Distinct Molecular Basis for Differential Sensitivity of the Serotonin Type 3A Receptor to Ethanol in the Absence and Presence of Agonist

Ethanol can potentiate serotonin type 3 (5-HT₃) receptor-mediated responses in various neurons and in cells expressing 5-HT₃A receptors. However, the molecular basis for alcohol modulation of 5-HT₃ receptor function has not been determined. Here we report that point mutations of the arginine at amino acid 222 in the N-terminal domain of the 5-HT₃A receptor can alter the EC₅₀ value of the 5-HT concentration-response curve. Some point mutations at amino acid 222 resulted in spontaneous opening of the 5-HT₃A receptor channel and an inward current activated by ethanol in the absence of agonist. Among these mutant receptors, the amplitude of the current activated by ethanol in the absence of agonist was correlated with the amplitude of the current resulting from spontaneous channel openings, suggesting that the sensitivity of the receptor to ethanol in the absence of agonist is, at least in part, dependent on the preexisting conformational equilibrium of the receptor protein. On the other hand, point mutations that conferred greater sensitivity to ethanol potentiation of agonist-activated responses were less sensitive or insensitive to ethanol in the absence of agonist. For these receptors, the magnitude of the potentiation of agonist-activated responses by ethanol was inversely correlated with the EC₅₀ values of the 5-HT concentration-response curves, suggesting that these mutations may modulate ethanol sensitivity of the receptor by altering the EC₅₀ value of the receptor. Thus, distinct molecular processes may determine the sensitivity of 5-HT₃A receptors to ethanol in the absence and presence of agonist.

The serotonin type 3 (5-HT₃) receptor is a member of a superfamily of ligand-gated ion channels that includes γ-aminobutyric acid type A (GABAₐ), glycine, and nicotinic acetylcholine receptors (1). Initial molecular cloning studies identified a subunit, the 5-HT₃A receptor, from different mammalian species (1, 2). Like other members of this superfamily, the 5-HT₃A receptor consists of a large N-terminal domain, four transmembrane domains (TM), and a large intracellular domain (1). In situ hybridization studies have detected the expression at high levels of the 5-HT₃A receptor subunit in the hindbrain, especially in the nucleus tractus solitarius, area postrema, substantia nigra, and ventral tegmental area (3–5). In some of these brain areas, activation of 5-HT₃ receptors appears to increase the release of neurotransmitters such as glutamate, GABA, and dopamine (DA) (6–8). Because DA is thought to play an important role in brain reward and reinforcement mechanisms, stimulation of DA release by activation of 5-HT₃ receptors may be of significance in the mechanisms involved in anxiety, psychosis, cognitive processes, and addiction (9, 10).

Accumulating evidence has indicated that the 5-HT₃ receptor is an important target for alcohol action in the central nervous system (9, 11, 12). Ethanol has been found to potentiate 5-HT₃ receptor-mediated currents in various neuronal cell lines (13), mammalian cell lines (14), and X. oocytes (15, 16) expressing recombinant 5-HT₃A receptors. Several lines of evidence suggest that 5-HT₃ receptors may play an important role in alcohol preference and reward mechanisms (9, 17). Recent clinical studies have provided evidence that ondansetron, a selective 5-HT₃ receptor antagonist, can reduce alcohol intake in early onset alcoholics (18, 19). However, the cellular and molecular mechanisms of ethanol action on 5-HT₃ receptor function are not fully understood. On the cellular level, the potentiation of 5-HT₃A receptor-mediated responses by ethanol was found to be inversely dependent on agonist concentration. The potentiation increased with decreasing agonist concentrations and was not observed in the presence of high agonist concentrations (16, 20). Recent studies showed that ethanol slowed the desensitization rate of the current activated by 5-HT and increased maximal amplitude of current activated by DA, a partial agonist of the 5-HT₃ receptor, suggesting that ethanol may act on channel gating through increasing the probability of channel opening (21, 22).

Many recent investigations into the molecular mechanism of alcohol action have focused on GABAₐ and glycine receptors (23–25). Point mutations of several specific amino acids located in the second and third TMs of glycine and GABAₐ receptors have been shown to alter the sensitivity of the receptor to ethanol, suggesting that these residues may be crucial for the allosteric modulation of receptor function by ethanol. However, mechanistic studies of ethanol action in the absence of agonist have not been reported for ligand-gated ion channels. In addition, although some of the point mutations that alter the sensitivity of GABAₐ and glycine receptors to ethanol have been also found to be sensitive to ethanol in the absence of agonist...
site-directed Mutagenesis—Point mutations of a cloned mouse 5-HT$_{3A}$ receptor were introduced using a QuickChange site-directed mutagenesis kit (Stratagene). The authenticity of the DNA sequence through the mutation sites was confirmed by double strand DNA sequencing using an ABI Prism 377 automatic DNA sequencer (Applied Biosystems).

