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Novel Modulation of a Nicotinic Receptor Channel Mutant Reveals that the Open State Is Stabilized by Ethanol

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ABSTRACT

Ethanol enhances the gating of a family of related ligand-gated ion channels including nicotinic acetylcholine, serotonin type 3, γ-aminobutyric acid-A, and glycine receptors. This common action may reflect shared molecular and kinetic mechanisms. In all of these receptors, ethanol enhances multichannel currents elicited with low agonist concentrations, but not with high agonist concentrations. A single mutation in the nicotinic receptor β subunit, βT263I, causes ethanol to enhance multichannel currents elicited with both low and high acetylcholine concentrations. Based on the ratios of acetylcholine EC_{50}s in the presence and absence of ethanol, this mutant's sensitivity to enhancement is similar to wild type. Ethanol enhancement of βT263I receptor activation shows no voltage dependence. In the presence of ethanol, the apparent single-channel conductance of the βT263I receptor is reduced and the apparent channel lifetime is lengthened. Both the 28% increase in maximal current and the 2-fold reduction in EC_{50} observed at 300 mM ethanol are quantitatively predicted by simulation of a simple kinetic scheme in which ethanol increases by 4-fold the ratio of microscopic opening rate (β) to closing rate (α) for acetylcholine-bound βT263I receptors. We conclude that ethanol enhancement of βT263I currents reflects stabilization of its open-channel state relative to agonist-bound closed states. Ethanol effects in wild-type receptors can also be explained by this mechanism.

Agonist-induced activation of both peripheral and neuronal nicotinic acetylcholine receptors (nAChRs) is enhanced by ethanol (EtOH). (Gage, 1965; Bradley et al., 1980; Forman et al., 1989; Nagata et al., 1996). Similar EtOH enhancement is observed in related ligand-gated channels such as γ-aminobutyric acid-A, 5-hydroxytryptamine-3, and glycine receptors and it is thought that enhancement of the function of these receptors plays a role in EtOH's behavioral effects (Leidenheimer and Harris, 1992; Aguayo and Pancetti, 1994; Machu and Harris, 1994; Deitrich et al., 1997). The kinetic mechanism underlying activation enhancement by ETOH is uncertain.

In peripheral nAChRs from *Torpedo* electroplaque and muscle, EtOH enhancement of ACh-gated currents is observed with low concentrations of ACh, but currents elicited by saturating ACh concentrations are unaffected by up to 300 mM EtOH (Forman et al., 1989; Wu et al., 1994). Thus, ACh-response relationships are shifted toward lower concentrations, a phenomenon known as leftward (or sinistral) shift.

The leftward shift of ACh responses by EtOH could be achieved by altering several different steps in the gating mechanism of nAChR. The most obvious possibility is that EtOH might enhance the affinity of agonist binding sites. Secondly, EtOH might increase the probability of channel opening (p_{open}) after agonist binding. Increasing p_{open} is predicted to result in leftward shift of ACh responses, but because nAChRs with both agonist sites occupied by ACh are estimated to be open more than 97% of the time, increasing p_{open} will not dramatically increase peak responses at saturating ACh concentrations. Another possible mechanism, reported for benzodiazepine enhancement of γ-aminobutyric acid-A receptors, is that nAChR single-channel conductance could be increased by EtOH, although this mechanism should also increase peak current responses at high ACh (Eghball et al., 1997). Furthermore, in experiments where desensitization or agonist channel block moderates the overall measured response, leftward shifts in agonist responses could occur if EtOH reduces these actions.

Nicotinic receptors formed from wild-type α, γ, and δ subunits and β subunits containing a channel mutation, βT263I, are affected by EtOH in a manner that has not been previously reported. ACh-induced currents from βT263I receptors are enhanced by EtOH at both low and high ACh concentrations. A detailed examination of EtOH effects on βT263I mutant receptors supports a model where enhanced activa-

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ABBREVIATIONS: ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; EtOH, ethanol.

tion in the presence of EtOH is due to an increased opening probability of ACh-bound nAChRs.

Materials and Methods

Materials. cDNAs encoding wild-type α, β, γ, and δ subunits and the αY198F mutant in pSP64T vectors were provided by Dr. James McLaughlin (Tufts Medical School, Boston, MA) and βT263I mutant cDNA in pGEM2-SP6 was provided by Dr. Cesar Labarca (California Institute of Technology, Pasadena, CA). Acetylcholine chloride (ACh), EtOH, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

***Xenopus* Oocyte Expression.** Wild-type and mutant nAChRs were expressed on the surface of *Xenopus* oocytes after injection of oocytes with messenger RNA mixtures encoding the four nAChR subunits. Detailed methods were previously described (Forman et al., 1995). All procedures with frogs were approved by the Massachusetts General Hospital Animal Care Committee. After microinjection, oocytes were incubated for 48 to 96 h, then manually stripped of their vitelline membranes and used for electrophysiology.

