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Ethanol Modulation of Nicotinic Acetylcholine Receptor Currents in Cultured Cortical Neurons

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ABSTRACT

Ethanol, at physiologically relevant concentrations, significantly enhanced high-affinity neuronal nicotinic acetylcholine receptor (NnAChR) currents insensitive to α-bungarotoxin (α-BuTX-ICs) in cultured rat cortical neurons in a fast and reversible manner, as determined by standard whole-cell patch-clamp recording techniques. The enhancement was (mean $\pm$ S.D.) 7.7 $\pm$ 5% to 192 $\pm$ 52% upon coapplication of 3 to 300 mM ethanol with 1 to 3 μM ACh. No plateau for this ethanol-induced enhancement of α-BuTX-ICs was reached. The maximal α-BuTX-IC evoked by very high concentrations of ACh also was increased upon coapplication of ethanol. In contrast, ethanol weakly inhibited low-affinity NnAChR currents sensitive to α-BuTX (α-BuTX-SCs) (5 $\pm$ 4% to 29 $\pm$ 6% inhibition by 10 to 300 mM ethanol at 300 to 1000 μM ACh). This neuronal preparation also enabled comparison of ethanol action on NnAChRs with its action on *N*-methyl-D-aspartate receptor currents and γ-aminobutyric acid receptor currents within the same neurons. Ethanol (100 mM) was more potent at enhancing NnAChR α-BuTX-ICs (61 $\pm$ 9% enhancement) than it was at enhancing γ-aminobutyric acid receptor current (3 $\pm$ 3% enhancement—not statistically significant) or at inhibiting *N*-methyl-D-aspartate receptor currents ($\sim$35 $\pm$ 7% inhibition). Thus, NnAChRs, particularly those insensitive to α-BuTX, may be sensitive conduits through which ethanol can mediate some of its actions in the brain.

In animals, tolerance to some of the reinforcing effects of ethanol can be induced by chronic nicotine administration, and tolerance to some of the effects of nicotine can be induced by chronic ethanol administration (Zacny, 1990; Collins, 1996). Such cross-tolerance infers the involvement of central nervous system (CNS) nicotinic acetylcholine receptors (NnAChRs) in the mediation of ethanol action in the brain. Ethanol is known to modulate the function of numerous neurotransmitter-gated receptor-ion channels important in the CNS, including inhibition of *N*-methyl-D-aspartate receptors (NMDARs), enhancement of γ-aminobutyric acid receptors ($GABA_A$Rs), enhancement of type III 5-hydroxytryamine (or serotonin) receptors ($5\text{-}HT_3$Rs), as well as inhibition of voltage-activated calcium channels (Mullikin-Killpatrick and Treistman, 1993; Crews et al., 1996; Lovinger, 1997). Although ethanol modulation of the nicotinic receptors at the neuromuscular junction is well documented (Miller et al., 1991), the very limited reports concerning the direct action of acute application of ethanol on NnAChRs have been restricted to our previous study of ganglionic-type NnAChRs natively expressed by PC12 cells (Nagata et al., 1996) and a recent study by Covernton and Connolly (1997) using cloned NnAChRs recombinantly expressed in *Xenopus* oocytes.

Our previous study (Nagata et al., 1996) indicated variable effects on NnAChR current amplitude upon application of low concentrations of ethanol, whereas Covernton and Connolly (1997) reported enhancement of NnAChR currents upon application of high concentrations of ethanol. Covernton and Connolly (1997) also reported variable effects of enhancement and inhibition at the lower ethanol concentrations for the $\alpha3\beta4$ subtype NnAChR, which is considered to be a major NnAChR subtype in PC12 cells (Rogers et al., 1992). Considering that ethanol and nicotine are the two most used and abused drugs, and that their effects are most certainly manifest in the brain, more effort to delineate the molecular and cellular mechanisms of their effects on NnAChRs expressed in central neurons is of crucial importance.

However, because of diffuse expression of NnAChRs in the brain and the resultant difficulty of obtaining NnAChR responses in primary neuronal cultures, the effects of ethanol on the primary NnAChRs expressed in central neurons (generally thought to be the $\alpha4\beta2$ and $\alpha7$ subtypes) have not been forthcoming. This has led to attempts to establish and characterize cloned NnAChRs in recombinant expression systems—an approach also seemingly plagued with difficulties.

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ABBREVIATIONS: NnAChR, neuronal nicotinic acetylcholine receptor; α-BuTX, α-bungarotoxin; α-BuTX-SC, α-BuTX-sensitive current; α-BuTX-IC, α-BuTX-insensitive current; DHβE, dihydro-β-erythroidine, DMPP, dimethylphenylpiperazinium; GABA, γ-aminobutyric acid; $GABA_AR$, $GABA_A$ receptor; NMDA, *N*-methyl-D-aspartate; NMDAR, NMDA receptor.

Even when achieved it has been indicated that cloned NnAChRs expressed either transiently in *Xenopus* oocytes or stably in mammalian cell lines may not accurately represent native NnAChRs in neurons (Cooper and Millar, 1997; Lewis et al., 1997; Sivilotti et al., 1997).

In the present study, long-term cultures of rat cortical neurons were established from which a tangible number of neurons were found to exhibit nicotinic acetylcholine currents. These ACh-evoked NnAChR currents had sufficient amplitude to enable apt investigation of ethanol action on *central* NnAChR-mediated ion channel activity. The primary findings were that ACh-evoked NnAChR currents sensitive to α-bungarotoxin (α-BuTX-SCs) were modestly inhibited by ethanol, whereas ACh-evoked NnAChR currents insensitive to α-bungarotoxin (α-BuTX-ICs) were significantly enhanced by low concentrations of ethanol. The neuronal preparation also made it possible to compare ethanol sensitivities of NnAChRs, NMDARs, and $GABA_A$Rs within the same neuron. Such comparisons demonstrated that the potency of ethanol in enhancing NnAChR α-BuTX-ICs was greater than that for the enhancement of $GABA_A$R currents and that for the inhibition of NMDAR currents exemplifying the diversity of outcomes that ethanol could produce even at the single neuron level.

Materials and Methods

Cell Preparation. Rat cortical neurons were isolated and cultured by a procedure slightly modified from that described elsewhere (Marszalec and Narahashi, 1993). In brief, rat embryos were removed from 17-day pregnant Sprague-Dawley rats under methoxyflurane anesthesia. Small wedges of frontal cortex were excised and subsequently incubated in phosphate-buffered saline solution containing 0.25% (w/v) trypsin (Type XI; Sigma-Aldrich, St. Louis, MO) for 20 min at 37°C. The digested tissue was then mechanically triturated by repeated passages through a Pasteur pipette, and the dissociated cells were suspended in neurobasal medium with B-27 supplement (GiBCO, Gaithersburg, MD) and 2 mM glutamine. The cells were added to 35-mm culture wells at a concentration of 100,000 cells/ml. Each well contained five 12-mm coverslips (previously coated with poly-L-lysine) overlaid with confluent glia that had been plated 2 to 4 weeks earlier. The cortical neuron/glia cocultures were maintained in a humidified atmosphere of 90% air/10% CO_2 at 37°C. Cells used for electrophysiological experiments were cultured for 1 to 9 weeks.

Electrophysiological Recordings. All chemicals were reagent grade or higher from Sigma-Aldrich unless otherwise specified. The standard external solution consisted of: 140 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 15 mM acid-HEPES, 10 mM Na-HEPES, with 3 μM $LaCl_3$, 0.2 μM TTX, and 0.3 μM atropine sulfate, pH 7.3. Two different pipette solutions were used: one with high calcium-buffering capacity, and a second with 5 mM Mg-ATP added. The two different solutions were used to evaluate the possible effects of internal Ca^{++} or energy processes on NnAChR activity. The pipette solution with high calcium-buffering capacity consisted of: 140 mM cesium-gluconate, 15 mM NaCl, 5 mM potassium-gluconate, 15 mM acid-HEPES, 10 mM sodium-HEPES, 35 mM cesium-BAPTA [1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetate; free BAPTA $\cong$ 20 mM], 12 mM calcium-gluconate (free $Ca^{++} \cong 100$ nm), 4 mM magnesium-gluconate (free $Mg^{++} \cong 1$ mM). The ATP-containing pipette solution consisted of: 140 mM cesium-gluconate, 15 mM NaCl, 5 mM potassium-gluconate, 1 mM $MgCl_2$, 15 mM acid-HEPES, 10 mM sodium-HEPES, 11 mM EGTA, 1 mM $CaCl_2$, 5 mM magnesium-ATP, and 0.2 mM sodium-GTP. Pipettes were pulled from borosilicate glass capillaries (Kimax-51; 1.8 mm o.d., 1.5 i.d.; Kimble Glass Co., Vineland, NJ) or Clark Patch Glass (PG120T; 1.2 o.d., 0.93 i.d.; Warner Instruments, Hamden, CT) and lightly fire-polished to a final resistance of 2 to 4 MΩ when filled with the internal solution.