Preparation of cRNA and Expression of Receptor—Complementary RNA (cRNA) was synthesized in vitro from a linearized template cDNA with a mMACHINE RNA transcription kit (Ambion Inc.). The oocytes of mature Xenopus laevis frogs were isolated as described previously (28). Each oocyte was injected with a total of 20 ng of RNA in 20 nl of diethylpyrocarbonate-treated water and was incubated at 19 °C for 18 h in Modified Barth’s solution (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO$_3$, 0.05 mM CaCl$_2$, 0.8 mM MgSO$_4$, 10 mM HEPES, pH 7.4).

Two-electrode Voltage-Clamp Recording—After incubation for 2–5 days, the oocytes were studied at 20–22°C in a 90-μl chamber. The oocytes were superfused with modified Barth’s solution at a rate of ~6 ml/min. Agonists and antagonists were diluted in the bathing solution and applied to the oocytes for a specified time, using a solenoid valve-controlled superfusion system (Automate Scientific). Membrane currents were recorded by two-electrode voltage-clamp at a holding potential of −70 mV, using a Gene Clamp 500 amplifier (Axon Instruments, Inc.). Data were routinely recorded on a chart recorder (Gould 2300S).

Values are expressed as mean ± S.E.

**Single-oocyte Ligand Binding**—To determine $[^{3}H]$5-HT binding, we used the single-oocyte binding method (29) with modifications. Briefly, oocytes injected with the cRNA of the wild type (WT) and mutant 5-HT$_{3A}$ receptors were prescreened by two-electrode voltage-clamp at −70 mV. Oocytes with current amplitudes from 4 to 6 μA in response to 100 μM 5-HT were selected and held individually by suction at the end of a Pasteur pipette tip. The oocytes were first incubated at 20–22°C for 30 s in 300 mM $[^{3}H]$5-HT solution (specific activity = 20 Ci/mmol; Amersham Biosciences), and then rinsed for 6 s in 150 ml of ice-cold modified Barth’s bathing solution to remove free $[^{3}H]$ ligands from the oocyte surface. The nonselective binding was determined by incubation in 300 mM unlabeled 5-HT. The specific binding was determined by subtracting the nonspecific binding from the total binding. Each sample is the average from three or four oocytes, and each data point is the average from at least three or four separate experiments. The radioactivity (cpm) of each sample was determined in a 1409 DSA Wallac liquid scintillation counter (PerkinElmer Life Sciences). The association and dissociation rate constants were calculated using the following equation.

$$k_{a} = k_{a0} - k_{d}[C]$$

$$(k_{a} = k_{a0} - k_{d}[C])$$

(Eq. 1)

$k_{a0}$ is the observed rate constant (s$^{-1}$), determined from fitting a one-phase exponential association equation: $Y = Y_{\text{max}}(1 - e^{-k_{a}t})$, $[C]$ is the concentration of radioligand used, and $k_{d}$ is the dissociation rate constant. $k_{a0}$ (BC$_{50}$), $k_{a0}$, and $k_{d}$ were determined by equilibrium and competitive binding data using Prism Software (GraphPad).

**Data Analysis**—Statistical analysis of concentration-response curves was performed using the following form of the Hill equation.

$$I = I_{\text{max}} / (1 + (EC_{50}/A)^{n})$$

(Eq. 2)

$I$ is the peak current at a given concentration of agonist $A$, $I_{\text{max}}$ is the maximal response, $EC_{50}$ is the half-maximal concentration, and $n$ is the slope factor (apparent Hill coefficient). Data were statistically compared by the unpaired $t$ test or ANOVA analysis. Correlation analysis was carried out using nonparametric regression or linear regression (Statistica, StatSoft).