Rapid Perfusion Patch-Clamp Electrophysiology. Electrophysiology recordings were made at room temperature (20–22°C). Borosilicate patch pipettes were polished to give open tip resistance of 2–5 MΩ. For rapid perfusion studies, oocyte membrane patches were pulled in the outside-out configuration and held at −50 mV. Inside and outside buffers were symmetrical K-100 (97 KCl mM, 1 $MgCl_2$ mM, 0.2 EGTA mM, and 5 K-HEPES mM, pH 7.5). Currents through the patch-clamp amplifier (Axopatch-200A; Axon Instruments, Foster City, CA) were filtered (8-pole bessel, 2 kHz) and digitized at 5 to 10 kHz using a 586-class PC, a 12-bit A/D converter (National Instruments, Austin, TX), and custom software.

Submillisecond ACh jumps at the experimental patch surface were achieved using a computer-activated, piezo-driven theta tube. Patches were continuously superfused with control solution (K-100 with or without EtOH) through one lumen of the theta tube. A computer signal actuated the piezo to rapidly position the other theta tube lumen (ACh in K-100 with or without EtOH) before the patch. Superfusate exchange times (open pipette junction current method) were 0.2 to 0.5 ms. The ACh exposure period was usually 300 ms and patches were "recovered" in control solution for 5 to 15 s between ACh exposures. EtOH effects were assessed with drug present both in control and ACh superfusates.

Single-Channel Studies. Recordings were made at room temperature using excised inside-out patches held at 150 mV. Pipettes were 2 to 5 MΩ resistance and uncoated. Pipette and bath solutions were symmetrical K-100 and ACh in the pipette was 0.2 to 1.0 μM. EtOH, when present, was added to the pipette solution only. Continuous recordings were acquired at 10 kHz digitization with 5 kHz filtering using the FETCHEX program in pClamp6.0 (Axon Instruments).

Data Analysis. In rapid concentration-jump studies, each patch studied under a given set of ACh/EtOH conditions was exposed to these drugs sequentially 8 to 16 times with a recovery period in between each exposure. The ensemble of current traces were averaged. Control ensemble average currents (saturating ACh without EtOH) were assessed before and after experiments where patches were exposed to EtOH. Data was not analyzed if the two control peak currents differed by more than 10%. Experimental peak currents were normalized to the average peak from the two bracketing control measurements in the same patch. For concentration-response studies, normalized data from at least three patches from different oocytes were pooled and averaged for each EtOH concentration studied.

Exponential functions (eq. 1) were fitted to the decay portion of current data.

$$I = (I_{peak} - I_{\infty})e^{-t/\tau_{des}} + I_{\infty} \quad (1)$$

Agonist (ACh) concentration-response data were analyzed by fitting logistic functions (eq. 2) to control-normalized data using Origin (Microcal Inc., Northampton, MA) software on a 586-class PC. All results, except where noted, are reported as mean ± S.D.

$$\frac{I_{peak}}{I_{peak}^{Max}} = \frac{[ACh]^{nH}}{[ACh]^{nH} + EC_{50}^{nH}} \quad (2)$$

Amplitude and open-time histograms from single-channel recordings were constructed, then fitted with gaussian and exponential functions by the Levenberg-Marquardt least-squares minimization method, using pClamp6.0 software.

Kinetic Simulations. MATLAB software (The Mathworks, Natick, MA) was used to both generate simulated nAChR currents by the Q-matrix method and to identify peak current amplitudes in the simulations.

Results

EtOH enhances βT263I currents at high ACh. Oocyte membrane patches expressing nicotinic receptors containing the βT263I mutation produced ACh-induced currents very similar to those of wild-type receptors. Upon exposure to submillisecond ACh concentration jumps, multichannel inward currents rapidly peaked and then desensitized monoexponentially (Fig. 1A, 1 mM ACh trace). As previously reported, βT263I mutant nAChRs are characterized by an ACh EC_{50} that is about 3-fold higher than that for wild type (56 ± 2 μM; Fig. 2), and maximal ACh-induced desensitization proceeds at a rate similar to that for wild-type receptors (Forman, 1997).