Whole-cell currents recorded at room temperature (20–25°C) were filtered at 1 to 10 kHz (4-pole Bessel, −3 dB) via either an Axopatch 200 amplifier or an Axopatch 1-C amplifier (Axon Instruments, Foster City, CA). The holding potential was –70 mV, unless otherwise specified. Recorded currents were directly digitized at 1 to 10 kHz, acquired to the hard disk of the microcomputer via a Digidata 1200 ADC/DAC interfaced to a microcomputer under control of the ClampEx module of the Pclamp6 software package (Axon Instruments). Agonists and test compounds were applied to cells via a modified U-tube system (Marszalec and Narahashi, 1993) with a solution-exchange time of 10 to 15 ms as measured by change in junction potential. Dihydro-β-erythroidine·HBr (DHβE) and mecamylamine·HCl were obtained from Research Biochemicals International, Natick, MA. In the present study, the term "coapplication" refers to the short (0.5–3 s), simultaneous application of effector(s) with agonist via the U-tube only (the application solution contains both the effector(s) and agonist), not via perfusion through the external bathing solution. The term "pre-exposure" refers to the application of effector(s) via perfusion through the external bathing solution. The ethanol used in these experiments was nondenatured, absolute (200 proof) ethyl alcohol USP (Midwest Grain Products, Weston, MO) bought and stored in glass containers.

Analysis. Current records initially were analyzed via the ClampFit module of the Pclamp6 to assess whole-cell current amplitudes and decay kinetics. Cumulative concentration-response results subsequently were compiled for graphical analysis in SigmaPlot. Analysis of variance and/or Student's *t* tests were performed to assess significance of differences between test and control measurements.

Results

Description of Cortical Neurons in Culture. The neuronal cultures consisted of a variety of differently shaped neurons appearing as pyramidal, irregularly rounded, ovoid, and rectangular. After about 1 week in culture, most neurons projected from two to as many as five or more neurites. The ovoid cells generally projected two major neurites, the pyramidal cells projected three to four, and the irregularly rounded and rectangular cells projected four or more. After about 2 weeks, a dense and complex network of neuronal processes was established. At this time and later, it became apparent that synapses between these cultured neurons had "reformed." This was evidenced by spontaneous activity that was recorded as excitatory and/or inhibitory postsynaptic currents, primarily mediated by NMDA and GABA receptors (Marszalec et al., 1998). After about 4 to 5 weeks, many of the pyramidal cells began to show signs of degeneration (blebbing and neurite degradation), while the irregularly rounded, ovoid, and rectangular cells remained relatively healthy looking until about 8 to 9 weeks in culture. Also more apparent after 4 to 5 weeks in culture were persistent, miniature spontaneous excitatory and inhibitory currents, which were not blocked by tetrodotoxin. Because such activity potentially could cause interference in assessing evoked NnAChR currents, 6-cyano-7-nitroquinoxaline-2,3-dione (1–3 μM), 2-amino-5-phosphonopentanoic acid (30 μM), and bicuculline (30 μM) or picrotoxin (30 μM) often were added to the external solution to reduce these miniature spontaneous excitatory and inhibitory currents. Inclusion of these agents in the extracellular solution reduced only the background activity and had no observable effect on the ACh- or nicotine-evoked currents.

Properties of NnAChR Currents. ACh in the presence of 0.1 to 0.3 μM atropine was routinely used to assess NnAChR activity in cultured cortical neurons. In general, in the absence of α-BuTX all neurons that responded to short applications of ACh (at concentrations $\geq$300 μM) always exhibited a quickly decaying current. In many neurons (pyramidal-shaped neurons in particular, although not restricted to this type of neuron) this quickly decaying current was the only type of current observed (Fig 1A). Some neurons exhibited a slowly decaying current component along with the quickly decaying current (Fig 1B). However, in general, neurons did not exhibit the slowly decaying current by itself.

In detail, the quickly decaying current was characterized by fast activation (3.2 $\pm$ 0.5 ms, 10–90% rise time at 3000 μM ACh) and fast decay (20 $\pm$ 2 ms, 10–90% decay time at 3000 μM ACh) kinetics that could be completely abolished upon 6- to 10-min perfusion of 30 to 100 nm α-BuTX through the external bathing solution for (Fig. 1A). These α-BuTX-sensitive currents (α-BuTX-SCs) displayed low ACh affinity (EC_{50} = 330 $\pm$ 30 μM ACh with a Hill coefficient of 1.3; n = 7) (Fig. 1F) and showed some rundown that could be attenuated by inclusion of Mg-ATP and Na-GTP in the pipette solution. No difference in the character of these currents was observed when high or low Ca^{++}-buffering-capacity pipette solutions was used, indicating no interference by calcium-dependent currents in these assessments. Such properties generally are attributed to homomeric α7 subtype NnAChRs, and, in all likelihood, the α-BuTX-SCs are mediated by α7-type NnAChRs.

