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ADDITIVE EFFECTS OF ENDOGENOUS CANNABINOID ANANDAMIDE AND ETHANOL ON α_7-NICOTINIC ACETYLCHOLINE RECEPTOR-MEDIATED RESPONSES IN *XENOPUS* OOCYTES.

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ABBREVIATIONS: ACh, acetylcholine; AEA, arachidonoylethanolamide; EtOH, ethanol; 5-HT, serotonin; FAAH, fatty acid amide hydrolase; Δ^9-THC, Δ^9-tetrahydrocannabinol; nACh, nicotinic acetylcholine; BAPTA, 1,2-bis (*o*-aminophenoxy) ethane-*N*, *N*, *N'*, *N'*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl) piperazineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride.

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ABSTRACT

The interaction between the effects of the endogenous cannabinoid receptor agonist anandamide and ethanol on the function of homomeric α_7-nicotinic acetylcholine (nACh) receptors expressed in *Xenopus* oocytes were investigated using the two-electrode voltage-clamp technique. Anandamide and ethanol reversibly inhibited currents evoked with ACh (100 μM) in a concentration-dependent manner. Coapplication of anandamide and ethanol caused a significantly greater inhibition of α_7-nACh receptor function than anandamide or ethanol alone. The IC_{50} value of 238 ± 34 nM for anandamide inhibition decreased significantly to 104 ± 23 nM in the presence of 30 mM ethanol. The inhibition of α_7-mediated currents by coapplication of anandamide and ethanol was not altered by phenylmethylsulfonyl fluoride, an inhibitor of anandamide hydrolyzing enzyme, or AM404 (N-(4-hydroxyphenyl)-arachidonylamide), an anandamide-transport inhibitor. Analysis of oocytes by matrix-assisted laser desorption/ionization technique indicated that ethanol treatment did not alter the lipid profile of oocytes and there is negligible, if any, anandamide present in these cells. Results of studies with chimeric α_7-nACh-5-HT_3 receptors comprised of the amino-terminal domain of the α_7-nACh receptor and the transmembrane and carboxyl-terminal domains of 5-HT_3 receptors suggest that while ethanol inhibition of the α_7-nACh receptor is likely to involve the N-terminal region of the receptor, the site of action for anandamide is located in the transmembrane and carboxyl-terminal domains of the receptors. These data indicate that endocannabinoids and ethanol potentiate each other's inhibitory effects on α_7-nACh receptor function through distinct regions of the receptor.

Endogenous cannabinoids (endocannabinoids) are a group of signaling lipids consisting of amides and esters of long-chain polyunsaturated fatty acids. In recent years, several studies provided evidence for the modulatory role of endocannabinoids in alcohol abuse and addiction (for a review, Basavarajappa and Hungund, 2002). For example, chronic ethanol intake has been shown to increase endocannabinoid levels in various brain regions involved in drug addiction (Gonzalez et al., 2002; 2004). In addition, the exposure of neuronal cell lines or cerebellar granular neurons to chronic ethanol resulted in an increased accumulation of endocannabinoids (Basavarajappa and Hungund, 1999; 2002). Furthermore, persistent stimulation of neuronal cannabinoid receptor (CB_1) by enhanced endocannabinoid levels has been shown to induce down-regulation of density and the function of the receptor in chronic ethanol-exposed mouse brain (Basavarajappa and Hungund, 2002).

However, increased levels of endocannabinoids by ethanol can also facilitate the interaction of these molecules with ethanol on common target proteins other than cannabinoid receptors. Several reports indicate that endocannabinoids produce effects that are not mediated by the activation of the cloned CB_1 and/or CB_2 receptors. For example, it has been demonstrated that endocannabinoids such as anandamide can inhibit the function of voltage-dependent-Ca^{2+} channels (Oz et al., 2000, 2004a; Chemin et al., 2001), Na^+ channels (Nicholson et al., 2003), various types of K^+ channels (Poling et al., 1996; Maingret et al., 2001), 5-HT_3 receptor function (Barann et al., 2002; Oz et al., 2002a, Godlewski et al., 2003), and nicotinic ACh receptors (Oz et al., 2003a; 2004b), suggesting that additional molecular targets for endocannabinoids exist in the CNS (for a recent review Oz, 2005).

Similar to endocannabinoids, ethanol also interacts directly with voltage-dependent-Ca^{2+} channels (Oz et al., 2001; 2002b), Na^+ channels (Oz and Frank, 1995; Shiraishi and Harris, 2004), K^+ channels (Lewohl et al., 1999; Oz et al., 2003b), 5-HT_3 receptors (Lovinger and Zhou, 1998), and

nicotinic ACh receptors (Yu et al., 1995; Cardoso et al., 1999).

Nicotinic acetylcholine (nACh) receptors containing the α_7 subunit belong to the ligand-gated ion channel super family (Lindstrom et al, 1996). The potential involvement of these receptors in pharmacological actions of ethanol has been reported in several earlier studies (for a review Narahashi et al, 1999). Since both ethanol and endocannabinoid, anandamide, have been shown to modulate the function of α_7-nACh receptors, the present study was performed to investigate if there is an interaction between direct effects of anandamide and ethanol on the functional properties of α_7-nACh receptors. We found that anandamide and ethanol cause an additive inhibition on the function of α_7-nACh receptor by interacting with distinct regions of the receptor.

MATERIALS AND METHODS

Mature female *Xenopus laevis* frogs were purchased from Xenopus laevis I (Ann Arbor, MI) and were housed in dechlorinated tap water at 19-21^{o}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; $NaHCO_3$, 2.4; $MgSO_4$, 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; $NaHCO_3$, 2.4; $Ca(NO_3)_2$, 0.3; $CaCl_2$, 0.9; $MgSO_4$, 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 3-5 ml/min. The bathing solution consisted of (in mM): NaCl, 95; KCl, 2; $CaCl_2$, 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 5-6 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.

Compounds were applied externally by addition to the superfusate. All chemicals used in preparing the solutions were from Sigma-Aldrich (St. Louis, MO). Anandamide, (-)-nicotine, AM404, and α-bungarotoxin were from Sigma-RBI (St. Louis, MO). Procedures for the injections of BAPTA (50-100 nl, 100 mM) were performed as described previously (Oz et al., 1998). BAPTA was prepared in Cs_4-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 0.3 % (v/v) in MBS solutions, a concentration twice as high as that resulting from the most concentrated application of the agents used.