When multichannel βT263I receptor currents were elicited

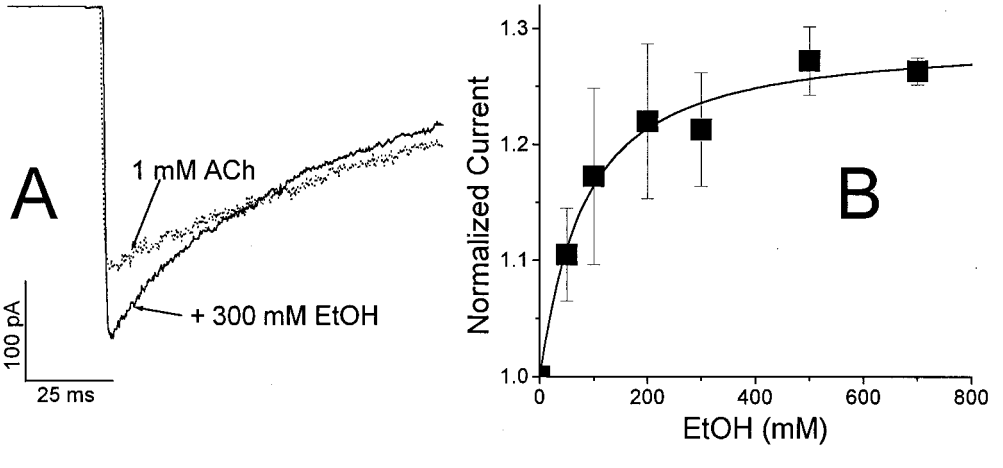

Fig. 1. EtOH enhances maximal currents in βT263I mutant nAChRs. A, multichannel currents from an outside-out oocyte patch expressing βT263I nAChRs were stimulated with 1 mM ACh (18 × EC_{50}). Control currents rise rapidly to a peak and then desensitize monoexponentially with a time constant of 140 ± 13 ms. In the presence of 300 mM EtOH, peak current is enhanced by 29% and the desensitization time constant is reduced to 90 ± 8 ms. B, EtOH-dependent enhancement of βT263I currents elicited with 1 mM ACh are shown. Each point represents the average ± S.D. of at least three measurements. The line through the data represents a logistic function: maximal enhancement = 29 ± 3.5%, half-effect EtOH concentration = 80 ± 21 mM, n_H = 1.1 ± 0.37.

with high ACh concentrations in the presence of EtOH, we unexpectedly observed currents that were higher than those elicited by ACh alone (Fig. 1A). In a series of five patches, currents elicited with 1 mM ACh were enhanced up to 29% in the presence of high EtOH concentrations (Fig. 1B). We also observed significant EtOH-dependent enhancement of currents at low EtOH concentrations associated with inebriation. At 50 mM EtOH, ACh-induced currents were enhanced 11 ± 3%. A logistic fit to the EtOH-dependent enhancement data gave half-maximal enhancement at 80 ± 21 mM. EtOH also increased the apparent ACh-induced desensitization rate by nearly 50% (Fig. 1A).

EtOH Shifts βT263I ACh Response Leftward. The enhancing action of EtOH was quantified by determining the extent of EtOH-induced leftward shift in agonist response curves. In wild-type *Torpedo* and mouse nAChRs, 300 mM EtOH causes the ACh EC_{50} to decrease about 2-fold (Forman et al., 1989; Zhou and Forman, submitted for publication.). We therefore measured ACh concentration responses in patches expressing βT263I nAChRs both in the absence and presence of 300 mM EtOH.

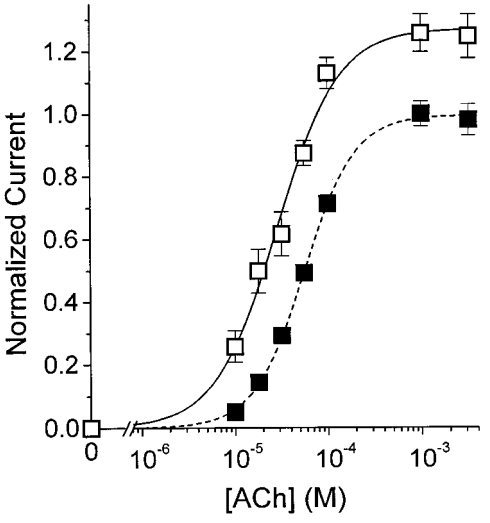

Fig. 2. EtOH effect on ACh concentration-response relationship for βT263I nAChRs. Currents were elicited from outside-out oocyte patches expressing βT263I nAChRs. Data points represent average (±S.D.) of at least three peak currents measured from separate patches and normalized to the peak current measured in the same patch at 1 mM ACh in the absence of EtOH. Lines represent logistic equations fitted to data by nonlinear least squares. No EtOH (■): Max = 1.0 ± 0.01; EC_{50} = 56 ± 1.1 μM; n_H = 1.6 ± 0.05. 300 mM EtOH (○): Max = 1.27 ± 0.037; EC_{50} = 29 ± 2.5 μM; n_H = 1.3 ± 0.15.

In the presence of 300 mM EtOH, ACh-activated multichannel current responses were shifted leftward and demonstrated increased maximal response (at ACh ≥1 mM). The fitted EC_{50} for ACh in the presence of 300 mM EtOH was 29 ± 3.1 μM, approximately half of its value in the absence of EtOH. Thus, the magnitude of EtOH-induced leftward shift in βT263I nAChR responses is the same as that observed in both wild-type mouse and *Torpedo* nAChRs.

EtOH Enhancement Shows No Voltage Dependence. ACh can act as a voltage-sensitive channel blocker (self-inhibition) as well as an agonist. In wild-type nAChRs, channel block is observed at ACh concentrations above 1 mM and at membrane potentials below −50 mV (Sine and Steinbach, 1984). If ACh is a potent blocker of βT263I nAChRs, a possible mechanism to explain EtOH enhancement at high ACh is that EtOH weakens channel block by ACh (or other ions). We tested this hypothesis by determining whether EtOH enhancement of both wild-type and βT263I mutant channels was dependent on the membrane holding potential.

In wild-type nAChRs, a small degree of inward rectification of macroscopic currents elicited with 0.5 mM ACh was observed, with a null potential near 0 mV in symmetrical solutions (Fig. 3A). The linearity of the current-voltage (I-V) relationship at negative membrane potentials demonstrates that very little self-inhibition occurs in wild-type receptors at this ACh concentration. EtOH (300 mM) caused only a small change in the wild-type nAChR I-V relationship, enhancing currents at positive holding potentials by less than 10%.