The current characterized by slow decay showed no sensi-

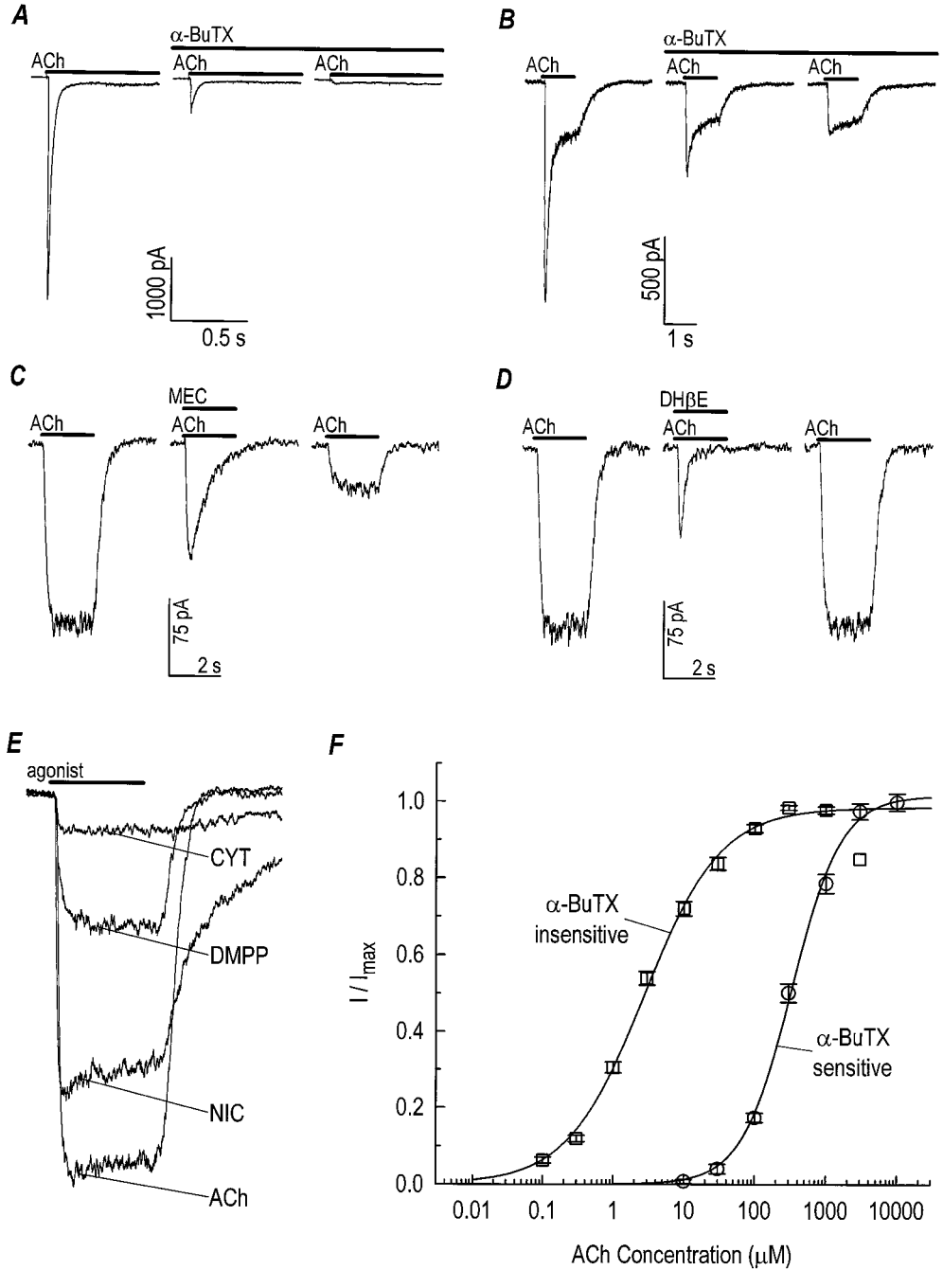

Fig. 1. Two types of ACh-activated currents in cultured rat cortical neurons. A, sequential whole-cell currents evoked by 3000 μM ACh (solid bar under effector labels indicate application times) at 3-min intervals exhibiting fast onset and decay. This type of current is sensitive to α-BuTX, as indicated by the slow, but irreversible inhibition after perfusion of 100 nM α-BuTX through the external bath solution for ~6 min. B, sequential whole-cell currents evoked by 300 μM ACh at 3-min intervals exhibiting both a fast-decaying component inhibited after ~6 min perfusion of 100 nm α-BuTX through the external bath solution and a slowly decaying α-BuTX-insensitive component remaining after such α-BuTX perfusion. C and D, sequential whole-cell currents evoked by 3 μM ACh at 3-min intervals in the presence of 30 nM α-BuTX demonstrating that the α-BuTX-insensitive steady-state current is blocked by short coapplications of 10 μM mecamylamine (MEC) or 0.1 μM DHβE. E, superimposed whole-cell α-BuTX-ICs evoked by 3 μM concentrations of ACh, nicotine (NIC), DMPP, and cytisine (CYT), all from the same neuron and in the presence of 30 nM α-BuTX. F, Agonist concentration relationships for ACh activation of α-BuTX-SCs (○) and α-BuTX-ICs (□). The data points are the mean peak current amplitudes with S.E.M. expressed as a fraction of the maximal current obtained at 10,000 μM ACh for α-BuTX-sensitive currents and at 300 μM ACh for α-BuTX-ICs. Data are from 7 to 23 neurons. The EC_{50} for α-BuTX-SC activation was 330 $\pm$ 30 μM ACh with a Hill coefficient of 1.3. The EC_{50} for activation of α-BuTX-ICs was 2.7 $\pm$ 0.2 μM ACh with a Hill coefficient of 0.8.

tivity to α-BuTX and persisted well after (>10 min) exposure to the toxin (remaining current in Fig. 1B). In the constant presence of 30 to 100 nM α-BuTX both perfused through the bath and included in the application solutions, the α-BuTX-ICs could be inhibited by mecamylamine (Fig. 1C) or DHβE (Fig. 1D). These α-BuTX-ICs displayed a relatively high ACh affinity (EC_{50} = 2.7 ± 0.2 μM ACh with a Hill coefficient of 0.8; n = 23) (Fig. 1F), although considerable cell-to-cell variance in the calculated ACh EC_{50} was indicated by the individual ACh concentration-response relationships, which ranged from 1.3 to 14 μM ACh. The α-BuTX-ICs exhibited no significant rundown whether or not Mg-ATP or Na-GTP was included in the pipette solution. No significant difference in current character was observed when using either the high or the low Ca^{++}-buffering capacity pipette solution.

α-BuTX-ICs could also be activated by nicotine, dimethylphenylpiperazinium (DMPP), and cytisine. At a concentration of 3 μM for all agonists (a concentration chosen so as not to introduce complexities due to desensitization) and in the presence of 30 to 100 nM α-BuTX, the α-BuTX-ICs were most effectively activated by ACh, followed closely by nicotine (88 ± 16% that of ACh; n = 5), with DMPP being modestly effective (40 ± 6% that of ACh; n = 5), and cytisine being least effective (13 ± 3% that of ACh; n = 4) (Fig. 1E). Perhaps noteworthy is the very apparent slow inactivation of current evoked by either nicotine or cytisine. This was not an incidental application/removal methodological effect and was always observed when currents were evoked with these two agonists, possibly suggesting differences in affinity or mechanisms of activation/inactivation for nicotine and cytisine versus ACh and DMPP.

The properties of high ACh affinity, nanomolar to low micromolar potency of the DHβE inhibition, and poor cytisine activation exhibited by the α-BuTX-ICs indicates that they most closely resemble α4β2-subtype NnAChRs (Luetje and Patrick, 1991; Buisson et al., 1996; Chavez-Noriega et al., 1997; Fenster et al., 1997). However, contributions by other NnAChR subunits, such as β4 and α5, or even α2 or α3 contribution cannot be ruled out at present in the absence of immunological data and considering the variance in the ACh sensitivity indicated by the relatively large range in the individual cell-to-cell ACh EC_{50} values.

Ethanol on α-BuTX-SCs. To assess the effects of ethanol on α-BuTX-SCs separately from α-BuTX-ICs, only those ACh currents exhibiting low affinity (little or no response at ACh concentrations ≤30 μM) that quickly decayed back to the baseline (very fast desensitization) within 1 to 3 s of ACh application were deemed α-BuTX-SCs and used to further assay effects of ethanol.

The α-BuTX-SCs evoked by concentrations of 300 μM ACh (near its EC_{50} for this type of current) showed weak inhibition of the peak amplitude upon coapplication of ethanol at concentrations of 10 to 300 mM. To verify that more prominent effects of ethanol were not missed as a consequence of the very fast onset and decay of the α-BuTX-SCs, triplet comparisons of control (Fig 2A, first set) were compared with that upon coapplication of 100 mM ethanol (Fig. 2A, second set), with that upon pre-exposure to 100 mM ethanol via perfusion through the external bath solution for 3 to 6 min (Fig. 2A, third set), and with that upon washout of the ethanol (Fig. 2A, fourth set). The pre-exposure protocol did result in slightly more inhibition. However, pre-exposure likely sacrifices some specificity in terms of direct action of ethanol on α-BuTX-SC, because ethanol is known to affect many intracellular regulatory systems (Diamond and Gordon, 1997). Thus, the more prolonged presence of ethanol increases the possibility of inclusion of indirect effects of ethanol. Still, the detectable effect of ethanol on α-BuTX-SCs was quite modest. Yet, although only modest, the inhibition observed upon coapplication of ethanol was statistically significant at concentrations of ethanol ≥30 mM (Table 1). However, over the entire 3 to 300 mM ethanol concentration range, a half-maximal inhibition was never attained using the coapplication protocol (Fig. 2B; Table 1). Furthermore, although a small decrease in the I_{max} and a small increase in the ACh EC_{50} upon coapplication of 100 mM ethanol were indicated, as illustrated in Fig. 2C, these changes did not pass tests for statistical significance. No effects on the kinetics of current onset or decay were detectable.

Ethanol on α-BuTX-ICs. The assessment of the effects of ethanol on α-BuTX-ICs separately from α-BuTX-SCs was technically more straightforward. Only those ACh-evoked currents remaining after constant perfusion of 30 to 100 nm α-BuTX exhibiting high affinity (considerable response at ACh concentrations ≤30 μM) that never decayed back to the baseline (slow desensitization) within 1 to 3 s of ACh application were deemed α-BuTX-ICs and used to further assay effects of ethanol.

The α-BuTX-ICs evoked by concentrations of 3 μM ACh (near its EC_{50} for this type of current) exhibited significant amplitude enhancement upon coapplication of ethanol at concentrations of 3 to 300 mM (Table 2). No consistent effects on the kinetics of current decay were found. Shown in Fig. 3A is enhancement by coapplication of 10, 30, and 100 mM ethanol, which are at the low, medium, and high range of physiological relevance. Pre-exposure to ethanol was not necessary for this effect of ethanol on α-BuTX-ICs to be observed, and only coapplication of ethanol with ACh was used to assay this effect further so as to minimize the inclusion of possible indirect effects of ethanol. However, for comparative purposes, a few experiments were conducted whereby ethanol (100 mM) was perfused through the external bath solution for 3 to 6 min (Fig. 3B). Using this pre-exposure protocol, enhancement of the α-BuTX-ICs similar to that upon simultaneous coapplication was observed. Although, after washout of bath-perfused ethanol, the current often did not fully recover, which again may be indicative of additional, less direct actions of ethanol on α-BuTX-insensitive NnAChRs.

The compiled concentration–enhancement relationship (Fig. 3C) indicated that coapplication (no pre-exposure) with 3 μM ACh caused enhancement of α-BuTX-ICs that were statistically significant down to 3 mM ethanol (Table 2). Very small enhancements of α-BuTX-ICs were sometimes observed upon coapplications of 1 mM ethanol, but were not statistically significant. The marked enhancement at 300 mM ethanol showed no apparent trend toward a maximum. This notion was corroborated by even greater enhancement upon coapplication of 1 M ethanol (Fig. 3D). However, note that the coapplication of 1 M ethanol appears to induce changes in the kinetics of current decay, which may suggest additional mechanisms of ethanol action at such high ethanol concentrations.

Ethanol was able to enhance α-BuTX-ICs at both low and high concentrations of ACh (Fig. 4A). The ethanol-induced

enhancements were greater at lower than at higher concentrations of ACh (Fig. 4B, inset), yet coapplication of ethanol still resulted in a somewhat substantial increase in the calculated I_{max} (32 ± 11% increase; $P < .004$) (Fig. 4B). Coapplication of ethanol also caused a small but significant decrease in the calculated ACh EC_{50} (1.3 ± 0.4 μM decrease; $P < .01$) without significantly changing the Hill coefficient (0.02 ± 0.1 decrease; $P < .9$). The higher variance in ethanol enhancement at low concentrations of ethanol (Fig. 4B, inset) likely reflects the variation in cell-to-cell ACh sensitivity noted above.