Synthesis of cRNA and chimeric construct: The cDNA clones of the chick nAChα$_7$ subunit and 5-HT_{3A} subunit were provided by Dr. Lindstrom (University of Pennsylvania, PA) and Dr. David Julies (University of California, San Fransisco, 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-nACh - 5-HT_{3A} receptor was constructed as described previously (Eisele et al., 1993, Yu et al., 1996).

Anandamide analysis: A voyager De-Pro matrix-assisted laser desorption/ionization (MALDI) time-of-flight instrument (PE-Biosystem, Framingham, MA) was used in this study 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 8 to 9 oocytes in controls (in MBS solution) or in 100 mM ethanol

containing MBS solutions. The individual oocytes were then fractured and spread across the target surface. The 2,5-Dihydroxybenzoic acid (DHB) matrix was deposited directly onto the sample prior to insertion into the mass spectrometer. DHB was prepared in a 50:50 ethanol:water solution. Anandamide was used as a standard and was prepared a 3 mM in ethanol and was subsequently diluted in distilled water.

Data analysis: Average values were calculated as mean ± standard error (SE). 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,

$$y = E_{max}/(1+[x/EC_{50}]^{-n}),$$

where x and y are concentration and response, respectively, E_{max} is the maximal response, EC_{50} is the half-maximal concentration, and n is the slope factor (apparent Hill coefficient).

RESULTS

Xenopus oocytes injected with distilled water (n=4) did not demonstrate ion currents when 1-3 mM ACh in the presence of 1 μM atropine was applied. In oocytes injected with α_7-nAChR mRNA, a 4-5 s application of ACh activated a fast inward current that desensitized rapidly. These ACh-induced inward currents were elicited at 10 min intervals to avoid receptor desensitization, and were irreversibly abolished by 10 nM α-bungarotoxin (n=3, data not shown), indicating that these responses were mediated by neuronal α_7-nACh receptor-ion channels.

In earlier studies, ethanol and anandamide were shown to release intracellular Ca^{2+} in endothelial cells and *Xenopus* oocytes (Wafford et al., 1989; Howlett and Mukhopadhyay, 2000). In the oocyte expression system, the increased level of intracellular Ca^{2+} can be detected by Ca^{2+}-activated Cl^- channels and concomitant alterations in the membrane input resistance. For this reason, we examined the effects of ethanol and anandamide coapplication on membrane resistance (R_m), membrane capacitance (C_m) and resting membrane potential (V_m) in oocytes injected with α_7-nACh receptor mRNA. In control and in the presence of anandamide together with ethanol, the values for the means of R_m, C_m, and V_m values were 1.1 ± 0.3 and 1.0 ± 0.2 MΩ, 194 ± 17 and 201 ± 18 nF, and -34.3 ± 2.9 and -31.7 ± 3.2 mV, respectively (n=8-9, ANOVA, $P>0.05$). Thus, the data indicate that coapplication of anandamide (300 nM) and ethanol (100 mM) did not cause a significant effect on passive membrane properties of oocytes.

In previous studies we showed that anandamide alone and ethanol alone inhibited the function of α_7-nAChRs expressed in *Xenopus* oocytes with IC_{50} values of 229 nM (Oz et al., 2003a) and 58 mM (Yu et al., 1996), respectively. In the present study, we investigated the interaction between the inhibitory effects of anandamide and ethanol by their coapplication to oocytes. Application of 30 mM

of ethanol for 10 min inhibited ACh (100 μM)-induced ion currents in oocytes expressing α_7-nACh receptors (first and second traces from left; Fig. 1A). Perfusion of oocytes for 20 min with extracellular solution containing 30 mM ethanol and 100 nM anandamide caused a further inhibition of ACh-induced currents (third trace from left; Fig. 1A). Following 30 min recovery, 20 min application of 100 nM anandamide alone caused a significant inhibition of ACh-induced currents in the same oocyte (fourth and fifth traces from left; Fig. 1A). Results of experiments demonstrating the time courses of the effects of anandamide, ethanol and anandamide + ethanol applications on the mean amplitudes of the ACh-induced currents (normalized to current amplitudes induced by 100 μM ACh) from 4-5 oocytes are presented in figure 1B. Results summarizing the effects ethanol, anandamide, and ethanol + anandamide on ACh-induced responses are demonstrated in figure 1C.

In the next series of experiments, we examined the effect of a constant ethanol concentration (30 mM) on the concentration-dependency of anandamide-inhibition of nicotinic receptors. In the presence of 30 mM ethanol, inhibitory effects of anandamide on the nicotinic receptor-mediated response was enhanced significantly and IC_{50} values of 238 ± 34 nM for anandamide alone shifted to 104 ± 23 nM in the presence of anandamide + ethanol (Fig. 2A; n= 4-5, ANOVA, $P<0.05$). Data plotted in figure 2A were also analyzed after subtraction of tonic ethanol inhibition (30-35 % for 30 mM ethanol) and normalized to maximal inhibition in each group (*inset* to Fig. 2A). Reexamination of IC_{50} values from this data set indicated that the difference between anandamide (IC_{50} = 235 ± 32 nM) and anandamide + ethanol (IC_{50} = 102 ± 24 nM) was statistically significant (n=4-5; ANOVA, $P<0.05$); i.e., coapplication of anandamide and ethanol increased the potency of anandamide on nicotinic receptors (*inset* to Fig. 2A). In a similar study, we tested the effects of increasing concentrations of ethanol in the presence of a constant (100 nM) anandamide concentration (Fig. 2B).

In the presence of anandamide, efficacy of ethanol increased from 66 ± 5 % to 98 ± 4 % inhibition of controls (n=4-5). Although the IC_{50} value of 52 ± 5 mM for ethanol decreased significantly to 12 ± 4 mM during coapplication of ethanol and anandamide (Fig. 2B; n= 4-5, ANOVA, $P<0.05$), re-analysis of data by subtracting the tonic inhibition caused by anandamide and normalizing to maximal inhibition in each group indicated that the IC_{50} value remained unaltered in the presence of anandamide. In the presence of ethanol alone and ethanol + anandamide, IC_{50} values for normalized concentration-inhibition curves were of 22 ± 6 mM and of 20 ± 5 mM, respectively (*inset* to Fig. 2B).

