Ethanol Is a Fast Channel Inhibitor of P2X4 Receptors

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Received November 5, 2010; accepted January 5, 2011

Abstract

P2X receptors (P2XRs) are ion channels gated by synaptically released ATP. The P2X4 is the most abundant P2XR subtype expressed in the central nervous system and to date is the most ethanol-sensitive. In addition, genomic findings suggest that P2X4Rs may play a role in alcohol intake/preference. However, little is known regarding how ethanol causes the inhibition of ATP-gated currents in P2X4Rs. We begin to address this issue by investigating the effects of ethanol in wild-type and mutant D331A and M336A P2X4Rs expressed in human embryonic kidney (HEK) 293 cells using whole-cell patch-clamp methods. The results suggest that residues D331 and M336 play a role in P2X4R gating and ethanol inhibits channel functioning via a mechanism different from that in other P2XRs. Key findings from the study include: 1) ethanol inhibits ATP-gated currents in a rapid manner; 2) ethanol inhibition of ATP-gated currents does not depend on voltage and ATP concentration; 3) residues 331 and 336 slow P2X4 current deactivation and regulate the inhibitory effects of ethanol; and 4) ethanol effects are similar in HEK293 cells transfected with P2X4Rs and cultured rat hippocampal neurons transduced with P2X4Rs using a recombinant lentiviral system. Overall, these findings provide key information regarding the mechanism of ethanol action on ATP-gated currents in P2X4Rs and provide new insights into the biophysical properties of P2X4Rs.

Introduction

P2X receptors (P2XRs) are a superfamily of cation-permeable ligand-gated ion channels activated by synaptically released extracellular ATP. P2Xs are ubiquitous in mammalian organisms and are expressed in virtually all types of tissues (Khakh, 2001; Rubio and Soto, 2001; North, 2002). ATP induces nonselective cationic currents through homomeric and heteromeric channels formed by seven subunits (P2X1-P2X7) (Torres et al., 1999; North, 2002). P2X subunits consist of two transmembrane (TM) domains connected by a large extracellular domain (ectodomain) (North, 2002; Burnstock, 2008). Crystallographic investigations have confirmed that P2XRs have a trimeric structure with a large rigid ectodomain and an α-helical segment (TM2) lining the pore (Kawate et al., 2009).

P2XRs are becoming a focus of investigation in ethanol studies because of building evidence suggesting that P2XRs are important mediators of ethanol-induced effects (Li et al., 2000; Davies et al., 2006; Asatryan et al., 2008; Tabakoff et al., 2009). ATP-gated currents are inhibited by ethanol at intoxicating and anesthetic concentrations when measured in P2X2Rs and P2X4Rs expressed in Xenopus oocytes (Xiong et al., 2000; Davies et al., 2002, 2005), whereas ATP-gated currents are potentiated by ethanol in P2X3Rs (Davies et al., 2005). Moreover, preliminary investigations found that ethanol inhibits ATP-gated currents of P2X7Rs expressed in oocytes.

The P2X4 subtype is the most abundant P2XR subtype expressed in the central nervous system (Buell et al., 1996; Soto et al., 1996). Moreover, P2X4Rs are the most ethanol-sensitive P2XRs identified to date; in the oocyte expression system ethanol inhibits P2X4R currents starting at concentrations below 10 mM (Xiong et al., 1999, 2000, 2005; Davies et al., 2002, 2005; Popova et al., 2010). In addition, findings from a genomic investigation suggest that P2X4Rs may play a role in alcohol intake and preference in rats. Specifically,
whole brain expression of the p2rx4 gene in naive animals was inversely related to innate 24-h alcohol preference, and P2X4 mRNA expression was decreased in alcohol-prefering rats (Tabakoff et al., 2009). In support of this notion, we have shown that null mutant P2X4 male mice drink significantly more alcohol than their wild-type (WT) littermates (D. Davies, unpublished preliminary findings).