Specificity of Ethanol Enhancement of α-BuTX-ICs. When applied in the absence of ACh, ethanol at concentrations >300 mM sometimes generated small currents, again perhaps insinuating that ethanol may exert additional mechanisms of action at very high concentrations. However, it generally was found that little or no current could be observed upon applications of ethanol ≤300 mM (Fig. 5A, first record). Such currents, when observed, were very small (<10% of control) and could not explain the large enhancement of α-BuTX-ICs when ethanol was coapplied with ACh. Furthermore, the enhancements of α-BuTX-ICs at ≤300 mM ethanol were specific to the ACh-evoked α-BuTX-ICs as demonstrated by the enhancement of ACh-evoked α-BuTX-ICs induced by 300 mM ethanol being inhibited by coapplication of DHβE (Fig. 5A).

Comparison of Ethanol on α-BuTX-ICs with Those Evoked by NMDA and GABA. As described in the introductory remarks, ethanol can affect many different types of receptor channels. Much importance of ethanol action in the

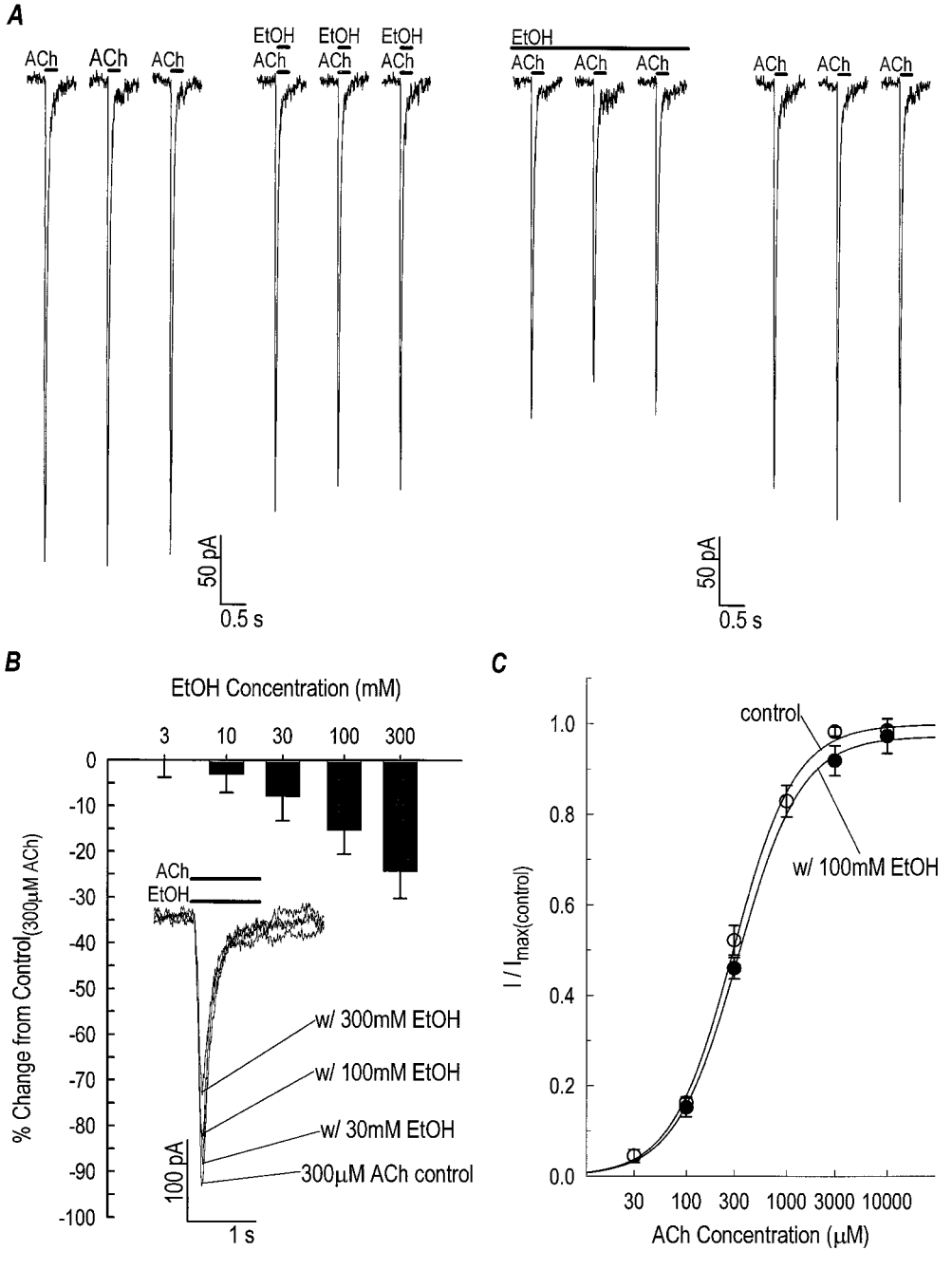

Fig. 2. Apparent inhibition of α-BuTX-SCs by ethanol. A, a sequence of triplet whole-cell α-BuTX-SCs evoked by 1000 μM ACh at 3-min intervals showing (from left to right) control responses (no ethanol), the slight inhibition of response upon coapplication of 100 mM ethanol, the somewhat greater inhibition of the response upon perfusing 100 mM ethanol through the external bath solution in addition to coapplying it, and, finally, the nearly full reversibility of the inhibition upon washing out the ethanol. B, the compiled ethanol concentration–enhancement relationship for α-BuTX-SCs evoked by 300 μM ACh over a 3 to 300 mM ethanol (coapplied) concentration range. The vertical data bars are the mean peak current amplitudes expressed as a percentage over the control current with S.D. Data are from eight neurons. Inset, sequential whole-cell α-BuTX-ICs evoked by 300 μM ACh from the same neuron showing increased inhibition with increasing concentrations of coapplied ethanol superimposed over control. Note that the inhibition never reaches half-maximal even at the extreme upper limit of the physiologically relevant concentration of 300 mM ethanol. C, Response versus ACh concentration relationships for α-BuTX-SCs with no ethanol (○) and when 100 mM ethanol was coapplied (●). The data points are the mean peak current amplitudes with S.E.M. expressed as a fraction of the maximal control current (3000 μM ACh in the absence of ethanol). Data are from at least 5 neurons (different from those used for B). The ACh EC_{50} in controls was 300 ± 34 μM ACh with a Hill coefficient of 1.3. The ACh EC_{50} upon coapplications of 100 mM ethanol was 322 ± 15 μM ACh with a Hill coefficient of 1.4.

brain has been attributed to its effects on NMDARs and $GABA_A$Rs. Therefore, experiments were conducted to compare the effect of ethanol on ACh-, GABA-, and NMDA-evoked currents in the cortical neuron preparation. To avoid cell-to-cell variations and to provide inherent controls for ethanol action on each receptor type, the effects of ethanol were compared between GABA and ACh responses or between NMDA and ACh responses that were evoked from the same neuron. As depicted in Fig. 5B, GABA-evoked currents were not significantly enhanced by coapplication of 100 mM ethanol, but could be modestly enhanced by coapplications of 300 mM ethanol. ACh-evoked α-BuTX-ICs in the same neurons were markedly enhanced by the same concentrations of ethanol. As shown in Fig. 5C, NMDA-evoked currents were inhibited by coapplications of 100 mM ethanol, whereas ACh-evoked α-BuTX-ICs in the same neuron were enhanced by coapplications of 100 mM ethanol. Such actions of ethanol on $GABA_A$R and NMDAR function depicted in these results are not new findings. However, coupling these effects of ethanol with that on NnAChRs at the intraneuron level illustrates that the overall effect ethanol exerts on neuronal activity would be quite dependent on the receptor constituency of each individual neuron.

Discussion

The results in the present study indicated that ethanol, at physiologically relevant concentrations, weakly inhibited ACh-evoked α-BuTX-SCs, whereas it significantly enhanced α-BuTX-ICs. These results exemplify the diverse effects ethanol can have on two types of NnAChRs and involve complexities that are best discussed by considering the effects on each separately.

Ethanol on α-BuTX-SCs. The results in the present study indicated statistically significant inhibition of α-BuTX-SCs upon coapplication of concentrations of ethanol $\geq$30 mM with 300 μM ACh (near its EC_{50}). However, the findings that an actual IC_{50} for ethanol inhibition was never observed, and that changes in the I_{max} and ACh EC_{50} for the response were not statistically significant indicate that the inhibition should not be interpreted without careful consideration of the limitations of the experimental methods used in this assessment. That is, the 10- to 15-ms solution exchange time estimated for the $\cup$-tube application system utilized was longer than the 3- to 4-ms rise time to reach 10 to 90% of the peak α-BuTX-SCs, and was merely about equal to the 18- to 22-ms decay for this current. Thus, accurate resolutions of the peak amplitude and/or any small change in current onset or decay, particularly at higher ACh concentrations, were in all likelihood not achieved. Even if the measured peak current appears consistent in controls, problems with interpreting changes still remain. For example, if ethanol actually enhances the amplitude of α-BuTX-sensitive NnAChR current, but also enhances the rate of current fast decay (often interpreted as acute desensitization), the combined effect causes the peak current to be even shorter lived. This shorter-lived peak, although greater in amplitude than the control, would actually appear to be lesser in amplitude due to it being even more inadequately resolved than the control. Therefore, the inhibition observed in the present can only be characterized as "apparent inhibition" and awaits employment of a faster effector application system that can accurately and consistently resolve the α-BuTX-SC response. Further interpretations of the effects of ethanol on the α-BuTX-SCs would be premature at this time.