Fatty acid ethyl esters are the major nonoxidative metabolites of ethanol in humans, representing the predominant ethanol metabolites in human brain after ethanol ingestion (Bora and Lange, 1993). Perfusion of tissue cultures by ethanol is known to cause production of fatty acid ethyl esters from arachidonic acid. These fatty acid ethyl esters such as ethyl arachidonate reported to have pharmacological effects on ion channels (Gubitosi-Klug and Gross, 1996). Since the hydrolysis of anandamide by fatty acid amide hydrolase FAAH would produce arachidonic acid, in the presence of ethanol in our extracellular solution, ethyl arachidonate produced from these molecules could mediate additive inhibitory effects of ethanol and anandamide on α_7-nACh receptor-mediated currents. To evaluate a role of anandamide hydrolysis by FAAH in our system, we compared the extent of anandamide + ethanol induced inhibition of nACh responses in the presence and absence of phenylmethylsulfonyl fluoride (PMSF; 0.2 mM), an inhibitor for FAAH. The amount of the inhibition by anandamide + ethanol on α_7-nACh receptor-mediated currents was not altered significantly in the presence of PMSF (Fig 3A).

In addition to FAAH, anandamide transport through cell membrane could also be a target for ethanol actions, as anandamide levels increase due to inhibition of anandamide transport during

chronic ethanol applications in mammalian cells (Basavarajappa et al., 2003). For this reason, we investigated the inhibitory effects of anandamide and ethanol coapplication in the presence of AM404 (1 μM), an anandamide-membrane transport inhibitor. The extent of additive inhibitory effects of anandamide and ethanol were not altered in the presence of AM404 (Fig. 3A).

Both anandamide and ethanol have been shown to enhance intracellular Ca^{2+} concentrations (Wafford et al., 1989; Howlett and Mukhopadhyay, 2000). Since activation of α_7-nACh receptors permit sufficient Ca^{2+} entry to activate endogenous Ca^{2+}-dependent Cl^- channels in *Xenopus* oocytes (Sands et al., 1993), we determined whether or not coapplication of anandamide + ethanol can 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 nACh α_7 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 + ethanol produced the same level of inhibition (73 ± 7 % in controls versus 76 ± 6 % in BAPTA injected group) of ACh-induced currents when compared to control oocytes (Fig. 3B).

Examination of the voltage-dependence of the inhibition by anandamide + ethanol coapplication indicated that the degree of inhibition of the ACh (100 μM)-induced currents did not vary with membrane potential (Fig. 3C and 3D). In addition, there was no change on the reversal potential of the ACh-activated ion currents (4 ± 2 mV in controls versus 6 ± 3 mV in anandamide + ethanol), indicating that neither the ionic selectivity of the channel nor the driving force on Na^+ and Ca^{2+} were affected by these molecules.

To study possible effects of ethanol on endogenous anandamide levels, we looked for

quantitative detection of anandamide in control and 100 mM ethanol-treated oocytes by matrix-assisted laser desorption/ionization (MALDI) analysis. In order to test the sensitivity of our technique, several standard solutions of different anandamide concentrations were detected. As shown in figure 4A, anandamide was easily detectable down to 150 fM. The two dominant mass spectrums corresponded to the protonated anandamide and its sodium adduct. In contrast, anandamide was not observed in mass spectra of either MBS (Fig. 4B) or 100 mM ethanol (Fig. 4C) containing MBS solution. Moreover, lipid distribution was not altered by ethanol treatment (Figs 4B and 4C).

In earlier studies we found that anandamide inhibited α_7-nACh receptor-mediated responses with a potency that was at least one order of magnitude higher than at 5-HT_3 receptors expressed in *Xenopus* oocytes (Oz et al., 2002a; 2003a). Thus, in these studies, the IC_{50} values for anandamide were 229 nM and 3.7 μM at α_7-nACh and 5-HT_3 receptors, respectively. The development of chimeric α_7-nACh-5HT_3 receptors (Eisele et al., 1993, Yu et al., 1996; Zhang et al., 1997) and the differences in binding sites for anandamide and ethanol at these chimeric receptors provided an opportunity to evaluate further the effect of dissociating these binding sites on the interaction between ethanol and anandamide. Therefore, we utilized a functional chimeric receptor-ion channel constructed with the N-terminal domain of the α_7-nACh receptor and the C-terminal and transmembrane domains of the 5-HT_3 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-nACh receptor with regard to the potency and efficacy of ACh except that the chimeric receptors display slower activation and inactivation kinetics than that of the native α_7-nACh receptors. In agreement with our earlier results (Oz et al., 2003a), applications of 100 nM anandamide and 30 mM ethanol inhibited the ACh-induced currents mediated by α_7-nACh receptors in a noncompetitive

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manner to 62 ± 5 % and 59 ± 6 %, respectively (Fig. 5A). Coapplication of anandamide and ethanol further inhibited ACh-induced currents to 36 ± 4 % of controls. The EC_{50} values for ACh in controls and in the presences of anandamide alone, ethanol alone and anandamide + ethanol were 103 ± 12, 110 ± 14, 99 ± 12 and 107 ± 10 μM (n=5-6), respectively. There was no statistically significant difference between these values (n=4-5; ANOVA, $P>0.05$). Consistent with our previous observations (Oz et al., 2002a), this same concentration of anandamide (100 nM) had no significant effect on 5-HT_3 receptor-mediated currents in oocytes injected with cRNA coding for this receptor (Fig. 5B) and coapplication of anandamide and ethanol (30 mM) did not cause an alteration on the maximal amplitudes of 5-HT_3 receptor-mediated currents. Similarly, the abilities of anandamide and anandamide + ethanol to inhibit ACh-induced currents mediated by the α_7-nACh-5-HT_3 chimeric receptors were examined (Fig. 5C). In line with our earlier results (Oz et al., 2004b, Yu et al., 1996), although anandamide (100 nM) did not cause a significant alteration, ethanol (30 mM) inhibited chimeric receptor-mediated ion currents to 58 ± 6 % of controls (n=5). Coapplication of ethanol and anandamide also resulted in suppression of ACh-induced currents mediated by the α_7-nACh-5-HT_3 chimeric receptors (Fig. 5C). Maximal ACh-induced responses were inhibited to 61 ± 5 % and 64 ± 6 % of controls in the presences of anandamide and anandamide + ethanol, respectively. There was no statistically significant difference between these values (n=4-5; ANOVA, $P>0.05$). The EC_{50} values for ACh in controls and in the presences of anandamide alone, ethanol alone and anandamide and ethanol were 37 ± 5, 41 ± 4, 35 ± 5, 38 ± 4 μM (n=5-6), respectively.