Investigations are beginning to shed light on potential sites of ethanol action in P2X4Rs. Previous findings suggested that the ectodomain and the TM interfaces in P2X4Rs contained targets for ethanol action (Xiong et al., 2005; Asatryan et al., 2008; Yi et al., 2009). Additional support for this hypothesis comes from a report that mutations at positions 331 or 336 in the ectodomain-TM2 region of P2X4Rs significantly reduced or eliminated the modulatory effects of ethanol (Popova et al., 2010).

Less is known regarding the mechanism of ethanol action on P2X4Rs. To date, the majority of electrophysiological investigations exploring the sites and mechanisms of ethanol action on P2XRs have used two-electrode voltage clamp on Xenopus oocytes (Weight et al., 1999; Li et al., 2000; Davies et al., 2002, 2005; Popova et al., 2010). However, there are some limitations to the use of oocytes for the study of drug effects on ion channels. The large size of the oocyte and the relatively slow perfusion rates typically used in these studies make it difficult to carry out detailed biophysical characterization of ligand-gated currents.

Whole-cell patch-clamp electrophysiology provides a higher level of resolution and insights into the biophysical properties of the receptor and the mechanism of drug-receptor interaction. However, at this time, few studies have used this approach to investigate the effects of ethanol on P2XRs. The work that is available has focused on mouse hippocampal and bullfrog DRG neurons (Li et al., 1998, 2000). These early neuronal studies found that ATP-gated currents were inhibited by ethanol in a concentration-dependent manner and that ethanol affected the rate of deactivation of the currents. Moreover, the authors of the latter study suggested that ethanol acted by altering the affinity of ATP. Overall, the findings from these initial patch-clamp studies were consistent with findings reported in oocytes (Weight et al., 1999; Li et al., 2000; Davies et al., 2002, 2005; Popova et al., 2010).

The current study represents the first comprehensive investigation that focuses on the mechanism of ethanol action on P2X4Rs expressed in a mammalian cell system using whole-cell patch-clamp technique combined with a mutagenesis approach. We found that ethanol inhibits channel gating by affecting the functioning of the receptor around residues D331 and M336 that are thought to play a role in P2X4R gating. To gain insight into the physiological consequences stemming from the ethanol effects on P2XRs, we overexpressed P2X4R in cultured rat hippocampal neurons using a recombinant lentiviral system. Investigations on these neurons revealed a similar degree and pattern of ethanol inhibition compared with those of transfected HEK293 cells. Overall, the results provide key insights into the biophysical properties of P2X4Rs and new knowledge regarding the mechanism of ethanol-receptor interaction.

**Materials and Methods**

**Site-Directed Mutagenesis.** The cDNA of rat P2X4R (GenBank accession no. X87763) was subcloned into pcDNA3 vector (Invitrogen, Carlsbad, CA). Mutagenesis was performed to introduce single point mutations using the QuikChange IIXL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The sequences of the sites of potential effects on P2X4Rs, the role in P2X4R gating. To gain insight into the physiological consequences stemming from the ethanol effects on P2XRs, the work that is available has focused on mouse hippocampal and bullfrog DRG neurons (Li et al., 1998, 2000). These early neuronal studies found that ATP-gated currents were inhibited by ethanol in a concentration-dependent manner and that ethanol affected the rate of deactivation of the currents. Moreover, the authors of the latter study suggested that ethanol acted by altering the affinity of ATP. Overall, the findings from these initial patch-clamp studies were consistent with findings reported in oocytes (Weight et al., 1999; Li et al., 2000; Davies et al., 2002, 2005; Popova et al., 2010).

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**HEK293 Cell Culture.** HEK293 cells, obtained from the American Type Culture Collection (Manassas, VA), were grown in Dulbecco’s modified Eagle’s medium/F12 media (Invitrogen) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT) and penicillin/streptomycin in O2/CO2 (95:5%) atmosphere. Cells were seeded in six-well plates 1 day before transfections that were performed using 0.2 to 8 μg of DNA and 5 μl of Lipofectamine 2000 reagent in OPTIMEM (Invitrogen). Four days after transfection, cells were trypsinized and plated in 35-mm single dishes in growth medium. Patch-clamp recordings were performed after 24 to 48 h.