If α-BuTX-SCs can be considered as mediated by the α7-subtype NnAChR, and because similar activation (6–7 ms time-to-peak) and desensitization (12–20-ms decay) kinetics for the cloned α7 subtype NnAChRs have been reported in other studies (Puchacz et al., 1994; Gopalakrishnan et al., 1995), inconsistencies in results obtained by different investigators studying this type of receptor might be expected considering the problem of accurately resolving the response.

TABLE 1
Statistics for ethanol-induced inhibition of α-BuTX-sensitive currents

Analysis of variance	% Change over Control (300 μM ACh)				
	3 mM EtOH	10 mM EtOH	30 mM EtOH	100 mM EtOH	300 mM EtOH
Mean	1.0	−3.1	−8.0	−15	−24
Maximum	−6.1	−8.1	−18	−24	−32
Minimum	9.4	2.6	−3	−8.7	−15
S.D.	±4.8	±4.0	±5.3	±5.1	±5.9
S.E.M.	±1.7	±1.4	±1.9	±1.9	±2.1
n	8	8	8	8	8
t tests					
P	<0.6	<0.07	<0.004		

TABLE 2
Statistics for ethanol-induced enhancement of α-BuTX-insensitive currents

Analysis of variance	% Change over Control (3 μM ACh)				
	3 mM EtOH	10 mM EtOH	30 mM EtOH	100 mM EtOH	300 mM EtOH
Mean	+3.9	+14	+27	+56	+141
Maximum	+7.0	+17	+30	+63	+164
Minimum	+2.0	+10	+21	+52	+128
S.D.	±2.1	±2.7	±3.4	±3.6	±12
S.E.M.	±0.7	±1.0	±1.2	±1.3	±4
n	8	8	8	8	8
t tests					
P value	<0.002				

For instance, the lack of obtaining a relevant ethanol IC_{50} for observed inhibition of α-BuTX-SCs is in contrast to the reported 33 mM IC_{50} for ethanol inhibition of cloned α7 NnAChRs expressed in *Xenopus* oocytes (Yu et al., 1996). This could be an indication of substantial differences between recombinant and native NnAChRs; however, it may simply be due to differences in effector application methodology, because the solution exchange time is generally on the order of seconds to even minutes in oocyte experiments, which is even less than the 10- to 15-ms solution exchange time in the present study. This comparison actually may suggest that the observed inhibition of α-BuTX-SCs or α7-mediated currents by ethanol is only apparent inhibition and that the true effects remain to be determined.

Ethanol on α-BuTX-ICs. Over the 3 to 300 mM ethanol concentration range tested, ethanol's consistent effect was only to enhance the amplitude of the α-BuTX-ICs, without significantly affecting the decay kinetics, with the threshold concentration for observing this effect at concentrations of 1 to 3 mM ethanol. This is in contrast to our previous results whereby the more consistent effect of ethanol at low millimolar to high micromolar concentrations was to increase the rate of decay of NnAChR whole-cell currents in PC12 cells while causing varied inhibition and enhancement of the current amplitude (Nagata et al., 1996). Although this contrast certainly deserves further attention, it may yet again exemplify the diversity of ethanol action on different subtypes of NnAChRs, because the primary functional NnAChRs in PC12 cells are most likely α3-containing NnAChRs in combination with β4 and/or probably β2 and α5, whereas the α-BuTX-ICs in cortical neurons of the present study, according to their pharmacological profile, appear most likely to be

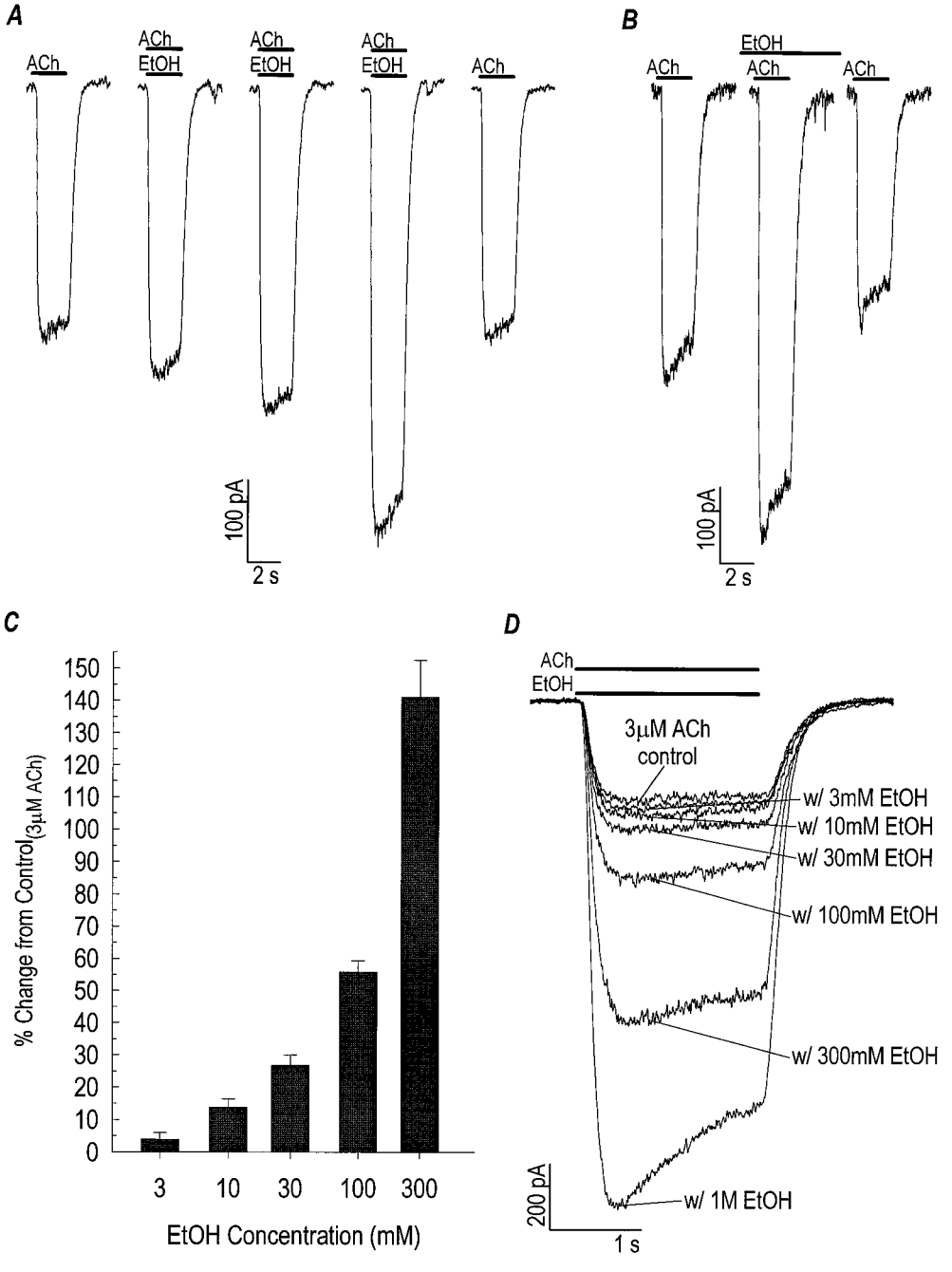

Fig. 3. Ethanol concentration-enhancement relationship for α-BuTX-ICs. A, a sequence of whole-cell α-BuTX-ICs evoked by 3 μM ACh at 3-min intervals from the same neuron showing (from left to right) the control response (no ethanol), the enhancement of the response upon coapplication of 10, 30, and 100 mM ethanol, followed by the full reversibility of the enhancement back to control level (no ethanol). B, a sequence of whole-cell α-BuTX-ICs evoked by 3 μM ACh at 6-min intervals from the same neuron showing (from left to right) the control response (no ethanol), the enhancement of the response upon perfusing 100 mM ethanol through the external bath solution starting right after the control response in addition to coapplying it, and, finally, the reversibility of the enhancement back to a level somewhat less than the initial control upon washing out the ethanol. C, the compiled ethanol concentration–enhancement relationship for α-BuTX-ICs evoked by 3 μM ACh over a 3 to 300 mM ethanol concentration range. The vertical data bars are the mean peak current amplitudes expressed as a percentage over the control current with S.D. Data are from eight neurons. D, Sequential whole-cell α-BuTX-ICs evoked by 3 μM ACh from the same neuron showing increased enhancement with increasing concentrations of coapplied ethanol superimposed over control. Note that the enhancement is not maximal at the extreme upper limit of the physiologically relevant concentration of 300 mM ethanol, as evidenced by the extensive enhancement at a concentration of 1 M ethanol coapplied.

mediated by α4-containing NnAChRs in combination with β2. Perhaps recent studies on known combinations of cloned NnAChRs provide more insight into such a contention. That is, Covernton and Connolly (1997) reported that the current amplitude of cloned $\alpha3\beta4$ NnAChR subtypes expressed in *Xenopus* oocytes was the most sensitive of all of the cloned NnAChR subtypes they tested, being affected by ethanol at concentrations of as low as 1 mM. Also in that study, the effect of ethanol at very low concentrations sometimes was seen as enhancement and at other times was seen as inhibition. No indication of changes in kinetics were mentioned in that study, but the effector application exchange rate in *Xenopus* oocyte experiments generally limits accurate assessment of such kinetic parameters. However, Cardoso et al. (1998) reported the current amplitude of cloned $\alpha3\beta4$ NnAChRs expressed in *Xenopus* oocytes to be enhanced by ethanol, but it was found to be the among the least sensitive of the cloned NnAChRs they tested. Thus, it appears that ethanol action on $\alpha3\beta4$ NnAChRs is in need of more detailed investigation and may be the culprit in terms of the contrast between the present results of ethanol action on NnAChRs in cortical neurons and our previous results on NnAChRs in PC12 cells.