DISCUSSION

In the present study we provide evidence indicating that endogenous cannabinoid anandamide and ethanol have additive inhibitory effects on the function of neuronal α_7-nACh receptors expressed in *Xenopus* oocytes. Our results also suggest that sites of actions for ethanol and anandamide are different and there is no allosteric cooperation between ethanol and anandamide on α_7-nACh receptors.

Alterations of intracellular Ca^{2+} levels by ethanol and anandamide have been reported in several cell types (Wafford et al., 1989; Howlett and Mukhopadhyay, 2000). In oocytes, Ca^{2+}-activated Cl^- channels are highly sensitive to intracellular levels of Ca^{2+} (for a review, Dascal, 1987). Under voltage-clamp conditions, these alterations in intracellular Ca^{2+} levels can be detected by changes in holding currents. However, during our experiments, the coapplication of anandamide and ethanol to oocytes expressing α_7-nACh 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 ethanol and anandamide. In addition, passive membrane properties of oocytes were not significantly altered by anandamide and ethanol, suggesting that coapplication of these reagents to oocytes did not disrupt the integrity of the lipid membrane.

Endogenous cannabinoids, at the concentration range used in this study, have been shown to activate cannabinoid receptors (for a review, Howlett et al., 2002). However, binding studies conducted indicate that cannabinoid receptors are not expressed endogenously in *Xenopus* oocytes (Henry and Chavkin, 1995). In addition, our previous work has demonstrated that the CB_1 receptor antagonist SR-141716A and the CB_2 receptor antagonist SR144528 did not affect the anandamide-induced inhibition of α_7-nACh receptors expressed in oocytes, nor did pertussis toxin alter the

inhibition by anandamide (Oz et al., 2003a), suggesting a direct effect of anandamide on the α_7-nACh receptors. Based on these observations, we propose that additive inhibitory effect of anandamide and ethanol on the function of neuronal α_7-nACh receptors expressed in oocytes is also independent of the participation of cannabinoid receptors.

Chronic application of ethanol to mammalian cells has been shown to increase extracellular concentration of anandamide by activating *de novo* synthesis of anandamide and inhibiting its intracellular transport (Basavarajappa and Hungund, 1999; 2002). Although in our ethanol experiments, oocytes were only acutely exposed to ethanol for 20 to 30 min of duration, we set up several experiments to evaluate if ethanol-induced changes in anandamide levels could account for the additive inhibitory effect of anandamide and ethanol on the function of neuronal α_7-nACh receptors expressed in oocytes. As a first step, we compared the spectra profiles of oocyte preparations under control conditions and after exposure to ethanol for 30 min, and found that anandamide was not detectable in any of these preparations. Thus, ethanol-induced changes in anandamide levels do not account for the additive inhibitory effects of anandamide and ethanol on the function of neuronal α_7-nACh receptors expressed in oocytes. In additional studies, we demonstrated that the additive effects of ethanol and anandamide did not change when the anandamide transport inhibitor AM404 was present in bathing solution, suggesting that inhibition of anandamide transport does not mediate the additive inhibitory effect of anandamide and ethanol on the function of neuronal α_7-nACh receptors.

In our earlier experiments, we have demonstrated that inhibition of neuronal α_7-nACh receptors by anandamide is not mediated by its metabolic products (Oz et al., 2003a; 2004b). One of the products of anandamide hydrolysis is arachidonic acid (Cravatt and Lichtman, 2002), and the perfusion of tissue cultures by ethanol is known to cause production of pharmacologically active

compounds such as ethyl arachidonate (fatty acid ethyl ester) from arachidonic acid (Gubitosi-Klug and Gross, 1996). However, inhibition of FAAH activity by PMSF did not prevent the additive actions of anandamide and ethanol on the function of neuronal α_7-nACh receptors, suggesting that the production of arachidonic acid ethyl esters from arachidonic acid and ethanol does not mediate the additive inhibitory effects of anandamide and ethanol in oocyte expression system.

Analysis of the inhibition of α_7-nACh receptors at different anandamide concentrations indicated that in the presence of ethanol, the IC_{50} value for anandamide decreased significantly. In contrast, in the presence of anandamide, alteration of the IC_{50} value for ethanol was not observed (Fig. 2). It is likely that in the presence of ethanol, this change in the IC_{50} value for anandamide is due to the different efficacies of anandamide (complete inhibition) and ethanol (approximately 65 % inhibition of α_7-nACh receptor-mediated currents). Thus, in the presence of ethanol, maximal inhibition of ACh-induced responses occurred at a lower anandamide concentration. As a result, the IC_{50} shifted to lower values without a change in anandamide efficacy. On the other hand, only in the presence of anandamide was ethanol able to inhibit ACh-induced responses completely (increased efficacy without causing an alteration on IC_{50} value). Both anandamide and ethanol are allosteric inhibitors of α_7-nACh receptors, as these molecules bind to sites topographically distinct from the agonist binding sites for ligand-gated ion channels. Recently, allosteric interactions of ethanol with neurosteroids and membrane cholesterol on the function of ion channels have been investigated and both positive and negative allosteric interaction between ethanol and these modulators have been reported (Akk and Steinbach, 2003; Crowley et al., 2003). Similarly, ethanol has been shown to potentiate anandamide activation of TRPV1 receptor-mediated currents in HEK293 cells (Trevisani et al., 2002).

Earlier studies have demonstrated positive or negative cooperation between different allosteric

modulators for various ligand-gated ion channels (for reviews; Changeux and Edelstein, 1998; Christopoulos, 2002). Here we report for the first time that ethanol and anandamide have additive inhibitory effects on the function of α_7-nACh receptors. This additive effect did not show cooperativity between the binding sites for ethanol and cannabinoids on the α_7-nACh receptors. The lack of cooperativity between the binding sites for these allosteric modulators was confirmed in experiments employing the chimeric α_7-nACh-5-HT_3 receptor. The functional chimeric α_7-nACh-5-HT_3 receptor consisted of an N-terminal domain of the α_7-nACh receptor and the transmembrane and C-terminal domains of the 5-HT_3 receptor (Eisele et al., 1993, Zhang et al., 1997). We have demonstrated previously that anandamide and ethanol have allosteric binding sites on the α_7-nACh receptor (Yu et al, 1997; Oz et al., 2003a, 2004b).