**RESULTS**

**Functional Characterization of Point Mutations at Residue 222 of 5-HT$_{3A}$ Receptors**—Although molecular cloning has identified two subtypes of 5-HT$_{3}$ receptors, 5-HT$_{3A}$ and 5-HT$_{3B}$ (1, 30), the 5-HT$_{3A}$ receptor is thought to be an essential component of all serotonin-gated ion channels. Fig. 1A shows three consecutive agonist residues at 220, 221, and 222 of the 5-HT$_{3A}$ receptor. These arginines, which are highly conserved across 5-HT$_{3A}$ receptors from different species, were predicted by computational modeling to be important structural elements for activation of the 5-HT$_{3}$ receptor (31). To examine the functional role of these arginines, each of the residues (positions 220–222) was replaced with alanine. The R221A mutant receptor was not functional (data not shown), indicating that the arginine at 221 is critical for activation of the 5-HT$_{3}$ receptor. In contrast, the R220A and R222A mutant receptors were functional when expressed in Xenopus oocytes. The maximal amplitudes of currents activated by 5-HT were not significantly different among the WT, R220A, and R222A mutant receptors ($p > 0.05$; Table I). However, the alanine substitution at amino acid 222, but not at 220, significantly decreased the $EC_{50}$ value of the 5-HT concentration-response curve (Fig. 1B; Table I). Next, we examined whether the R222A mutation alters the sensitivity of the receptor to 2-methyl-5-HT (2-Met-5-HT), a partial agonist of the 5-HT$_{3}$ receptor. Fig. 1B shows that the R222A mutation also significantly shifted the 2-Met-5-HT concentration-response curve to the left. The $EC_{50}$ values of the 5-HT and 2-Met-5-HT concentration-response curves for the R222A mutant receptors were approximately 70- and 14-fold lower, respectively, than those of the WT 5-HT$_{3A}$ receptor ($n = 6$; $p < 0.01$). The $EC_{50}$ value and the Hill coefficient for 2-Met-5-HT were 13 ± 0.4 μM and 1.8, respectively, for the WT receptors and 1.9 ± 0.2 μM and 1.6, respectively, for the R222A receptors. The R222A mutation increased the efficacy of 2-Met-5-HT from 45 ± 3% to 99 ± 6% of the maximal 5-HT-activated response; these values were significantly different (unpaired $t$ test, $p < 0.01$, $n = 5–7$). To determine whether the R222A mutation affects receptor binding, we conducted receptor-binding experiments using a single-oocyte ligand-binding method described previously (29). The time constants of $[^{3}H]$5-HT association were 9.1 ± 1.3 s for the WT receptors and 7.2 ± 1.1 s for the R222A receptors, and the dissociation rates were 0.9 ± 0.3 s for the WT receptors and 0.8 ± 0.1 s for the R222A receptors; these values were not significantly different (unpaired $t$ test, $p > 0.6$). Fig. 1C illustrates receptor-binding data for the WT and R222A mutant 5-HT$_{3A}$ receptors. The 5-HT concentration-response curves of 0.3 μM $[^{3}H]$5-HT binding for the WT receptors (open circles) and for the R222A mutants (solid circles) are superimposed. The values of $BC_{50}$ and the Hill coefficient were 0.67 ± 0.07 μM and 1.8 ± 0.1, respectively, for the WT 5-HT$_{3A}$ receptors and 0.52 ± 0.06 μM and 1.7 ± 0.1, respectively, for the R222A mutant receptors; these values were not significantly different (unpaired $t$ test, $p > 0.1$). These results suggest that the R222A mutation decreases the $EC_{50}$ of 5-HT$_{3A}$ receptor through modulation of receptor gating. To understand the structure-function role of the amino acid residues at 222 of the 5-HT$_{3A}$ receptor, the arginine at 222 was replaced by various amino acids and the function of each mutant receptor was examined by a two-electrode voltage-clamp in Xenopus oocytes. Except for R222K (Lys), a positively charged amino acid residue, the other point mutations at 222
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Fig. 1. Alteration of 5-HT₃A receptor sensitivity to agonists by single amino acid mutations at Arg-222. A, the amino acid sequence of the pre-TM1 segments of the 5-HT₃A receptor containing three consecutive arginine residues (positions 220–222). B, concentration-response curves for 5-HT and 2-Met-5-HT for the WT and R222A mutant receptors. The amplitude of current activated by 2-Met-5-HT was normalized as percentage of the maximal response activated by 5-HT for each receptor. Each data point represents the mean ± S.E. of five to seven oocytes. The error bars not visible are smaller than the size of symbols. C, single-oocyte ligand binding assay for the WT and R222A mutant receptors. Specific [³H]5-HT binding for the WT (open circles) and R222A (solid circles) receptors was determined by subtracting nonspecific binding (determined with 300 μM nonradiolabeled 5-HT) from total binding. Each data point is the average cpm from 9–12 oocytes. D, 5-HT concentration-response curves for WT and Arg-220 and Arg-222 mutant receptors. Except for WT and R220A (Ala-220), the letters represent the various amino acids substituted at position 222. The curves shown are the best fit to Equation 2 under “Experimental Procedures.” The error bars not visible are smaller than the size of symbols.

TABLE I
Summary of the properties of the WT and mutant 5-HT₃A receptors expressed in Xenopus oocytes

| Receptor | 5-HT EC₅₀ (μM) | Hill slope | Iₘ₉max (pA) |
|----------|----------------|------------|-------------|
| WT       | 1.58 ± 0.1     | 1.6 ± 0.2  | 4.2 ± 0.5   |
| R220A    | 1.30 ± 0.1     | 1.7 ± 0.2  | 3.8 ± 0.4   |
| R222A    | 0.02 ± 0.01*   | 2.1 ± 0.2* | 4.5 ± 0.6   |
| R222E    | 0.04 ± 0.02*   | 1.5 ± 0.1  | 4.6 ± 0.4   |
| R222I    | 0.06 ± 0.01*   | 1.4 ± 0.4  | 2.5 ± 0.1*  |
| R222Q    | 0.07 ± 0.01*   | 1.2 ± 0.3  | 5.6 ± 1.3   |
| R222G    | 0.09 ± 0.01*   | 1.2 ± 0.1  | 3.9 ± 0.2   |
| R222F    | 0.09 ± 0.01*   | 0.9 ± 0.1* | 1.2 ± 0.3*  |
| R222D    | 0.13 ± 0.01*   | 1.9 ± 0.2  | 2.7 ± 0.4   |
| R222T    | 0.30 ± 0.01*   | 1.6 ± 0.1  | 2.8 ± 0.5*  |
| R222N    | 0.32 ± 0.01*   | 1.5 ± 0.1  | 3.1 ± 0.5   |
| R222H    | 0.60 ± 0.3*    | 1.3 ± 0.1  | 3.4 ± 0.3   |
| R222K    | 2.10 ± 0.4*    | 1.7 ± 0.1  | 3.5 ± 0.6   |