**Isolation of Hippocampal Neurons.** Timed-pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN) were sacrificed by CO2 inhalation (derived from a tank source). After euthanasia, embryonic day 18 (E18) rat pups were removed from the uterus and decapitated. Fetal hippocampi were dissected out, incubated in cold Hanks’ buffer solution (Invitrogen), and then subjected to enzymatic digestion in 0.02% trypsin solution at 37°C for 5 min. Digested tissue was rinsed three times with Hanks’ buffer solution and then dissociated with mechanical force by triturating through a series of different sizes of polished Pasteur pipettes. Cells were plated onto poly-L-lysine-coated culture dishes containing neurobasal media supplemented with B-27, primocin, and 25 μM glutamate. Media changes occurred every 3 to 4 days using supplemented neurobasal media without glutamate. The neurons were cultured for at least 5 days to allow for maturation before lentiviral infection.

**Lentivirus Production and Transduction of Neurons.** cDNA encoding rat WT P2X4R was subcloned into the pLVX-AcGFP-N1 lentiviral expression vector (Clontech, Mountain View, CA) that results in a C terminus-tagged P2X4-GFP fusion protein. The lentiviral vector construct was verified by enzymatic digestion and gel electrophoresis and DNA sequencing (USC/Norris DNA Core Facility). This construct was then mixed with Lentiv X HT proprietary packaging mix (Clontech) and used with Lentiphos HT transfection system (Clontech) to transfect 293T cells (50–60% confluence) for production of vesicular stomatitis virus G pseudotyped, replication-incompetent lentivirus. Viral titers were determined using HT-1080 fibroblastoama cells and through reverse transcription-polymerase chain reaction of viral RNAs. Neurons were infected at day in vitro 5 to 7 with 106 infectious units/ml and after 48 h, transduction confirmation of protein expression was visualized by GFP fluorescence.

**Patch-Clamp Recordings.** HEK293 cells and neurons were voltage-clamped at −50 mV at room temperature, and ATP-induced currents were acquired using an Axopatch 200B amplifier, Digidata 1320 interface, and pClamp 9.0 software (Molecular Devices, Sunnyvale, CA). Data were digitized at 5 kHz and filtered at 1 kHz. Composition of the external solution was 135 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, and 10 mM glucose with pH of 7.4 adjusted with NaOH. Osmolarity was adjusted to 315 mmol/kg with sucrose. Patch electrodes (2–6 MΩ) were filled with 140 mM KCl, 2 mM MgCl2, 2.5 mM EGTA, 2 mM TEA-Cl, 4 mM K2ATP, and 10 mM HEPES, pH 7.25 with KOH and osmolality of 310 mmol/kg. Agonist and drug containing solutions were prepared freshly on the day of an experiment. To avoid strong run-down in P2X4R currents, ATP was applied for the shortest period of time sufficient to reach the peak in most experiments. Time between applications was 15 to 120 s depending on cell recovery from desensitization. In experiments with ethanol preapplication, cells were
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Results

Ethanol Inhibits P2X4Rs Expressed in HEK293 Cells.

In the first investigation, ethanol (100 mM) significantly inhibited 3 μM ATP (EC₅₀) currents to 59.8 ± 3.2% of control with full recovery to 93.6 ± 7.3% upon washout (Fig. 1, A and B). Ethanol applied in the absence of ATP did not significantly alter baseline current (data not shown). To gain insight into the mechanism of ethanol action, we next conducted ATP concentration response studies in the presence and absence of 100 mM ethanol. As shown in Fig. 1C, ATP elicited a robust current in a concentration-dependent manner with EC₅₀ = 7.5 μM and Hill coefficient = 0.9. This finding agrees with previous published studies in oocytes and HEK293 cells (Xiong et al., 2000; Davies et al., 2005; Popova et al., 2010). Coapplication of 100 mM ethanol with ATP resulted in the ATP concentration-response curve with EC₅₀ = 9.2 μM, Hill coefficient = 1.0. It is noteworthy that the degree of ethanol inhibition was not significantly different when tested at lower versus higher concentrations (e.g., 1 μM versus 100 μM ATP; Fig. 1D). Because of the lack of concentration dependence, we selected to use 3 μM ATP for the remainder of our experiments because it yielded reasonable amplitudes of ATP currents without causing appreciable desensitization (when application was not prolonged), and these currents were reliably inhibited by 100 mM ethanol. The lack of an obvious ATP EC dependence differed with previous work on P2X4Rs when expressed in oocytes where exposure to 100 μM ATP surmounted the ethanol effect to a greater extent than at lower concentrations of ATP (Xiong et al., 2000; Davies et al., 2005; Popova et al., 2010).