In this study we have examined if the chimeric α_7-nACh-5-HT_3 receptor uncouples a positive or negative linkage between the allosteric modulators ethanol and anandamide. Previous studies have demonstrated that anandamide inhibits the function of α_7-nACh receptors with a potency that was an order of magnitude higher than at 5-HT_3 receptors in *Xenopus* oocytes (Oz et al., 2002a; 2003a). In the present study, we found that anandamide did not inhibit chimeric α_7-nACh-5-HT_3 receptor; however, coapplication of anandamide and ethanol caused a significant inhibition of the chimeric α_7-nACh-5-HT_3 receptor. Nevertheless, the extent of inhibition by the coapplication of anandamide and ethanol was not significantly different from the inhibition caused by ethanol alone. The lack of additive effects of anandamide and ethanol on the chimeric α_7-nACh-5-HT_3 receptor-mediated currents indicates that there is no cooperativity (positive or negative) inherent to topographically distinct binding sites between ethanol and anandamide, and that these molecules act on different sites of the nicotinic ACh receptor.

In several earlier studies, ethanol has been shown to modulate the release and metabolism of arachidonic acid and prostaglandins (George and Collins, 1985; Westcott and Collins, 1985; Basavarajappa et al., 1998). In addition to anandamide, fatty acids such as arachidonic acid and their oxygenated metabolites such as prostaglandins, also are allosteric inhibitors of nicotinic ACh receptors (Vijayaraghavan et al., 1995; Tan et al., 1998; Du and Role 2001; Oz et al., 2004b). Thus, by modulating fatty acid release and metabolism, ethanol may indirectly influence signaling via nicotinic ACh receptors.

To our knowledge, this is the first study investigating the interaction between two allosteric inhibitors on the function of α_7-nACh receptors; however, a similar study on nACh receptors from mouse skeletal muscle investigated the effects of arachidonic acid and prostaglandin D_2 on the desensitization of nACh receptor-mediated ion currents (Nojima et al., 2000). In this study, coapplication of arachidonic acid and prostaglandin D_2 caused a cooperative increase in their effects on desensitization of nACh receptor-mediated ion currents.

Because of the enhanced potency of the endocannabinoid modulation by ethanol and the presence of α_7-nACh receptors in these critical locations such as presynaptic terminals, it is possible that in the presence of ethanol the activity of the α_7-nACh receptors may be further modulated by endocannabinoids released from postsynaptic sites in situ. Considering that chronic ethanol treatments enhance anandamide synthesis, it is likely that ethanol-induced inhibition of α_7-nACh receptors would be more relevant due to already suppressed function of α_7-nAChRs by enhanced endocannabinoid tone.

In conclusion, our results indicate that co-administration of ethanol and anandamide causes additive inhibition of α_7 nACh receptor-mediated currents in *Xenopus* oocytes. The results of studies

with chimeric α_7-nACh-5-HT_3 receptor suggest that there are multiple allosteric modulatory sites for ethanol and anandamide. Collectively, these data suggest that α_7-nACh receptors may represent a novel link between endocannabinoid tone and ethanol in the intact nervous system.

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FIGURE LEGENDS

Figure 1. The effects of anandamide, ethanol and anandamide + ethanol on α_7-nACh receptor-mediated ion currents. **(A)** Currents activated by ACh (100 μM) and recorded from the same oocyte in control extracellular media (*first trace from the left*), at 10 min bath application of 30 mM ethanol (*second trace from the left*), and 20 min after coapplication of 30 mM ethanol and 100 nM anandamide (*third trace from the left*), following 30 min of recovery (*fourth trace from the left*), and after 20 min application of 100 nM anandamide alone (*the last trace from the left*). **(B)** Time-courses of the effects of anandamide, ethanol and anandamide + ethanol on the peak ACh-induced currents. Each data point represents the normalized means ± s.e.m. of 4 to 5 experiments. The duration of the anandamide or anandamide + ethanol application is indicated by the horizontal bar. **(C)** Bar graph comparing the inhibitory effects of anandamide, ethanol, anandamide + ethanol on the maximal amplitudes of ACh-induced currents. AEA and EtOH indicate anandamide and ethanol, respectively. Statistical significance at the level of $P< 0.05$ was presented with * (ANOVA).

Figure 2. The effects of ethanol and anandamide on concentration-response curves for anandamide and ethanol-induced inhibition of α_7-nACh receptor-mediated ion currents. **(A)** Concentration-response curve for anandamide in the absence and in the presence of 30 mM ethanol. Inset shows analysis of data after the subtraction of tonic inhibition caused by 30 mM ethanol and normalizing to maximal inhibition for each data set. Data points represent the mean ± s.e.m. of 4-6 oocytes. **(B)** Concentration-response curve for ethanol 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 ± s.e.m. of 4-6 oocytes. The curves are the best fit of the data to the logistic equation described in the methods.

Currents were activated by applying ACh (100 μM).

Figure 3. Additive effect of anandamide and ethanol on ACh-induced (100 μM) currents is not due to alterations in anandamide hydrolysis, anandamide membrane transport, endogenous Ca^{2+}-activated Cl^- channels and membrane-potential. **(A)** The effects of the inhibitions of anandamide hydrolysis by PMSF (*black bar*) and anandamide transport by AM404 (*gray bar*) on the suppression of ACh-induced currents by coapplication of anandamide and ethanol. **(B)** Comparison of the effects of anandamide + ethanol on the amplitudes of ACh-induced currents in the absence or presence of factors contributing to the activation of Ca^{2+}-dependent Cl^- channels. Oocytes were either injected with 50 nl distilled water and recorded in a 2 mM Ca^{2+}-containing MBS solution (*black bar*) or injected with 50 nl of 100 mM BAPTA (*gray bar*) and recorded in a 2 mM Ba^{2+}-containing MBS solution. Each data point represents the normalized mean ± s.e.m. of 5 to 6 experiments. **(C)** Current-voltage relationships of ACh-activated currents in controls, ethanol (30 mM), anandamide (100 nM), and anandamide + ethanol. Each data point represents the normalized means and s.e.m. of 3 to 5 experiments. **(D)** The effects of anandamide and anandamide + ethanol are presented as percent inhibition of ACh-activated currents at different voltages indicated in the figure.