In βT263I mutant nAChRs, inward rectification of macroscopic currents was much stronger than that seen in wild type, and again the null potential was near 0 mV (Fig. 3B). I-V relationships were linear at negative voltages, demonstrating that ACh block of βT263I nAChRs remains weak in the presence of the pore mutation. EtOH enhancement of βT263I multichannel currents was of equal magnitude at both negative and positive holding potentials. In the data shown in Fig. 3B, 300 mM EtOH enhanced inward multichannel currents by 42% and outward currents by 40%.

EtOH Reduces Single-Channel Conductance of βT263I nAChRs. We examined whether enhancement of βT263I multichannel currents was reflected in single-channel conductances by measuring single-channel conductances

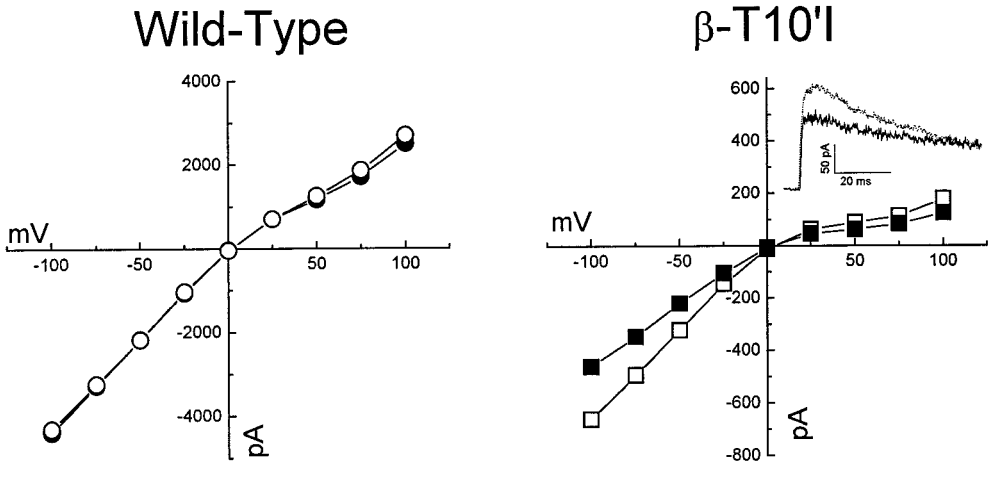

Fig. 3. The voltage dependence of multichannel nAChR currents in the presence and absence of EtOH. A, multichannel currents elicited with 500 μM ACh (●) or 500 μM ACh + 300 mM EtOH (○) from a single oocyte patch expressing wild-type nAChRs. Some inward rectification is apparent in both data sets. Currents are enhanced 8% by EtOH at +100 mV. B, Multichannel currents elicited with 500 μM ACh (■) or 500 μM ACh + 300 mM EtOH (□) from a single oocyte patch expressing βT263I nAChRs. Pronounced inward rectification is apparent in both data sets. Enhancement at negative holding voltages averages 42 ± 1.5% (n = 4 ± S.E.) and at positive holding voltages averages 40 ± 2.2% (n = 4 ± S.E.). Inset, currents recorded at +100 mV demonstrate 43% enhancement by 300 mM EtOH (solid line).

in excised inside-out patches using a low concentration of ACh (0.5 μM) in the pipette.

Single βT263I-channel openings were very brief and appeared to be of varying magnitude (Fig. 4, top left). Amplitude histograms from βT263I currents showed a baseline peak and a single opening peak with an average conductance (±S.E.M., $n = 4$) of 47 ± 1.0 pS (Fig. 4, top middle). In the presence of EtOH, single-channel openings appeared to have longer durations and were of more consistent amplitude (Fig. 4, bottom left). EtOH decreased the apparent single-channel conductance of βT263I nAChRs by 19 ± 1.0% (±S.E.M., $n = 4$) to 38 ± 1.6 pS (Fig. 4, bottom middle).

EtOH Increases Apparent Single-Channel Lifetime of βT263I nAChRs. In recordings where over 95% of opening events were single openings, open-time duration histograms revealed two distinct βT263I channel open lifetimes. Most openings had fitted mean lifetimes below 0.2 ms. (Fig. 4, top right, see legend for details). In the presence of 400 mM EtOH, both short and long opening times increased and there was a shift in the distribution toward more long openings (Fig. 4, bottom right). These EtOH effects on channel lifetimes were consistently observed in a total of eight patches.

Discussion

The major finding of this study is that EtOH enhances macroscopic currents from βT263I mutant nAChRs at both low and high ACh concentrations. This result is in contrast to EtOH actions on wild-type nAChRs, where current enhancement is seen with low ACh, but only weak inhibition is apparent with high ACh concentrations. We investigated the βT263I mutant's interactions with EtOH in detail to determine why EtOH affects it differently from wild-type nAChRs. Our analysis demonstrates that EtOH enhancement of nAChR function is due to an increase in the opening probability of agonist-bound receptors.