significantly reduced the EC₅₀ value of the 5-HT concentration-response curve by 5–100-fold (p < 0.01) (Fig. 1D). The values of EC₅₀, Hill coefficient, and Iₘ₉max for 5-HT are given in Table I. It should be noted that the R222A mutation appeared to be site-specific because replacing an arginine with an alanine at 220 (R220A) did not significantly alter the sensitivity of the receptor to 5-HT (Fig. 1D; Table I).

Some Mutant Receptors Were Sensitive to Ethanol in the Absence of Agonist—Whereas ethanol at 100 mM did not induce detectable current (Fig. 2A) in cells injected with cRNAs of the WT or R222K 5-HT₃A receptors, 100 mM ethanol activated an inward current in cells expressing mutant receptors replaced with Gly and Phe at position 222. In Fig. 2B, the inward current activated by 200 mM ethanol for the mutant receptors was normalized and presented as percentage of the maximal response activated by 5-HT (Fig. 2B). In the cells expressing the mutant receptors replaced with Phe, Ile, Gly, Gln, and Asp at 222, 200 mM ethanol activated an inward current in the absence of agonist; however, R220A, R222A/T/E/N/H/K, and WT receptors were less sensitive or insensitive to this concentration of ethanol in the absence of agonist. On average, the order of magnitude of the agonist-independent effect by 200 mM ethanol for the mutant receptors was: Phe (13 ± 1.1%) > Ile (9.5 ± 0.6%) > Gly (7.3 ± 1.2%) > Gln (5.0 ± 1.1%) > Asp (4.9 ± 1.1%) > Ala (1.0 ± 0.4%) > Thr (0.53 ± 0.2%) > Glu (0.43 ± 0.1%) = Asn (0.2 ± 0.2%) = His (0.1 ± 0.05%) = Lys (0 ± 0%) = WT (0 ± 0%). The inward currents induced by ethanol appeared to be mediated through the mutant 5-HT₃A receptors because MDL-72222 (MDL), a selective 5-HT₃ receptor antagonist, inhibited the currents activated by ethanol in cells expressing R222G receptors (Fig. 2C). In these cells, both MDL and another selective 5-HT₃ receptor antagonist, LY 278,584 (LY), potently reduced the amplitude of the inward current activated by ethanol in the absence of agonist in a concentration-dependent manner over a concentration range from 0.1 to 300 nM (Fig. 2D). The IC₅₀ values of MDL and LY inhibition were 6.7 ± 0.3 and 11 ± 1 nM, respectively; the slope factors were 1.3 ± 0.2 and 1.2 ± 0.1, respectively; and the maximal values of inhibition were 86 ± 6 and 79 ± 5%, respectively. To study further the mechanism of MDL inhibition, we examined the effect of 10 nM MDL on the inward current activated by various concentrations of ethanol (Fig. 2E). In cells expressing R222G receptors, MDL at 10 nM reduced the ampli-
tude of inward current induced by ethanol at concentrations of 30, 60, 100, and 200 mM by 63 ± 8, 59 ± 5, 65 ± 8, and 60 ± 6%, respectively. These values were not significantly different (p > 0.2, ANOVA, n = 5), suggesting that the inhibition by MDL is independent of ethanol concentration. These results indicate that some point mutations at 222 of the 5-HT3A receptor can increase the sensitivity of the receptor to ethanol in the absence of agonist.

The Agonist-independent Ethanol Action at Some Mutant Receptors Correlates with the Spontaneous Channel Opening—Previous studies have reported that point mutations in the TM domains of nicotinic acetylcholine α7 and GABAα receptors can result in spontaneously opening or constitutively active channels (32, 33). To determine whether point mutations at 222 of 5-HT3A receptors can induce spontaneously active channels, we applied MDL, a 5-HT3 receptor antagonist, to cells expressing WT and mutant 5-HT3A receptors. Fig. 3A shows that, as a result of MDL inhibiting spontaneous channel opening, 300 nM MDL (300 nM) induced inward current in cells expressing R222G or R222F mutants, but not in cells expressing WT or R222K receptors. The order (Fig. 3B) of the average amplitude of the inward current increased by MDL (300 nM) for those mutant receptors was: R222F (Phe) > R222I (Ile) > R222Q (Gln) > R222G (Gly) > R222D (Asp) > R222A (Ala) > R222N (Asn) = R222T (Thr) = R222E (Glu). In cells expressing WT (Arg-222), R222H, R222K, and R220A receptors, MDL (300 nM) did not activate detectable outward current. Because the amino acid residues at 222 of the WT (Arg-222), R222K, and R222H are positively charged, this result suggests that the positive charge at 222 may be critical for stabilizing the receptor channels in a closed state. The amplitude of the outward current activated by 300 nM MDL (Fig. 3C) was significantly correlated with the amplitude of the current activated by 200 mM ethanol (SR = 0.96; p < 0.003, nonparametric regression, Statistica), indicating that the magnitude of the ethanol-induced inward current in the absence of agonist correlates with the magnitude of the spontaneous openings of the mutant ion channels.