Figure 4. Lack of anandamide in *Xenopus* oocytes and the effect of ethanol on mass spectra of *Xenopus* oocytes measured with matrix-assisted laser desorption/ionization. **(A)** Anandamide standard (150 fM) was indicated in spectrum. Peaks correspond to the protonated anandamide and its sodium adduct. Mass spectra of oocytes in control **(B)** and in the presence of 100 mM ethanol **(C)**. Cholesterol and phosphatdylcholine species were detectable in both control and ethanol treated oocytes.

Figure 5. Effect of anandamide, ethanol and anandamide + ethanol on responses mediated by α_7-nACh receptors, 5-hydroxytryptamine type 3 receptors (5-HT_3), and chimeric α_7-nACh-5-HT_3 receptors. Agonist concentration-response curves for α_7-nACh receptors **(A)**, 5-HT_3 receptors **(B)**, the chimeric receptors **(C)**, and the effects of anandamide (100 nM) and anandamide + ethanol (30 mM) on the responses mediated by each of these receptors are shown. Each data point represents the average of 3 to 5 cells (mean ± s.e.m.). The error bars that are not visible are smaller than the size of the symbols. Paired concentration-response curves were constructed and responses normalized to the maximum response under control conditions. Current traces illustrating the effect of anandamide and anandamide + ethanol on currents mediated by α_7-nACh receptors, 5-HT_3 receptors, and chimeric receptors are presented as insets. The bar above each record indicates the period of agonist application. Vertical calibration bars for the α_7-nACh receptors, the 5-HT_3 receptors, and the chimeric receptors represent 100 nA, 200 nA, and 200 nA, respectively. Horizontal calibration bars indicate 2 s, 10 s, and 3 s, respectively. The α_7-nACh receptor and chimeric receptor-mediated currents were activated by 100 μM ACh; the 5-HT_3 receptor-mediated currents were activated by 1 μM 5-HT.

Figure 1

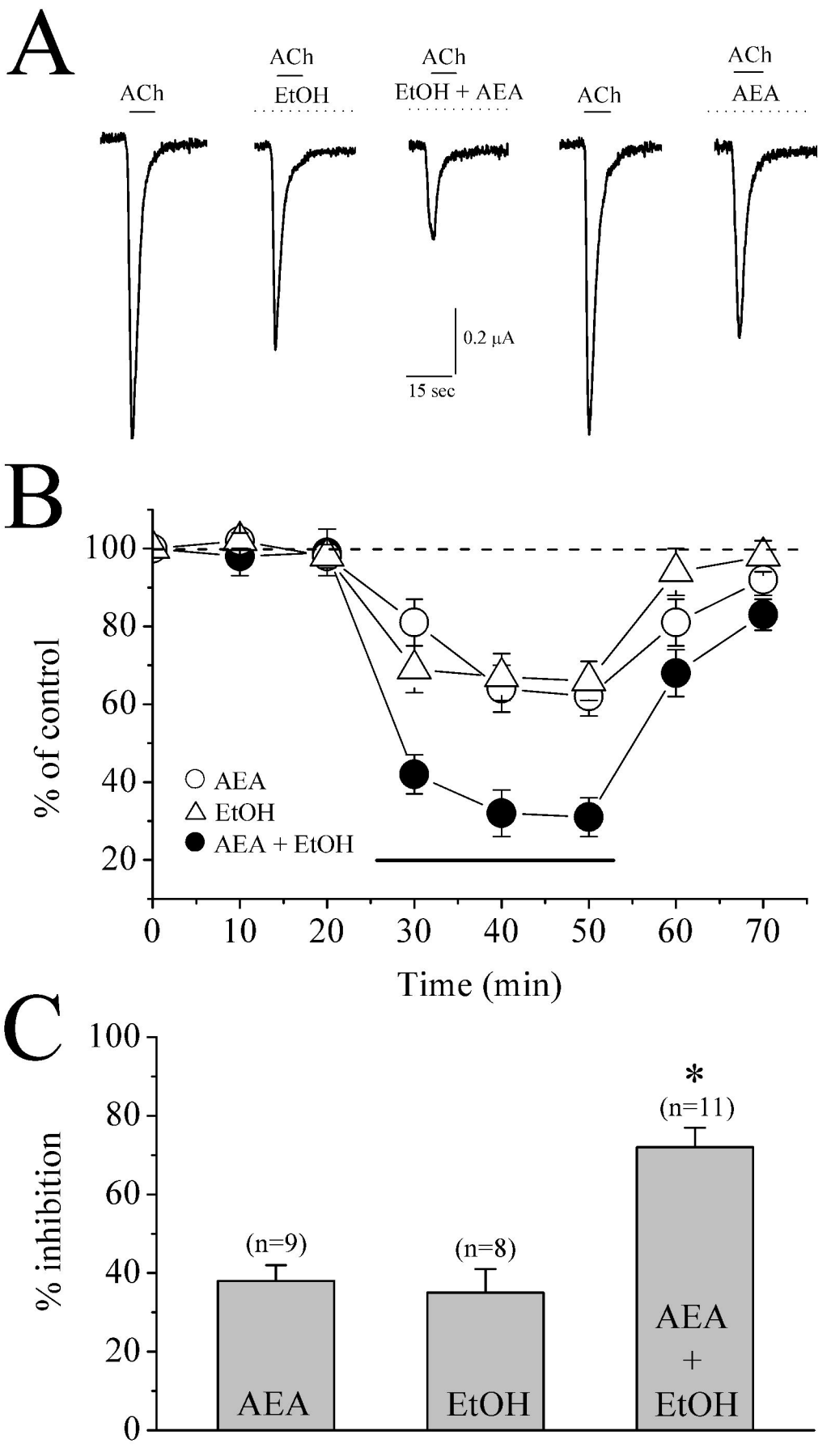
A
ACh
ACh
EtOH
ACh
EtOH + AEA
ACh
ACh
AEA
0.2 μA
15 sec
B
% of control
100
80
60
40
20
AEA
EtOH
AEA + EtOH
0
10
20
30
40
50
60
70
Time (min)
C
% inhibition
100
80
60
40
20
0
(n=9)
(n=8)
*
(n=11)
AEA
EtOH
AEA
+
EtOH