A number of different EtOH-associated changes in the nAChR gating mechanism could lead to enhancement of multichannel currents under different agonist conditions. A reaction scheme that incorporates the steps leading to channel opening, blocking, and desensitization is shown in Fig. 5. For simplicity, the two ACh binding sites are shown with equal microscopic affinities, because the arguments that follow would be equally valid for a model with distinct binding affinities. In wild-type mouse muscle nAChRs, microscopic rates for ACh binding to agonist sites (k_{on}) are diffusion limited, near 10^8 M^{-1} s^{-1}, whereas the rate for dissociation of ACh (k_{off}) is estimated to be >6000 s^{-1} (Lingle et al., 1992; Zhang et al., 1995). After binding of two ligands, opening (β) occurs at rates up to 60,000 s^{-1} (Zhang et al., 1995; Maconochie and Steinbach, 1998) and closing (α) at about 100 to 300 s^{-1} (Dilger et al., 1991; Zhang et al., 1995). At 21°C and −50 mV, the ACh open-channel blocking rate is about 5×10^6 M^{-1} s^{-1} and the unblocking rate is about 3000 s^{-1} (Sine and Steinbach, 1984). Agonist binding and channel opening and blocking are all very fast compared with desensitization ($k_d \approx 10$ s^{-1}), so that peak current after submillisecond concentration jumps should reflect only these steps. At saturating (but not blocking) ACh concentrations, all receptors rapidly enter the A_2R state and the resulting maximal macroscopic current will be the unit current, i, times the number of channels times the open probability of doubly ACh-bound receptors $p_{open} = \beta/(\alpha+\beta)$. Channel block by EtOH will also affect maximal currents by reducing the effective channel conductance.

Enhancement of multichannel nAChR currents could be associated with changes in agonist binding, single-channel opening probability, channel conductance, channel block, or desensitization kinetics. Our data directly rule out some of these possibilities. The use of rapid concentration jumps at excised membrane patches enables direct observation of enhancement in multichannel peak currents, which is clearly independent of desensitization (Fig. 1). With longer agonist applications, we can directly observe EtOH effects on the desensitization rate, k_d. Consistent with previous observations in studies of *Torpedo* nAChR (Forman et al., 1989; Wu and Miller, 1994), we find that EtOH accelerates nAChR

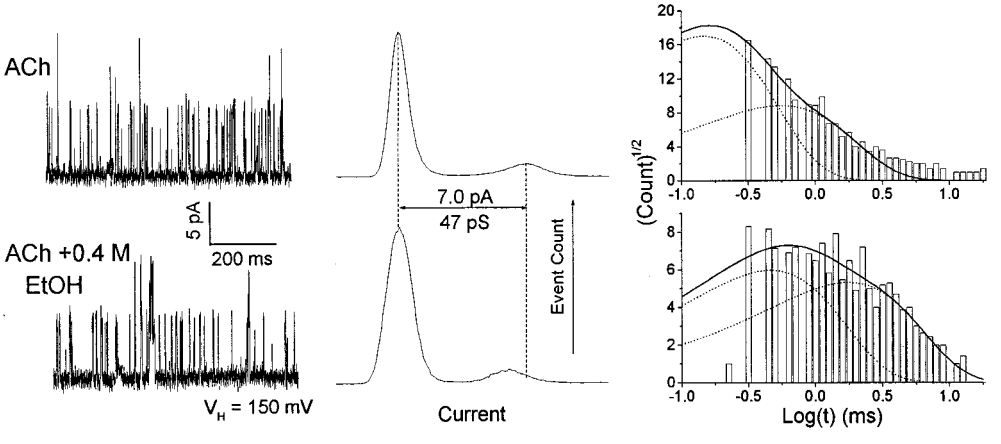

Fig. 4. EtOH reduces the single-channel conductance and increases mean open time of βT263I nAChRs. Top panels show current recordings with ACh alone, whereas the lower panels show data recorded in the presence of ACh and 400 mM EtOH in the recording pipette. Left, current recordings from two separate excised inside-out patches with 500 nM ACh (top) or 500 nM ACh plus 400 mM EtOH (bottom). The lower opening frequency in the bottom panel is due to a smaller number of channels. Middle, all-points amplitude histograms of the current traces shown in the left panels. With ACh alone, the histogram peaks are separated by 7.0 pA. Combined data from four patches gave an average (±S.E.) conductance of 47 ± 1.0 pS. In the presence of 400 mM EtOH, the histogram peaks are separated by 5.9 pA (average = 39 ± 1.6 pS, $n = 4$). Right, open time histograms derived from traces shown in the left panels (10 s total data each). Double exponential fits are overlaid on the histogram data (solid lines). For ACh alone, the data were fit with time constants of 0.15 ± 0.096 ms (79% of openings) and 0.6 ± 0.12 ms (21% of openings). For ACh plus 400 mM EtOH, data were fit with time constants of 0.5 ± 0.34 ms (56% of openings) and 1.8 ± 0.33 ms (44% of openings).

desensitization in the presence of both low and high ACh concentrations. In slower current assays, this effect should reduce, not enhance, measured peak currents.