Focusing on the details of the ethanol enhancement of α-BuTX-ICs in cortical neurons, it was found that no apparent plateau for ethanol enhancement of α-BuTX-ICs could be reached at concentrations of ethanol as high as 1000 mM, and α-BuTX-ICs were enhanced even at very high concentrations of ACh, which were maximal in controls. It is also noteworthy to point out that ethanol, at high concentrations,

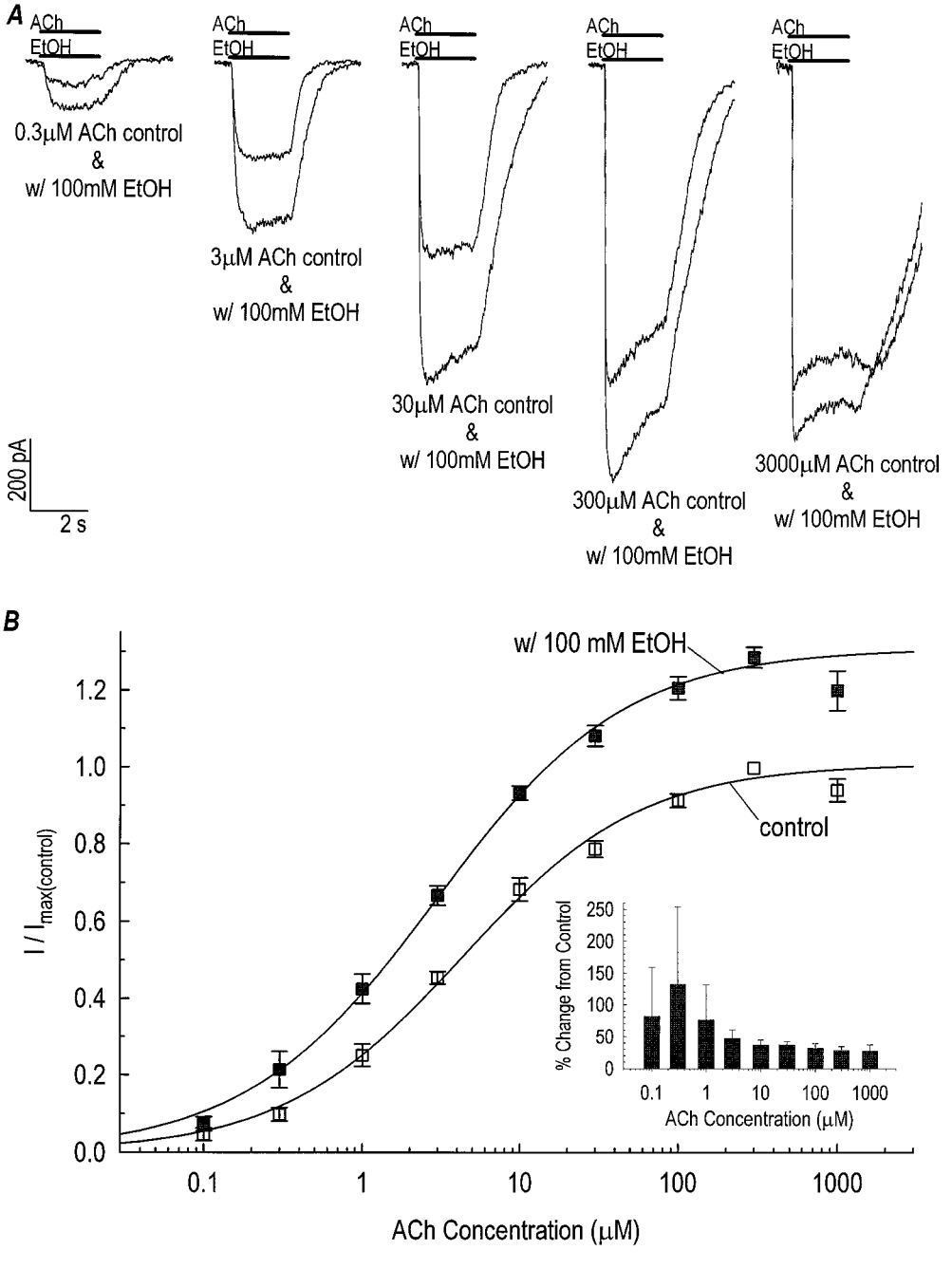

Fig. 4. Ethanol enhances α-BuTX-ICs at all concentrations of ACh. A, a series of superimposed sequential pairs of α-BuTX-ICs evoked by (from left to right) 0.3, 3, 30, 300, and 3000 μM ACh under conditions of control and upon coapplication of 100 mM ethanol. This illustrates that ethanol can induce enhancement of α-BuTX-IC response even at very high concentrations of ACh. B, response versus ACh concentration relationships for α-BuTX-ICs with no ethanol (□) and when 100 mM ethanol was coapplied (■). The data points are the mean peak current amplitudes with S.E.M. expressed as a fraction of the maximal control current (300–1000 μM ACh). Data are from at least five neurons (different from those used for Fig. 3C). The ACh EC_{50} in controls was 3.8 ± 0.4 μM ACh with a Hill coefficient of 0.80 ± 0.05. The ACh EC_{50} upon coapplications of 100 mM ethanol was 2.5 ± 0.3 μM ACh with a Hill coefficient of 0.78 ± 0.05. Inset, transformation of data in main plot to percent enhancement of α-BuTX-ICs by ethanol at varying concentrations of ACh showing that, in general, there is more enhancement by ethanol at lower evoking concentrations of ACh, yet at all ACh concentrations, enhancement is observed. The vertical data bars are the mean peak current amplitudes expressed as a percentage over the control current with S.D.

is known to produce anesthetic action. Yet, at all concentrations of ethanol tested in the present study, only enhancement was observed, which is in contrast to the inhibitory effects of general anesthetics on cloned α-BuTX-insensitive NnAChRs expressed in oocytes reported recently (Flood et al., 1997; Violet et al., 1997). Preliminary results obtained thus far indicate only inhibition of α-BuTX-ICs in cortical neurons by the volatile general anesthetic halothane, as well as the long-chain alcohol, *n*-octanol. Thus, apparently ethanol does not have the same mechanism(s) of action on α-BuTX-ICs as these general anesthetics.

Pertaining to mechanism of action, these aspects of the ethanol enhancement of α-BuTX-ICs could be interpreted in several different ways. One plausible way of interpreting the ethanol-induced enhancement of α-BuTX-insensitive currents is that ethanol increases α-BuTX-insensitive NnAChR channel conductance. This would be consistent with the result that ethanol caused enhancements of the responses evoked by concentrations of ACh that were maximal in controls. However, the enhancements induced by higher concentrations of ethanol are difficult to reconcile by a mechanism involving increases in channel conductance alone. If, for example, 25 pS is taken as a plausible single-channel conductance for α-BuTX-insensitive NnAChRs, and ethanol acts only to increase the open-channel conductance, the 400% enhancement over control induced by 1000 mM ethanol would correspond to a 75 pS increase in single-channel conductance. Such a large drug-induced conductance increase has never been reported, and changes in structure and function of a receptor channel sufficient to cause such an increase in conductance would be difficult to conceive. Nonetheless, such an action by ethanol cannot be completely ruled out at