Figure 2

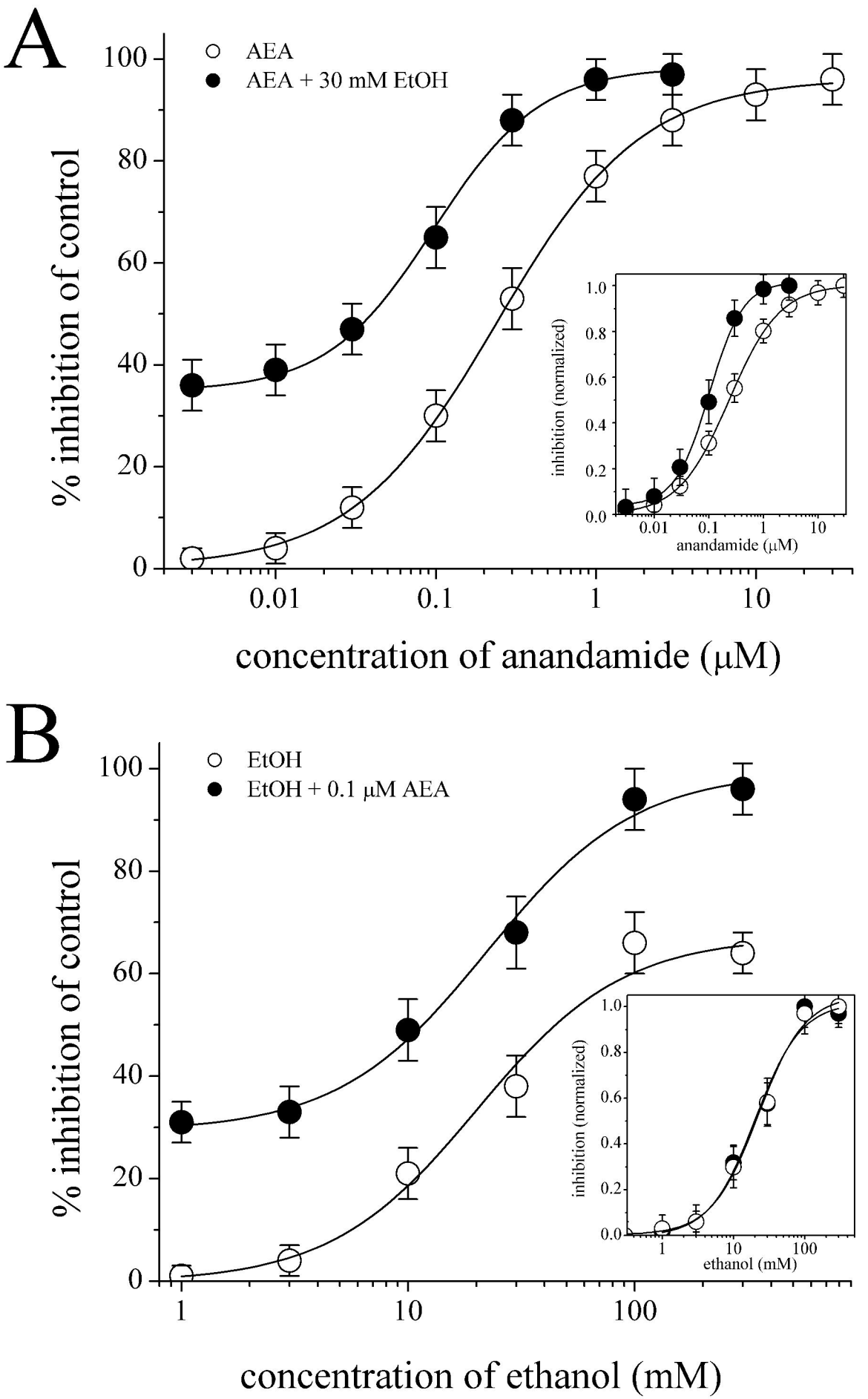
A
AEA
AEA + 30 mM EtOH
% inhibition of control
concentration of anandamide (μM)
inhibition (normalized)
anandamide (μM)
B
EtOH
EtOH + 0.1 μM AEA
% inhibition of control
concentration of ethanol (mM)
inhibition (normalized)
ethanol (mM)