It is noteworthy that in the first studies a consistent and significant “rebound” of the ATP-gated currents at the end of the ethanol/ATP applications appeared to occur (Fig. 1A, arrow). That is, at the termination of ethanol/ATP application there was a reduction in the degree of ethanol inhibition (shifting from approximately 60% of the control ATP current back to 80.0 ± 5.0%; Fig. 1B). This effect was more visible when cells were in a lifted-up mode. We reasoned that the rebound was probably caused by ethanol washing off faster than ATP, resulting in an immediate relief from the inhibition. To test this notion, we next varied the timing and order of ethanol and ATP exposures using a three-barrel flow pipe.

The Onset and Offset of Ethanol Effect on P2X4R Channels Is Rapid. As illustrated in Fig. 2A, in the presence of ATP, ethanol inhibition of P2X4R currents and washout was rapid with rates of onset comparable with the rates of solution exchange (~10–100 ms depending on lifted or attached cell mode). When applied with a delay to allow full channel opening, ethanol continued to cause inhibition of the ATP-gated current to the similar degree as when using a simultaneous coapplication protocol (Figs. 2, A and B and 1, A and B). In addition, simultaneous termination of both ethanol and ATP application resulted in a similar “rebound” as previously noted (Fig. 2A, right). Comparison of the two traces shown in Fig. 2A, illustrates that the maximum point of the rebound is similar in magnitude to the level of the
Ethanol Affects P2X4R Channels in Open State. We next investigated whether the effects of ethanol are use-dependent by comparing the degree of ethanol-induced inhibition using several different drug application protocols (Fig. 3). This included protocols with and without ethanol pretreatment before coapplication with agonist. Pretreatment of ethanol for 30 to 60 s before ethanol/ATP application did not significantly change the magnitude of ethanol inhibition (illustrated in Fig. 3A, third and fourth trace, and B). In addition, there was no subsequent inhibition after ethanol was applied during the rest and the deactivation but not open states of the channel (Fig. 3A, second trace and B). It is noteworthy that leaving ethanol in the buffer while washing out ATP resulted in the absence of the rebound (Fig. 3A, third trace and C). This observation provides additional support for the hypothesis that ethanol interacts with the channel faster than ATP.

Extending our investigation to gain further insight into the mechanism of ethanol inhibition, we performed a series of multiple applications of ethanol in the presence of ATP. As shown in Fig. 3C, there was no significant change in the magnitude of ethanol inhibition from one application of ethanol to the next, confirming that the onset of ethanol action is too fast to resolve the question of use dependence using multiple applications. As such, we tested whether the speed of ethanol offset of inhibition depended on the ATP concentration. This was accomplished by estimating the offset rates ATP-activated currents at this time point when tested in the absence of ethanol. This finding adds support to the hypothesis that the rebound is a result of faster ethanol washing out compared with ATP.

To further test this hypothesis, we changed the order of ethanol and increased the time of ethanol and ATP application. Changing the order of drug application did not affect the magnitude or the duration of the ethanol effect (Fig. 2C). However, continued application of ATP after termination of ethanol application resulted in ATP currents that were similar in magnitude compared with that of the control after the rebound (Fig. 2B). It is noteworthy that changing the order of drug application did not significantly alter the degree of ethanol inhibition or the rebound effect. A comparison of the degree of ethanol inhibition and rebound (where applicable) between the three protocols did not reveal any significant differences (Figs. 1B and 2, B and D). Taken together, the findings support the hypothesis that the observed “rebound” is caused by ethanol rapidly washing out from the receptor before ATP unbinding. Moreover, the experiments demonstrated that the time frame of our solution exchange was sufficient to allow equilibration of ethanol at its site of action. Overall, our results suggest that the effects of ethanol, as measured in P2X4Rs, are attributable to ethanol acting as a fast channel inhibitor.