We also show that voltage-dependent agonist blockade of nAChR channels is negligible under the experimental conditions used for this study. I-V relationships for both wild-type and mutant nAChRs show inward rectification due to voltage-dependent channel closing rate (Auerbach et al., 1996), but enhancement of βT263I currents by EtOH is equal at both positive and negative holding potentials (Fig. 3). In addition, our single-channel studies demonstrate that EtOH does not enhance single-channel nAChR conductance (Fig. 4), ruling this out as a possible mechanism.

The EtOH-induced leftward shift in agonist concentration responses could be caused by either enhanced agonist binding or by enhanced p_{open}. Because the p_{open} of ACh-bound wild-type nAChR is near 1.0, either of these actions would lead to an unchanged maximal current at saturating ACh. Partial agonists such as suberyldicholine, decamethonium, or nicotine bind to the ACh agonist sites on nAChR, but the probability of channel opening when these sites are occupied is low. EtOH and other short-chain alcohols enhance nAChR function at both low and high partial agonist occupancy (Wu and Miller, 1994; Tonner et al., 1992; Liu et al., 1994). These observations suggest that EtOH affects the probability of channel opening after ligands bind, but do not rule out a mechanism where EtOH affects affinity for the agonist site.

A critical problem with the partial agonists is that they are all potent channel blockers, and the overall maximal current observed with these agonists is a function of both channel opening and blockade. Enhancing ligand binding affinity at the agonist sites without affecting p_{open} or agonist channel blocking affinity of these compounds would increase maximal current (Tonner et al., 1992; Liu et al., 1994). In the absence of agonist channel block, as established for our results using ACh as the agonist, simply enhancing ligand binding affinity would result in a leftward shift in the concentration-response curve without changing maximal current. Because EtOH does not enhance single-channel conductance and the number of receptors in an excised patch is unlikely to change (especially on the rapid time scale of our agonist concentration jumps), the only mechanism that can account for the EtOH-induced increase in maximal βT263I multichannel currents is an increase in single-channel p_{open}.

To test whether we could quantitatively account for both the increased maximal current and the decreased EC_{50} observed in the presence of 300 mM EtOH, we simulated βT263I nAChR currents based on a modified scheme (Fig. 5) without agonist block (Fig. 6). Kinetic parameters for the simulation were those given above for wild-type receptors, except that we set the channel closing rate, $\alpha = 8000\ s^{-1}$ based on our open lifetime estimates (Fig. 4). We varied β and found that at $\beta = 20{,}000\ s^{-1}$ ($\beta/\alpha = 2.5$; Fig. 6 △), that the EC_{50} of the simulated concentration-response data was 53 μM, close to the actual value for βT263I receptors (Fig. 2). The maximal p_{open} in this simulation was 0.71. To simulate the effect of EtOH, we increased the β/α ratio by either increasing β or decreasing α (with similar results). At $\beta/\alpha = 10$ (Fig. 6 ▽), the EC_{50} of simulated data dropped to 25 μM and the maximal p_{open} increased to 0.91. Thus, a 4-fold increase in β/α caused a 2-fold decrease in EC_{50} and a 28% increase in maximal p_{open}. The remarkable correlation between the simulated data and our measurements in the absence and presence of EtOH is demonstrated in Fig. 6 (right panel), where simulated concentration-response curves are plotted with renormalized electrophysiologic data from Fig. 2.

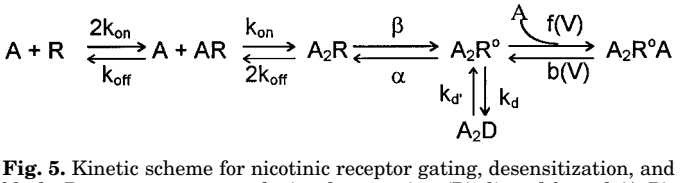

Fig. 5. Kinetic scheme for nicotinic receptor gating, desensitization, and block. Receptor states are depicted as resting (R), ligand bound (A_2R), open (A_2R^o), and desensitized (A_2D). Bimolecular ACh (A) binding rates and unimolecular rate constants for other state transitions are given in the text.

Our results confirm prior studies suggesting that EtOH acts on ligand-gated ion channels by stabilizing the open-channel state relative to the closed agonist-bound state (Aracava et al., 1991; Bradley et al., 1994; Wu and Miller, 1994; Zhou and Lovinger, 1998). Furthermore, our simulation of nAChR kinetics suggests that 300 mM EtOH increases β/α by about 4-fold. Indeed, the linear log-log relationship between predicted EC_{50} and β/α seen in Fig. 6 (left panel) has a slope near −0.5, indicating that EC_{50} depends on $(\beta/\alpha)^{-1/2}$. This result is also predicted by an approximate numeric solution for EC_{50} based on Fig. 5 (see Appendix).