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Figure 3

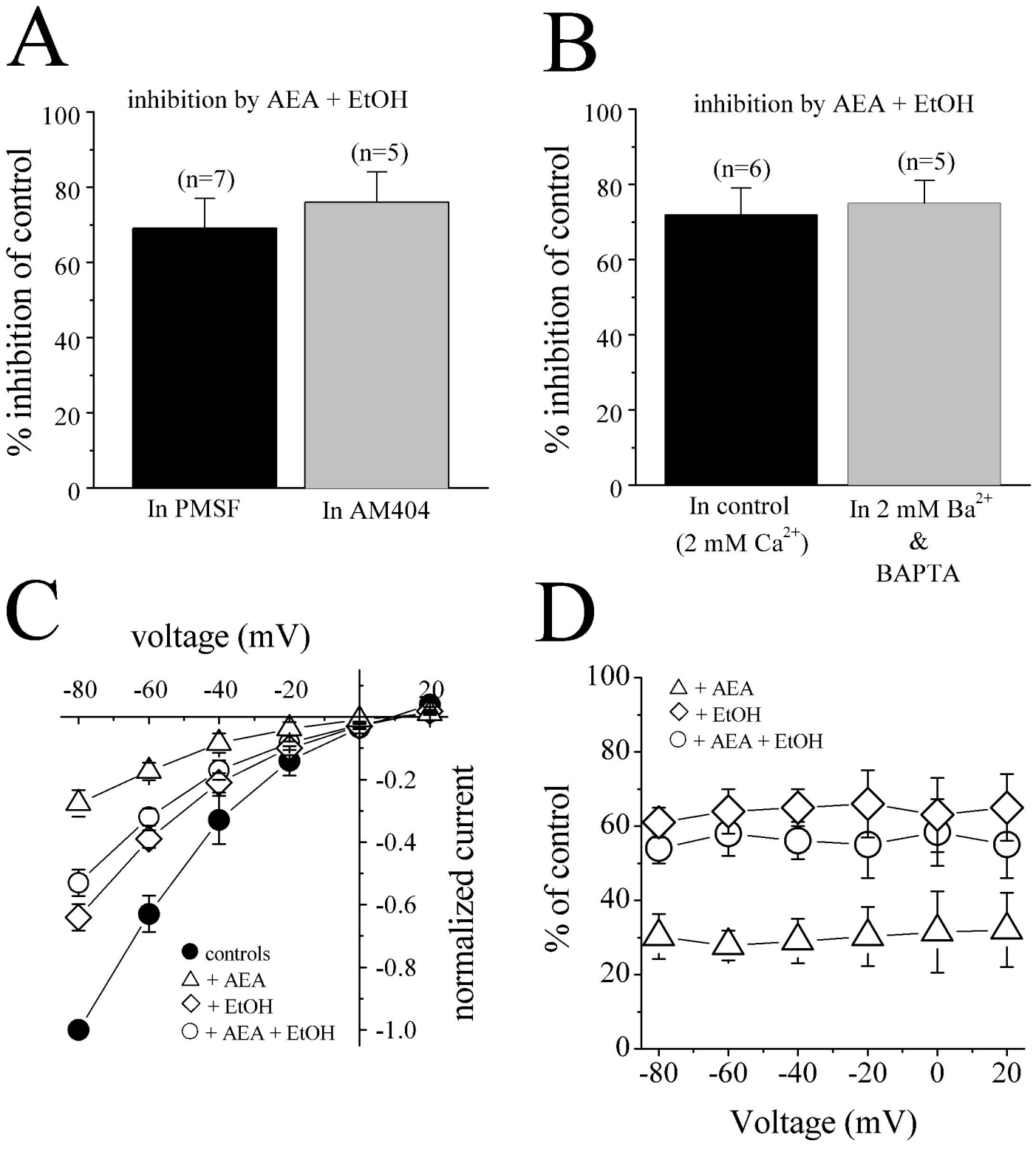

Figure 4

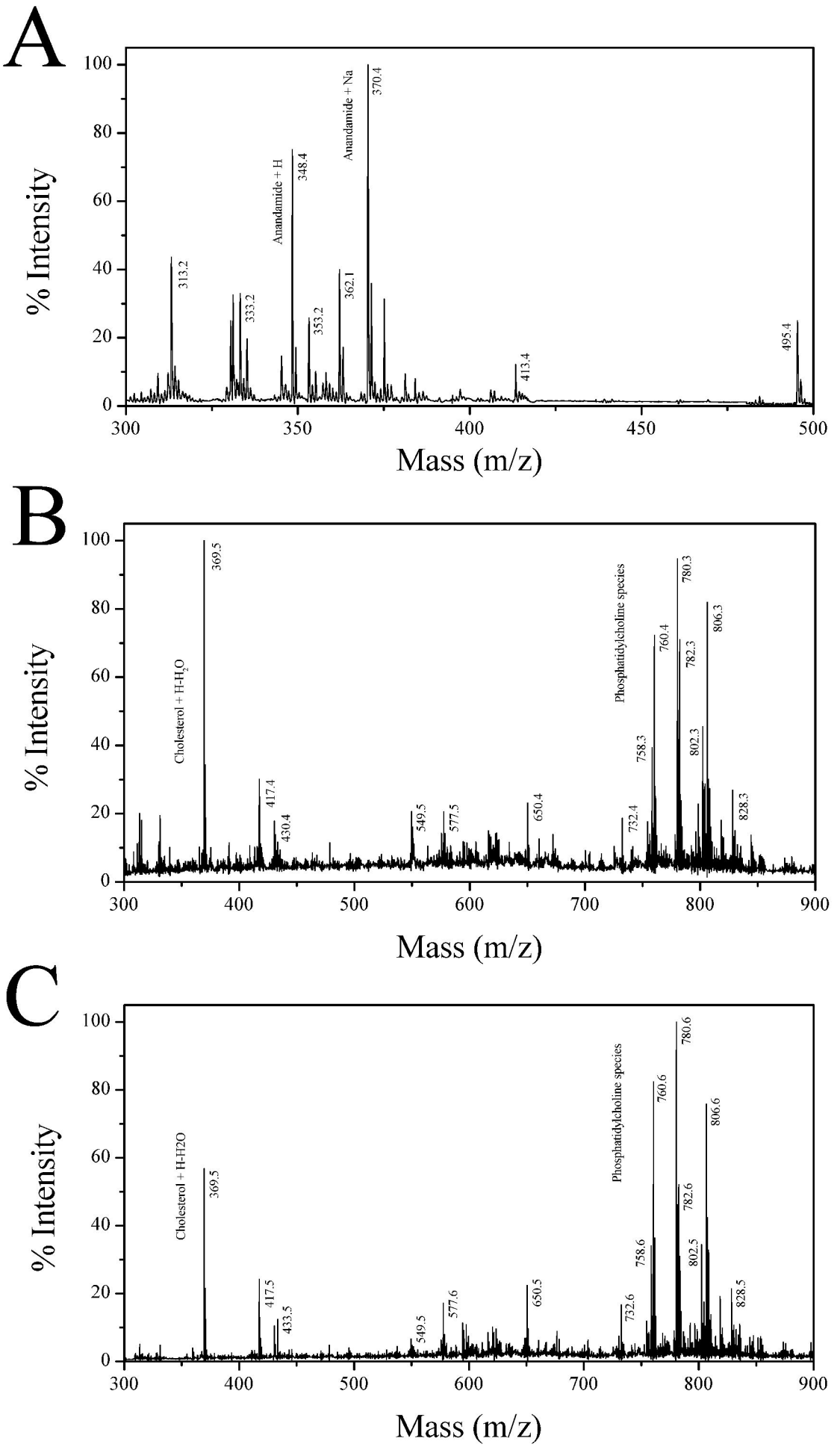
A
% Intensity
Mass (m/z)
313.2
333.2
Anandamide + H
348.4
353.2
362.1
Anandamide + Na
370.4
413.4
495.4
B
% Intensity
Mass (m/z)
Cholesterol + H-H2O
369.5
417.4
430.4
549.5
577.5
650.4
732.4
758.3
Phosphatidylcholine species
760.4
780.3
782.3
802.3
806.3
828.3
C
% Intensity
Mass (m/z)
Cholesterol + H-H2O
369.5
417.5
433.5
549.5
577.6
650.5
732.6
758.6
Phosphatidylcholine species
760.6
780.6
782.6
802.5
806.6
828.5

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Figure 5