Some Point Mutations of Arg-222 Can Alter Ethanol Potentiation of 5-HT Responses of 5-HT3A Receptors—Next, we examined whether or not point mutations of Arg-222 can affect the ethanol sensitivity of 5-HT responses of 5-HT3A receptors. The trace records in Fig. 4 illustrate the effect of ethanol at 100 and 200 mM on the 5-HT responses of WT, R222K, R222E, or R222A mutant receptors. The inward currents were activated by 5-HT at the EC50 concentration for that receptor. In cells expressing the WT receptors, ethanol potentiated the inward currents activated by 5-HT. The magnitude of the potentiation by ethanol decreased in cells expressing the R222K receptors. On the other hand, the magnitude of the potentiation by ethanol increased in cells expressing the R222E or R222A receptors. Thus, the 5-HT responses of these receptors are differentially sensitive to ethanol.

To determine the effects of mutations at 222 on ethanol modulation of the 5-HT3A receptors, we compared the ethanol-induced inward current in the absence of agonist with ethanol potentiation of 5-HT responses of the WT and mutant 5-HT3A receptors. As shown in Fig. 5A, ethanol-activated current for the mutant R222F/I/G/Q/D receptors was concentration-dependent over a concentration range of 10–200 mM. In contrast, Fig. 5B shows that the mutant R220A, R222A/E/N/T/H/K, and WT receptors were insensitive or relatively insensitive to ethanol concentrations up to 200 mM in the absence of agonist. In view of these results, we divided the WT and mutant receptors into two groups based on their sensitivity to ethanol in the absence of agonist. Group 1 included the receptors in which ethanol activated inward current in the absence of agonist. Group 2 included receptors in which ethanol did not activate significant inward current in the absence of agonist. On the other hand, we also found that the ethanol sensitivity of 5-HT-activated responses differed for the group 1 and group 2 receptors. Fig. 5C shows that ethanol, at concentrations from 10 to 200 mM, did not significantly affect...
the amplitude of current activated by low concentrations (EC₅₀) of 5-HT in cells expressing the group 1 receptors, whereas Fig. 5D shows that ethanol significantly enhanced responses activated by MDL for WT and different mutant 222 and 220 receptors. The current activated by MDL was normalized as percentage of the maximal response activated by 100 μM 5-HT. Each bar graph represents the average response from 5–7 cells. Except for WT and R220A (Ala-220), the rest of the symbols represent the various amino acids substituted at position 222. G, correlation between ethanol- and MDL-activated responses. The responses induced by ethanol and MDL were normalized as a percentage of the maximal response activated by 100 μM 5-HT. A linear regression fit to these data points revealed a strong positive correlation (SR = 0.96).

**FIG. 3.** Correlation of the amplitude of ethanol-activated current with the amplitude of current resulting from spontaneous channel openings. A, MDL at 300 nM activated outward current in the oocytes expressing R222G or R222F, but not WT or R222K receptors. B, bar graphs of the average amplitude of outward current activated by MDL for WT and different mutant 222 and 220 receptors. The current activated by MDL was normalized as percentage of the maximal response activated by 100 μM 5-HT. Each bar graph represents the average response from 5–7 cells. Except for WT and R220A (Ala-220), the rest of the symbols represent the various amino acids substituted at position 222. G, correlation between ethanol- and MDL-activated responses. The responses induced by ethanol and MDL were normalized as a percentage of the maximal response activated by 100 μM 5-HT. A linear regression fit to these data points revealed a strong positive correlation (SR = 0.96).

the amplitude of current activated by low concentrations (EC₅₀) of 5-HT in cells expressing the group 1 receptors, whereas Fig. 5D shows that ethanol significantly enhanced responses activated by 5-HT at EC₅₀ concentrations in cells expressing the group 2 receptors (p < 0.01). To determine whether the ethanol-induced inward current in the absence of agonist correlates with ethanol potentiation of agonist responses, we compared the sensitivity of the WT and mutant 5-HT₃A receptors to ethanol in the absence or presence of 5-HT (Fig. 5E). The result in Fig. 5E indicates that these receptors clearly differed in their sensitivity to ethanol in the absence and presence of agonist. There was no correlation between ethanol-induced inward current in the absence of agonist and ethanol potentiation of agonist responses (Fig. 5E; R = −0.41, p = 0.1, linear regression, n = 13).