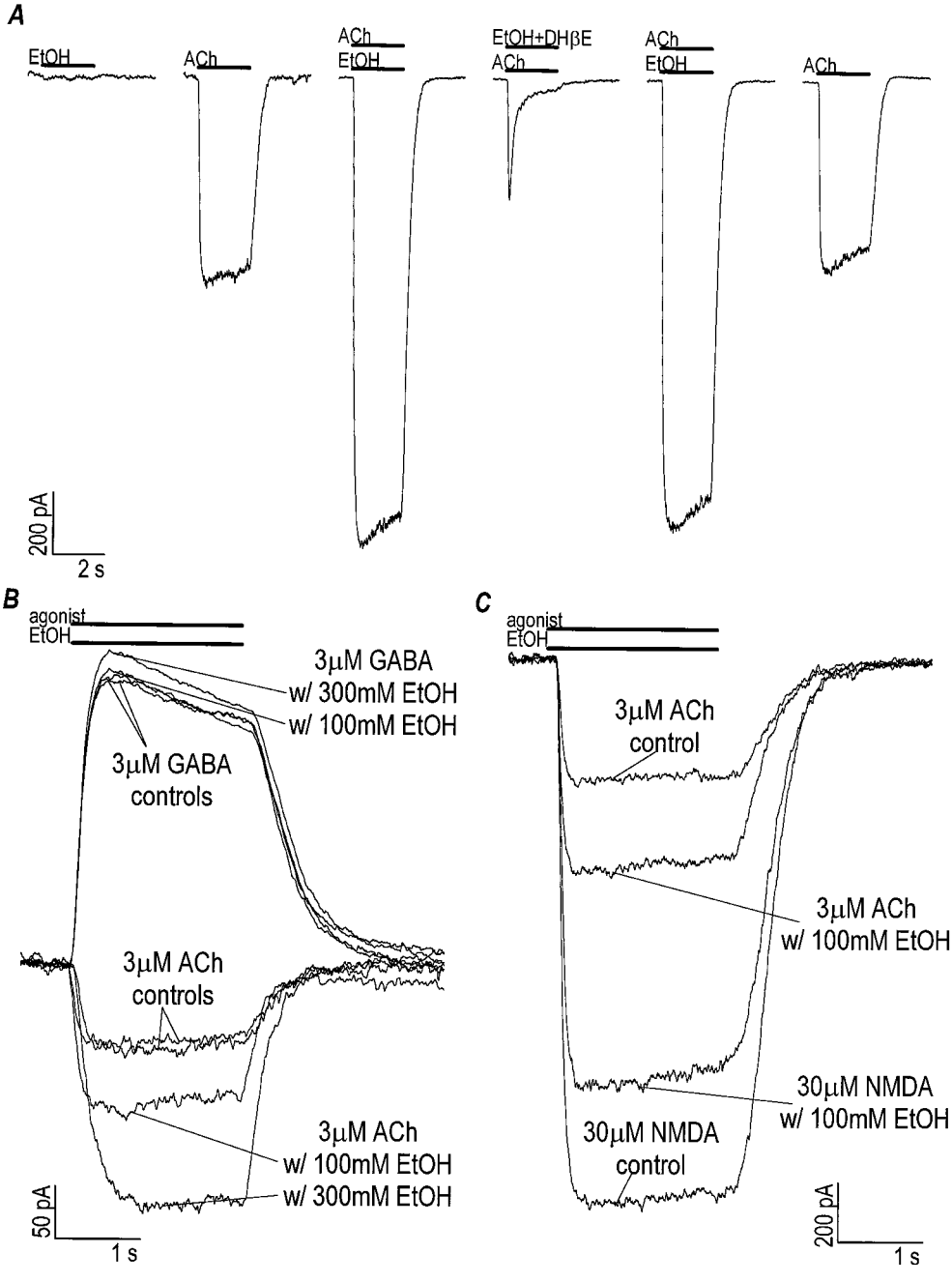

Fig. 5. Specificity of ethanol action on α-BuTX-ICs and intraneuron comparisons with NMDARs and $GABA_A$Rs. A, a whole-cell experiment showing a sequence of effector challenges applied at 3-min intervals from the same neuron showing (from left to right) the lack of response upon application of 300 mM ethanol in the absence of ACh, the control α-BuTX-IC response evoked by 3 μM ACh, the enhancement of the response upon coapplication of 300 mM ethanol, the nearly complete inhibition of the steady-state response upon additionally coapplying 1 μM DHβE with 300 mM ethanol and 3 μM ACh, the full reversibility of the DHβE inhibition back to the level of enhanced response upon coapplication of 300 mM ethanol, and, finally, the full reversibility of the enhancement back to control level. B, superimposed whole-cell α-BuTX-insensitive NnAChR and $GABA_A$R currents evoked from the same neuron with the membrane potential clamped at −25 mV. Under such conditions, GABA-evoked chloride currents (inward Cl^- flux) are outward (positive: up) and ACh-evoked α-BuTX-insensitive sodium/calcium currents are still inward (negative: down). GABA-evoked current is not significantly enhanced upon coapplication of 100 mM ethanol (3 ± 3% enhancement, $n = 8$) and only modestly enhanced upon coapplications of 300 mM ethanol (18 ± 8% enhancement, $n = 4$). ACh-evoked α-BuTX-ICs are substantially enhanced upon coapplications of both 100 mM ethanol (61 ± 9% enhancement, $n = 8$) and 300 mM ethanol (196 ± 63% enhancement, $n = 4$). C, superimposed whole-cell α-BuTX-insensitive NnAChR and NMDAR currents evoked from the same neuron (at –70 mV). As illustrated, whereas the ACh-evoked α-BuTX-IC is enhanced by coapplication of 100 mM ethanol (60 ± 10% enhancement, $n = 7$), the NMDA-evoked current is inhibited upon coapplication of the same concentration of ethanol (35 ± 7% inhibition, $n = 7$).

this time, and single-channel studies will be necessary to resolve this issue.

A more plausible interpretation of the results would be for ethanol to stabilize the open state of α-BuTX-insensitive NnAChRs. This has been suggested as a mechanism for ethanol action on *Torpedo* nAChRs by Wu et al. (1994), who asserted that the major effect of ethanol is to increase the open/closed equilibrium of nAChRs while causing only a minor change in their affinity for agonist. Such a mechanism is consistent with increased enhancement of α-BuTX-insensitive currents upon increasing ethanol concentration and with the small decrease in the EC_{50} for ACh activation exhibited by α-BuTX-insensitive responses in the presence of ethanol. It is also sufficient to explain the ethanol-induced enhancement of α-BuTX-insensitive maximal response if the open probability for NnAChRs is considerably low when using ACh as an agonist. Very low channel open probability (P_{open} = .006–.1) has been reported for α-BuTX-insensitive NnAChRs in chick lateral spiriform nucleus when using ACh as an agonist (Weaver and Chiappinelli, 1996). Thus, the results obtained in the present study in which ethanol enhances both submaximal and maximal α-BuTX-insensitive NnAChR responses while causing only small decreases in the ACh EC_{50} are consistent with a mechanism whereby ethanol increases the open/closed equilibrium of α-BuTX-insensitive NnAChRs mainly by increasing the probability of channel opening.

However, intricacies in the activity of α-BuTX-insensitive NnAChRs, such as the variation in the ACh dose-response evident in the present study, as well as evidence from other studies indicating that NnAChRs can enter multiple, long-lived desensitized states (Lester and Dani, 1994) and/or NnAChRs can exist on the surface of neurons as nonfunctional receptors (Margiotta et al., 1987) unquestionably make interpreting the action of any effector complicated. Therefore, further investigation into such details of NnAChR activity is absolutely necessary to elucidate more fully the actions of ethanol on NnAChRs.

Physiological Relevance. Many studies (Diamond, 1990) have established that in naïve subjects, the evident behavioral changes associated with increases in blood alcohol concentrations are as follows (behavior—associated blood alcohol concentration): altered mood, impaired attention—6 to 20 mM; impaired cognition and coordination, and sedation—20 to 40 mM; intoxication, ataxia—40 to 65 mM; severe stupor, coma, death—65 to 110 mM. In addition, subjects can achieve what is referred to as acute tolerance, whereby they exhibit intoxication at a blood alcohol concentration of 40 mM after 1.5 h of consuming ethanol, yet later appear sober with a 60 mM blood alcohol concentration after 4.5 h of continued consumption of ethanol. Heavy drinkers and alcoholics can achieve what is referred to as chronic tolerance whereby subjects can maintain blood alcohol concentrations of 30 to 120 mM ethanol after consuming ethanol for 6 h and still appear sober. According to such studies, severe alcoholics can attain blood alcohol concentrations as high as 330 mM and still remain conscious and alert.

Considering the above discussion, the results in the present study strongly suggest that enhancement of α-BuTX-insensitive NnAChR function is likely to be an important factor during acute intoxication, because the enhancement of α-BuTX-insensitive NnAChR function over a 10 to 30 mM ethanol concentration range was a significant 10 to 30% at EC_{50} ACh. The present study also revealed that at EC_{50} ACh, over a 100 to 300 mM ethanol concentration range, which may be reached in the brain under conditions of tolerance, 52 to 164% enhancement of α-BuTX-insensitive NnAChRs was evident (although the present study did not focus on whether NnAChRs might become tolerant). Moreover, the significant ethanol enhancement of α-BuTX-insensitive NnAChR function (30% increase in I_{max}) when activated by high concentrations of ACh is of considerable relevance taken in context of the possible ranges of neurotransmitter concentrations at synapses.