A direct implication of our observation that EtOH increases maximal currents in βT263I receptors is that the microscopic p_{open} of ACh-bound βT263I receptors must be significantly less than 1.0. The simulation shown in Fig. 6 suggests that p_{open} is near 0.7, but we can also estimate a value directly from our measurements. About 28% enhancement of maximal multichannel currents (at ACh ≥1 mM) in patches expressing βT263I receptors was observed at the highest EtOH concentrations we studied. Thus, assuming p_{open} for ACh-bound βT263I receptors in the presence of 300 to 700 mM EtOH is near 1.0, p_{open} in the absence of EtOH can be no more than 0.78 (1/1.28). Furthermore, the enhancing actions of EtOH overcome a modest EtOH-dependent reduction in βT263I single-channel conductance (Fig. 4). If we correct for the 19% inhibition of single-channel conductance at 400 mM EtOH, p_{open} in the absence of EtOH is estimated to be at most 0.63 (0.81/1.28).

In effect, ACh is a partial agonist at βT263I nAChRs, and our single-channel kinetic data suggest that the βT263I mutation destabilizes the open-channel state relative to that of the wild-type receptor. Single-channel βT263I currents recorded at low ACh show kinetic behavior consistent with this low opening probability estimate. Channel lifetimes for βT263I receptors are at least 20-fold shorter than wild-type channels, demonstrating that the closing rate of βT263I receptors is much higher than that of wild-type nAChRs. Although our concentration jumps are not fast enough to directly estimate opening rates, currents from rapidly perfused patches expressing βT263I nAChRs rise in under 1 ms (10–90% rise times are 0.5 ms in Fig. 1A), demonstrating that ACh binding and channel-opening rates are not dramatically slower than those in wild-type nAChRs. Of note, a homologous α subunit mutation, αS252I, does not confer a βT263I phenotype to nAChRs. Both the ACh EC_{50} and average channel lifetime for αS252I nAChRs are near those of wild type,

and EtOH does not increase maximal currents in patches expressing αS252I receptors, indicating that p_{open} is near 1.0 (Forman, 1997; Zhou and Forman, submitted for publication).

Finally, at least part of EtOH's effect on p_{open} is due to a decrease in channel closing rate (α), because apparent channel lifetimes were significantly longer in the presence of EtOH. This conclusion agrees with previous reports of single-channel kinetic analysis of EtOH effects in wild-type nAChRs (Aracava et al., 1991; Bradley et al., 1994).

We can closely simulate EtOH's effects on βT263I receptors by increasing β/α in as shown in Fig. 6, and this mechanism can also account for EtOH effects in wild-type nAChRs. Let us assume that the effects of EtOH on the βT263I activation mechanism are the same as those in wild-type receptors but adjust our model to incorporate the higher baseline p_{open} characterizing these channels. This situation is approximately represented by the simulated results in Fig. 6 (left panel) at a β/α ratio of 40 (■, derived from a simulation with $\beta = 20{,}000\ s^{-1}$ and $\alpha = 500\ s^{-1}$), giving $p_{open} = \beta/(\alpha+\beta) = 0.976$ and a fitted $EC_{50} = 12\ \mu M$. Assuming 300 mM EtOH causes a 4-fold increase in β/α to 160 (◇ and ◆, derived from simulation with $\beta = 20{,}000\ s^{-1}$ and $\alpha = 125\ s^{-1}$), Fig. 6 predicts that wild-type EC_{50} will drop about 2-fold to 6.4 μM while p_{open} rises to 0.994. Again, the model closely approximates experimental observations (Forman et al., 1989; Wu et al., 1994). To generalize, our model predicts that EtOH will have an equivalent effect on the EC_{50} derived from macroscopic current in these receptors, but EtOH's effect on currents at maximal agonist occupancy will depend on the p_{open} for the specific agonist/receptor pair as well as the degree of EtOH channel inhibition.

We confirmed this generalization in another mutant nAChR with a low p_{open}, αY198F. This mutation is in the agonist binding domain of nAChR (Tomaselli et al., 1991) and, like βT263I, is characterized by a low p_{open} and high ACh EC_{50}. Indeed, in the presence of 300 mM EtOH, ACh concentration responses from patches expressing αY198F are shifted leftward (2-fold reduction in EC_{50}) and maximum currents are enhanced by about 35% (data not shown). As seen with both wild-type and βT263I nAChRs, αY198F single-channel conductance is also inhibited by 19 ± 2.7% in the presence of 400 mM EtOH.

We conclude that the effects of EtOH on the gating kinetics of βT263I and αY198F receptors are the same as those in wild-type receptors. EtOH shifts ACh-response curves leftward by the same degree (about 2-fold at 300 mM EtOH) in wild-type and mutant nAChRs. The 2-fold leftward shift can be quantitatively accounted for by a 4-fold increase in β/α, but our data do not rule out a small additional direct EtOH enhancement of agonist binding. EtOH's differential effects on mutant and wild-type receptor currents at saturating ACh concentrations are explained by the different microscopic opening probabilities in the absence of EtOH. The increased p_{open} is associated with slowed channel closing rates, indicating a stabilized open state, and others have suggested that EtOH may also increase opening rates (Bradley et al., 1994). High-resolution single-channel burst analysis may define the relative contributions of opening and closing rate changes to EtOH's enhancing action.