**Correlational Analysis of Ethanol Sensitivity and Amino Acid Properties**—To gain insight into the structure-function relationship of the point mutations at 222, we used correlational analysis to compare the magnitudes of the direct action of ethanol and the ethanol potentiation with isoelectric point (pI) (34), polarity (35), hydropathicity (36), hydrophilicity (37), and volume (38) of the amino acid residues replaced at 222. Because the WT and mutant receptors clearly fall into two distinct groups based on their differential sensitivity to ethanol in the absence and presence of agonist, we analyzed the group 1 and 2 receptors separately. For the group 1 receptors, in which ethanol activated an inward current in the absence of agonist, the hydropathicity of the residues at 222 was significantly correlated with both the magnitude of the ethanol-induced inward current (Fig. 6A; SR = 0.87, p < 0.01, nonparametric analysis, n = 5) and the magnitude of the MDL-activated outward current (Fig. 6B; SR = 0.82, p < 0.01, nonparametric analysis, n = 5). For the group 2 receptors, in which ethanol potentiated agonist-activated responses, the pI of the amino acid residues at 222 (Fig. 6C) was inversely correlated with the magnitude of the ethanol potentiation (SR = 0.80, p < 0.01, nonparametric analysis, n = 7). In addition, a strong correlation was observed between the pI of the amino acid residue at 222 and the EC₅₀ values for the 5-HT concentration-response curves (Fig. 6D, SR = 0.92, p < 0.01, nonparametric analysis, n = 7). Thus, the correlation patterns differ for the group 1 and 2 receptors.

**Ethanol Potentiation Inversely Correlates with the 5-HT EC₅₀ Values of Group 2 Receptors**—In the light of the observation that pI at amino acid 222 correlates with the magnitude of ethanol potentiation of 5-HT-activated responses and the 5-HT EC₅₀ values for the group 2 receptors, it seemed possible that potentiation by ethanol might depend on agonist concentration
and correlate with the 5-HT EC_{50} value. Indeed, such a correlation was observed. As shown in Fig. 7A, the percentage increase in the group 2 receptor-mediated responses by 100 m\text{M} ethanol were maximal at the lowest agonist concentrations tested (0.003 \text{\mu M} for R222A receptors, 0.005 \text{\mu M} for R222E receptors, 0.01 \text{\mu M} for R222T and R222N receptors, 0.1 \text{\mu M} for R222H and WT receptors, 0.15 \text{\mu M} for R222K receptors, and 0.3 \text{\mu M} for R220A receptors), and decreased with increasing agonist concentration. The average increase in 5-HT-activated current was: 142\% for R222A, 91\% for R222E, 58.5\% for R222T, 60.5\% for R222N, 63\% for R222H receptors, 60\% for WT receptors, 36\% for R220A receptors, and 30\% for R222K receptors. To gain insight into the possible mechanism underlying ethanol potentiation of agonist responses for the group 2 receptor-mediated responses, we compared the percentage potentiation of agonist responses by 100 and 200 m\text{M} ethanol with the magnitude of the ethanol-induced inward current in the absence of agonist, the MDL-activated outward current and the EC_{50} value for 5-HT. Among all of these factors, the only variable that correlated (inversely) with ethanol potentiation of agonist responses was the EC_{50} value for 5-HT (Fig. 7B), suggesting that ethanol potentiation of 5-HT responses is dependent, at least in part, on the sensitivity of the receptors to agonist.

On the other hand, our understanding of ethanol potentiation of agonist responses for the group 1 receptors was less clear. Given the observation that group 1 receptor-channels can open spontaneously and may open further upon ethanol exposure, we may not be measuring the magnitude of ethanol potentiation of agonist responses at an equivalent extent of channel opening. To address this concern, we first tested whether ethanol potentiation of agonist responses of the group 1 receptor-mediated responses, we compared the percentage potentiation of agonist responses by 100 and 200 m\text{M} ethanol with the magnitude of the ethanol-induced inward current in the absence of agonist, the MDL-activated outward current and the EC_{50} value for 5-HT. Among all of these factors, the only variable that correlated (inversely) with ethanol potentiation of agonist responses was the EC_{50} value for 5-HT (Fig. 7B), suggesting that ethanol potentiation of 5-HT responses is dependent, at least in part, on the sensitivity of the receptors to agonist.