The enhancement of α-BuTX-insensitive NnAChR function perhaps becomes even more pertinent when considering the mounting evidence indicating that NnAChRs in the brain may be primarily presynaptic or perisynaptic and can modulate the release of many other neurotransmitters. These include dopamine, norepinephrine, GABA, and glutamate (Role and Berg, 1996; Colquhoun and Patrick, 1997; Lindstrom, 1997; Wonnacott, 1997). Thus, although many reports suggest that ethanol exerts its effects in the brain mainly via direct modulation of $GABA_A$Rs and NMDARs as well as voltage-gated calcium channels, the results obtained in the present study suggest reevaluation of this tenet so as to include central NnAChRs. This becomes especially apparent when considering the results of the present study, which indicate that within a single neuron, multiple ethanol-sensitive receptors can mutually exist, each with extremely different ethanol-induced modulation. Thus, the overall effect(s) of ethanol on such an immense neuronal network as the brain, which is ultimately determined by the complex summation of ethanol action at single neurons, would be extremely diverse. The findings that mecamylamine, a NnAChR antagonist, can counter ethanol-induced dopamine release in the nucleus accumbens (Blomqvist et al., 1997) in addition to countering ethanol-induced depression of cerebellar Purkinje neuron firing (Freund and Palmer, 1997) are recent examples indicating NnAChR involvement in the complexity and diversity of ethanol mediating its action in the brain.

Acknowledgments

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References

Blomqvist O, Ericson M, Engel JA and Soderpal MB (1997) Accumbal dopamine overflow after ethanol: Localization of the antagonizing effect of mecamylamine. *Eur J Pharmacol* **334:**149–156.

Buisson B, Gopalakrishnan M, Arneric SP, Sullivan JP and Bertrand D (1996) Human α4β2 neuronal nicotinic acetylcholine receptor in HEK 293 cells: A patch-clamp study. *J Neurosci* **16:**7880–7891.

Cardoso, RA, Valenzuela, CF, Chavez-Noriega, L, Harpold, M and Harris, RA (1998) Effects of ethanol on the function of neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. *Alcohol Clin Exp Res* **22 (Suppl):**46A.

Chavez-Noriega LE, Crona JH, Washburn MS, Urrutia A, Elliot KJ and Johnson, EC (1997) Pharmacological characterization of recombinant human neuronal nicotinic acetylcholine receptors hα2β2, hα2β4, hα3β2, hα3β4, hα4β2, hα4β4, and hα7 expressed in *Xenopus* oocytes. *J Pharmacol Exp Ther* **280:**346–356.

Collins AC (1996) The Nicotinic Cholinergic Receptor as a Potential Site of Ethanol Action, in *Pharmacological Effects of Ethanol on the Nervous System* (Detrich RA and Erwin VG eds) pp 95–115, CRC Press, Boca Raton, FL.

Colquhoun LM and Patrick JW (1997) Pharmacology of neuronal nicotinic acetylcholine receptor subtypes. *Adv Pharmacol* **39:**191–220.

Cooper ST and Millar NS (1997) Host cell-specific folding and assembly of the neuronal nicotinic acetylcholine receptor α7 subunit. *J Neurochem* **68:**2140–2151.

Covernton PJO and Connolly JG (1997) Differential modulation of rat neuronal nicotinic receptor subtypes by acute application of ethanol. *Br J Pharmacol* **122:** 1661–1668.

Crews FT, Morrow AL, Criswell H and Breese, G (1996) Effects of ethanol on ion channels. *Int Rev Neurobiol* **39:**283–367.

Diamond I (1990) Models for the Investigation of Alcohol Withdrawal Seizures, in *Alcohol and Seizures* (Porter RJ, Mattson RH, Cramer JA and Diamond I eds) pp 25–28, F. A. Davis Company, Philadelphia.

Diamond I and Gordon AS (1997) Cellular and molecular neuroscience of alcoholism. *Physiol Rev* **77:**1–20.

Fenster CP, Rains MF, Noerager B, Quick MW and Lester AJ (1997) Influence of subunit composition on desensitization of neuronal acetylcholine receptors at low concentrations of nicotine. *J Neurosci* **17:**5747–5759.

Flood P, Ramirez-Latorre J and Role L (1997) $\alpha 4\beta 2$ neuronal nicotinic acetylcholine receptors in the central nervous system are inhibited by isoflurane and propofol, but $\alpha 7$-type nicotinic acetylcholine receptors are unaffected. *Anesthesiology* **86:**859–865.

Freund RK and Palmer MR (1997) Ethanol depression of cerebellar Purkinje neuron firing involves nicotinic acetylcholine receptors. *Exp Neurol* **143:**319–322.

Gopalakrishnan M, Buisson B, Touma E, Giordano T, Campbell JE, Hu IC, Donnelly-Roberts D, Arneric SP, Bertrand D and Sullivan, JP (1995) Stable expression and pharmacological properties of the human $\alpha 7$ nicotinic acetylcholine receptor. *Eur J Pharmacol* **290:**237–246.

Lester RAJ and Dani JA (1994) Time dependent changes in central nicotinic acetylcholine channel kinetics in excised patches. *Neuropharmacology* **33:**27–34.

Luetje CW and Patrick J (1991) Both α- and β-subunits contribute to the agonist sensitivity of neuronal nicotinic acetylcholine receptors. *J Neurosci* **11:**837–845.

Lewis TM, Harkness PC, Sivilotti LG, Colquhoun D and Millar NS (1997) The ion channel properties of a rat neuronal nicotinic receptor are dependent on the host cell type. *J Physiol (London)* **505:**299–306.

Lindstrom J (1997) Nicotinic acetylcholine receptors in health and disease. *Mol Neurobiol* **15:**193–222.

Lovinger DM (1997) Alcohols and neurotransmitter gated ion channels: Past, present and future. *Naunyn-Schmiedeberg's Arch Pharmacol* **356:**267–282.

Margiotta JK, Berg DK and Dionne VE (1987) Cyclic AMP regulates the proportion of function acetylcholine receptors on chicken ciliary ganglion neurons. *Proc Natl Acad Sci USA* **84:**8155–8159.

Marszalec W and Narahashi T (1993) Use dependent pentobarbital block of kainate and quisqualate current. *Brain Res* **608:**7–15.

Marzalec W, Aistrup GL and Narahashi T (1998) Ethanol modulation of excitatory and inhibitory synaptic interactions in cultured cortical neurons. *Alcohol Clin Exp Res* **22:**1516–1524.

Miller KW, Wood SC, Forman SA, Bugge B, Hill WA and Abadji V (1991) The nicotinic acetylcholine receptor in its membrane environment. *Ann N Y Acad Sci* **625:**600–615.

Mullikin-Kilpatrick D and Treistman SN (1993) Electrophysiological studies on calcium channels in naïve and ethanol-treated PC12 cells. *Alcohol Alcoholism Suppl* **2:**385–389.

Nagata K, Aistrup GL, Huang CS, Marszalec W, Song J-H, Yeh JZ and Narahashi T (1996) Potent modulation of neuronal nicotinic acetylcholine receptor-channel by ethanol. *Neurosci Lett* **217:**189–193.

Puchacz E, Buisson B, Bertrand D and Lukas RJ (1994) Functional expression of nicotinic acetycholine receptors containing rat $\alpha 7$ subunits in human SH-SY5Y neuroblastoma cells. *FEBS Lett* **354:**155–159.

Rogers SW, Mandelzys A, Deneris ES, Cooper E and Heinemann S (1992) The expression of nicotinic acetylcholine receptors by PC12 cells treated with NGF. *J Neurosci* **12:**4611–4623.

Role LW and Berg DK (1996) Nicotinic receptors in the development and modulation of CNS synapses. *Neuron* **16:**1077–1085.

Sivilotti LG, McNeil DK, Lewis TM, Nassar MA, Schoepfer R and Colquhoun D (1997) Recombinant nicotinic receptors, expressed in *Xenopus* oocytes, do not resemble native rat sympathetic ganglion receptors in single-channel behavior. *J Physiol (London)* **500:**123–138.

Violet JM, Downie DL, Nakisa RC, Lieb WR and Franks NP (1997) Differential sensitivities of mammalian neuronal nicotinic acetylcholine receptors to general anesthetics. *Anesthesiology* **86:**866–874.

Weaver WR and Chiappinelli VA (1996) Single-channel recording in brain slices reveals heterogeneity of nicotinic receptors on individual neurons within the chick lateral spiriform nucleus. *Brain Res* **725:**95–105.

Wonnacott S (1997) Presynaptic nicotinic ACh receptors. *Trends Neurosci* **20:**92–98.

Wu G, Tonner PH and Miller KW (1994) Ethanol stabilizes the open channel state of the *Torpedo* nicotinic acetylcholine receptor. *Mol Pharmacol* **45:**102–108.

Yu D, Zhang L, Eisele J-L, Bertrand D, Changeux J-P and Weight FF (1996) Ethanol inhibition of nicotinic acetylcholine type $\alpha 7$ receptors involves the amino-terminal domain of the receptor. *Mol Pharmacol* **50:**1010–1016.

Zacny JP (1990) Behavioral aspects of alcohol-tobacco interactions. *Recent Dev Alcohol* **8:**205–219.

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