition of controls recorded in the same oocytes in the absence of these compounds. Each bar represents the average of 5 to 7 cells (mean $\pm$ S.E.M.).