Fig. 5. Comparison of ethanol-activated current in the absence of agonist with ethanol potentiation of agonist-activated responses. A, concentration-response curves for ethanol-activated current in the absence of agonist for the mutant R222F/I/G/Q/D receptors (group 1). The inward current activated by various concentrations (10–200 m\text{M}) of EtOH were normalized as a percentage of the maximal response activated by 100 \text{\mu M} 5-HT. Each data point represents the mean ± S.E. of seven oocytes. The curves are the best fit to Equation 2 under “Experimental Procedures.” B, concentration-response curves for ethanol-activated current for WT, R220A, and R222A/E/T/H/K receptors (group 2). For these receptors, ethanol activated little or no current in the absence of agonist. C, concentration-response curves of the group 1 receptors for ethanol potentiation of 5-HT responses. D, concentration-response curves of the group 2 receptors for ethanol potentiation of 5-HT responses. Each data point represents the mean ± S.E. of seven oocytes. The curves are the best fit to Equation 2 under “Experimental Procedures.” E, differential ethanol sensitivity of group 1 and group 2 receptors. The x axis represents the percentage potentiation of 5-HT-activated currents by 200 m\text{M} ethanol. The y axis represents the magnitude of current activated by 200 m\text{M} ethanol in the absence of agonist.

Fig. 6. Correlation of the group 1 and group 2 receptors with properties of amino acid substitution. A, for the group 1 receptors, a correlation was found between the hydropathicity of the amino acid residues at 222 and the magnitude of the ethanol-activated inward current in the absence of agonist. B, for the group 1 receptors, the hydropathicity was also correlated with the magnitude of MDL-activated outward current; these data were fitted using nonparametric analysis (Statistica). C, for the group 2 receptors, an inverse correlation was found between pI and the magnitude of ethanol potentiation of 5-HT responses. D, for the group 2 receptors, the pI values also correlated with the EC_{50} values of 5-HT concentration-response curves; these data were fitted using nonparametric analysis (Statistica).
equivalent basis that may represent the active conformational state of these receptors, we added up and normalized the amplitudes of the current activated by 5-HT at the apparent EC50 concentration, ethanol-induced current, and MDL-activated current (Fig. 8B). Using normalized response as "proportional opening" for each of the group 1 receptor channels (Fig. 8C), we found that the potentiation of agonist responses by ethanol of these receptors is inversely correlated with the percentage of the normalized maximal current ($p < 0.01$, linear regression, Statistica).

DISCUSSION

Mutagenesis studies have been valuable for identifying molecular determinants of alcohol sensitivity of neurotransmitter-gated membrane ion channels. In this study, we have observed that the mutant receptors that are sensitive to ethanol in the absence of agonist are also sensitive to the inhibition of spontaneous openings by 5-HT3 receptor antagonists, MDL and LY. These results are consistent, in general, with a previous study of GABA$_\alpha$ receptors (25). It is likely that some of the point mutations at 222 can reduce a free energy barrier that controls a transition from a closed state to an open state of the channel. As a result, these mutant receptors become sensitive to ethanol in the absence of agonist. It is particularly interesting that MDL and LY can block the ethanol-induced inward current of these receptors with IC50 values in a concentration range at $\sim 10$ nM (6.7 nM for MDL and 11 nM for LY). This concentration range is close to that of the receptor binding affinities for these antagonists (1, 39). This suggests that antagonist binding to

Fig. 7. For the group 2 receptors, the ethanol potentiation of 5-HT responses was inversely correlated with the EC50 values of 5-HT concentration-response curves. A, potentiation by 100 mM ethanol of current activated by various concentrations of 5-HT. The curves are the best fit to Equation 2 under "Experimental Procedures." The error bars not visible are smaller than the size of symbols. Each data point represents mean $\pm$ S.E. of 5–7 oocytes. B, for the group 2 receptors, a correlation was found between the magnitude of ethanol potentiation of 5-HT responses and the EC50 values for the 5-HT concentration-response curves. The data are the best fit to a linear regression (Statistica).

Fig. 8. For the group 1 receptors, the ethanol potentiation of 5-HT responses was inversely correlated with the extent of channel opening. A, agonist-concentration dependence of ethanol potentiation of 5-HT responses for R222G and R222F receptors. Each bar graph represents the average of five to seven cells. B, the open bar graphs show, sequentially from left to right, the average response activated by an EC5 concentration of 5-HT (left), 200 mM ethanol (middle) in the absence of agonist, or 300 nM MDL (right) in the absence of agonist in oocytes expressing R222F receptors. The solid bar graph (far right) shows the added responses. The bar graphs are normalized as percentage of the maximal response to 100 $\mu$M 5-HT. C, for group 1 receptors, the magnitude of ethanol potentiation of 5-HT responses is inversely correlated with the extent of channel opening (added responses). The data are the best fit to a linear regression (Statistica).
the receptor inhibits spontaneous opening of the channel. The observation that the magnitude of the antagonist inhibition of ethanol-induced inward current is not significantly different for different concentrations of ethanol suggests that the antagonist action is independent of ethanol concentration. It seems likely that the antagonists stabilize the channel in a closed state by increasing the free energy barrier for opening of the channel. These considerations suggest that the sensitivity of the receptor to ethanol in the absence of agonist is, at least in part, dependent on the preexistent conformational equilibrium of the receptor protein or a transition from a closed state to an open state of the receptor channel.