Appendix: Relationship between EC_{50} and β/α ratio in Fig. 5: A Steady-State Solution

Because neither agonist block nor desensitization limit peak current in our measurements, peak current can be approximated using a steady-state assumption. Figure 5, modified to remove both agonist block and desenstization,

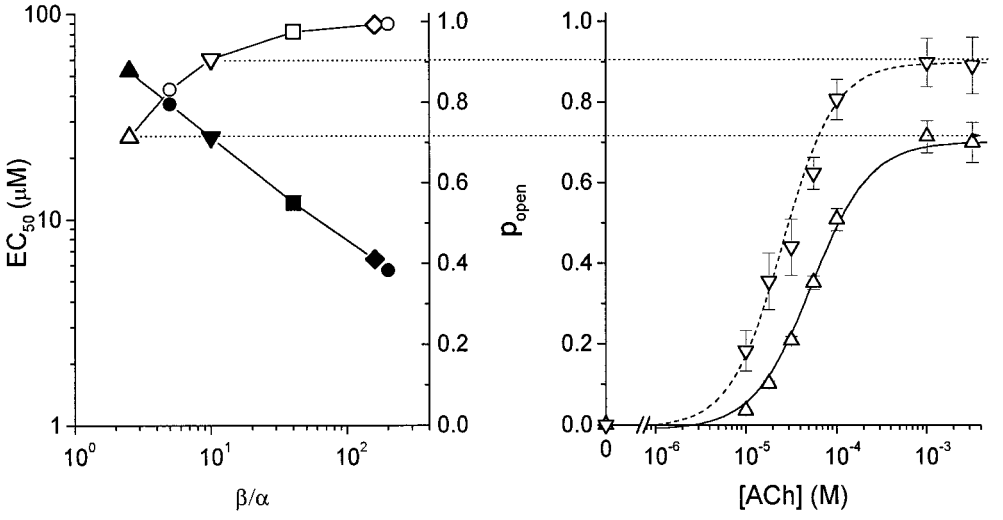

Fig. 6. Simulation of nAChR currents shows that increasing β/α ratio reproduces EtOH actions. Left, the kinetic parameters shown in Fig. 5 α and β were varied in Fig. 6 (modified to remove agonist block), which was used to generate simulated nAChR currents over a range of ACh concentrations. Maximal p_{open} values (open symbols) were calculated as $\beta/(\beta+\alpha)$ and EC_{50}s (filled symbols) were derived from logistic fits (eq. 2) to the simulated peak current versus [ACh] relationship at each β/α ratio. The curves drawn in the right panel were used to generate the parameters plotted for $\beta/\alpha = 2.5$ (△) and $\beta/\alpha = 10$ (▽). Parameters from simulations of wild-type receptor kinetics (see text) are represented by squares ($\beta/\alpha = 40$ at 0 EtOH) and diamonds ($\beta/\alpha = 160$ at 300 mM EtOH). Right, peak currents from two simulations are drawn as concentration-response curves. The solid line represents a simulation of βT263I receptor kinetics and was generated using $k_{on} = 10^8\ M^{-1}\ s^{-1}$, $k_{off} = 6000\ s^{-1}$, $\beta = 20{,}000\ s^{-1}$, $\alpha = 8000\ s^{-1}$, $k_d = 10\ s^{-1}$, and $k_{d'} = 1\ s^{-1}$, and was fitted with $EC_{50} = 53\ \mu M$, $p_{open}(max) = 0.71$, and $n_H = 1.4$. The dashed line used the same parameters, except $\alpha = 2000\ s^{-1}$ and was fit with $EC_{50} = 25\ \mu M$, $p_{open}(max) = 0.91$, and $n_H = 1.5$. Overlaid on the simulated curves are electrophysiological data from Fig. 2 that were renormalized by multiplying by 0.71, the βT263I maximum p_{open} derived from simulation. △ represent normalized βT263I peak currents measured in the absence of EtOH; ▽ represent currents measured in the presence of 300 mM EtOH.

predicts that the steady-state fraction of receptors open at a given agonist concentration is:

$$\frac{n_{open}}{n_{total}} = \frac{A^2}{A^2(1+\phi) + 2AK_A\phi + K_A^2\phi} \quad (A.1)$$

where n_{total} represents the number of activatable receptors in a patch, A is the agonist concentration, $K_A = k_{off}/k_{on}$, and ϕ is defined as α/β. At saturating agonist, this fraction approaches $(1+\phi)^{-1} = \beta/(\alpha+\beta) = p_{open}$.

At $A = EC_{50}$, $n_{open}/n_{total} = \frac{1}{2}(1+\phi)^{-1}$. Setting eq. A.1 equal to this value results in the following quadratic equation:

$$(1+\phi)A^2 - 2K_A\phi A - K_A^2\phi = 0 \quad (A.2)$$

The general solution for this quadratic (for positive EC_{50} values) is:

$$EC_{50} = K_A \times \frac{\phi + \sqrt{\phi + 2\phi^2}}{1+\phi} \quad (A.3)$$

When $\phi \ll 1$, which is the case for nAChRs with ACh as agonist, eq. A.3 is approximated by $EC_{50} = K_A \times \sqrt{\phi} = K_A \times (\beta/\alpha)^{-1/2}$ which is the result predicted by our simulation in Fig. 6.

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