The mechanisms underlying the magnitude of ethanol potentiation of agonist responses of mutant 5-HT₃₆ receptors appear to be more complicated. These mutant receptors are differentially sensitive to ethanol potentiation of agonist responses. For the group 2 receptors, the mutations in which ethanol-potentiated 5-HT-activated current had little or no ethanol-induced inward current in the absence of agonist. For these mutant receptors, the percentage potentiation by ethanol inversely correlated with the EC₅₀ values of the 5-HT₃ concentration-response curves, suggesting that mutation of arginine 222 may modulate the ethanol sensitivity of agonist responses by altering the EC₅₀ of the receptor. However, this result could also be explained in a number of different ways other than a simplistic conclusion that ethanol directly enhances the apparent agonist affinity. Given the observation that the point mutations at 222 do not affect receptor binding affinity, our results may be more consistent with a previous kinetic study of 5-HT₃ receptor in NCB-20 cells, which suggested that ethanol may modulate the gating of the receptor channels by favoring a stabilized open state (21). In this scenario, ethanol may increase the probability of opening of 5-HT₃ receptors, particularly under a circumstance when some point mutations at 222 reduce an energy barrier that constrains a transition of the ion channels from a closed state to an open state. Such a structural change could account for ethanol-inducing larger increases in current amplitude at low 5-HT concentrations in cells expressing some of the group 2 mutant 5-HT₃ receptors. A similar scenario could occur to the group 1 receptors in which ethanol exerted its maximal potentiation of 5-HT responses at the lowest active state of the receptor and with increasing proportional opening of the channel, ethanol potentiation of 5-HT responses decreased in magnitude. It is possible that group 1 mutations at 222 of the 5-HT₃ receptor stabilize the channel in an open state, as described by a previous study of a point mutation in the TM4 of Torpedo nicotinic acetylcholine receptor (40). Under this scenario, channels that were stabilized in an open state by the point mutations at 222 would become less sensitive to ethanol potentiation of 5-HT responses. Taken together, we hypothesize that the point mutations at 222 may modulate the ethanol potentiation of 5-HT responses by altering the EC₅₀ of the receptors, the gating of the channel or both.

Thus, distinct molecular processes may be involved in the ethanol-induced current in the absence of agonist and the ethanol potentiation of 5-HT responses based on the following supportive evidence. First, the mutant receptors exhibit opposite sensitivity to ethanol in the absence and presence of agonist. Second, whereas the ethanol-induced current is positively correlated with spontaneous openings of the channels, ethanol potentiation of 5-HT responses is inversely correlated with the 5-HT EC₅₀ values. Further, correlation analysis showed that the hydropathy and the positive charge of the amino acid residues at 222 were differentially correlated with the sensitivity of the receptor to ethanol in the absence and presence of agonist between the group 1 and 2 receptors. This hypothesis appears to be consistent with a previous study of ethanol action on GABA receptors (25), which showed that point mutations of S270W, V257W, and T262W in the TM2 domain of the GABA receptor α2 subunit reduced ethanol potentiation of the GABA-induced responses, and, on the other hand, produced an ethanol-induced current in the absence of GABA. Similar results are also reported in a recent mutagenesis study of anesthetic action on GABA receptors, in which point mutations in the TM2 domain of the β2 subunit differentially altered the sensitivity of the GABA receptor to anesthetics in the absence and presence of agonist (41). It is important to note, however, that we cannot rule out the possibility that ethanol may modulate 5-HT₃ receptor function through the same molecular process in the absence and presence of agonist. One of the alternative interpretations could be that ethanol, in the absence of agonist, may desensitize the receptor channel, thereby altering the sensitivity of the receptor to ethanol potentiation. This hypothesis, at least in part, appears to be inconsistent with some of our experimental findings. For instance, many of the mutant receptors in group 2, such as R222A and R222E, that were more sensitive than group 1 receptors to agonist exhibited little or no sensitivity to ethanol in the absence of agonist. In addition, the magnitudes of ethanol potentiation of 5-HT responses do not significantly differ between pre-incubation of ethanol and simultaneous application of ethanol with agonist (data not shown). Furthermore, there was no apparent desensitization occurring either in the currents evoked by ethanol in the absence of agonist (Fig. 2A) or in the currents activated by low concentrations of 5-HT with or without pre-incubation of ethanol (Fig. 4). In fact, ethanol was found to slow the desensitization of current activated by low concentrations of 5-HT in NCB-20 cells (21).

In summary, the study reported here provides evidence that the sensitivity of 5-HT₃ receptors to ethanol in the absence and presence of agonist may be mediated through distinct molecular mechanisms. Because the members of this ligand-gated ion channel superfamily are highly conserved in their amino acid sequences, our observations may provide general principles for future studies to look for the molecular basis of alcohol sensitivity of other neurotransmitter-gated ion channels in this superfamily.

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Distinct Molecular Basis for Differential Sensitivity of the Serotonin Type 3A Receptor to Ethanol in the Absence and Presence of Agonist
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