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Fig. 4. Ethanol does not affect activation and deactivation kinetics of P2X4R currents. A, representative traces of currents normalized to peak value. B, ethanol does not significantly affect current activation rates. \( \tau_{\text{on}} \) (ms) values for different pretreatment/treatment protocols were: NR/ATP, 194 ± 39; NR/ATP + EthOH, 199 ± 25; NR + EthOH/ATP, 168 ± 35; and NR + EthOH/ATP + EthOH, 222 ± 40. C, ethanol has little effect on the rates of current deactivation. \( \tau_{\text{off}} \) (ms): NR/ATP, 190 ± 25; NR/ATP + EthOH, 189 ± 23; NR + EthOH/ATP, 138 ± 17; NR + EthOH/ATP + EthOH, 174 ± 17. Significant differences were found only for the pair \( \tau_{\text{off}} \) control and \( \tau_{\text{off}} \) in the presence of ethanol during ethanol pretreatment (P < 0.01, Dunnett’s multiple comparison test). Currents were elicited by 3 \( \mu \)M ATP (n = 6).

at 3 and 100 \( \mu \)M ATP by monoeXponentially fitting the rebound curve. Indeed, as shown in Fig. 3D, the \( \tau_{\text{off}} \) was significantly lower at high agonist concentration \( \tau_{\text{off}} = 4.2 ± 0.4 \) ms at 3 \( \mu \)M ATP (n = 9) versus 1.9 ± 0.4 ms at 100 \( \mu \)M ATP (n = 3). This result suggested a use-dependent action of ethanol when the channel is in the conducting (open) state (i.e., higher percentage of open channels allows for faster equilibration of ethanol).

**Ethanol Does Not Affect Kinetics of P2X4R Currents.**

We also investigated the kinetics of current activation and deactivation using different ethanol application protocols. In accordance with previous observations (Li et al., 1998), ethanol did not change the rate of activation (Fig. 4, A and B). On the other hand, ethanol did not affect the rate of deactivation either (Fig. 4, A and C). These results contrast previous findings in bullfrog DRG neurons demonstrating that 50 to 100 mM ethanol accelerates channel deactivation (Li et al., 1994, 1998). It should be noted that DRG neurons express different P2X subunits, thus allowing for multiple receptor phenotypes (Kobayashi et al., 2005). The small, but statistically significant, difference for deactivation time constant \( \tau_{\text{off}} \) in the case when ethanol was present only in the bathing solution probably was caused by a rapid effect of ethanol during the course of deactivation as shown in Fig. 4A. These data also confirmed a rapid noncompetitive onset of ethanol action during channel open state but did not distinguish whether the action of ethanol is caused by an open-channel block mechanism.

**Ethanol Effect on P2X4Rs Is Voltage-Independent.**

Because our studies suggested that ethanol acts similarly to what one would expect from an open-channel blocker and because open-channel blockers commonly exhibit voltage-dependent block (Hille, 2001), we investigated the effect of ethanol on current-voltage relationship. In this experiment, we used a series of rapid ethanol applications where ethanol was applied during the same ATP pulse at different holding potentials (Fig. 5, A and B). Ethanol did not shift the reversal potential of ATP currents (~0 mV). There was no effect of ethanol at 0 mV caused by the absence of visible current. The inhibitory effect of ethanol on P2X4R currents was voltage-independent (Fig. 5C). This outcome was not surprising given the lack of change of ethanol molecules. Variability of ethanol effect on a cell-to-cell basis averaged from all tested voltages ranged from 55.8 to 74.8%; however, no significant difference was found (data not shown). Overall, these results are in accordance with earlier investigations of ethanol inhibition of recombinant and native P2XRs (Xiong et al., 2000).

**Residues 331 and 336 in P2X4Rs Play a Role in Channel Functioning and Ethanol Inhibition.**

Previous investigations found that amino acid substitutions at nonconserved positions 331 (i.e., D331) and 336 (i.e., M336) in P2X4Rs significantly reduced or eliminated the effects of ethanol when tested in Xenopus oocytes using two-electrode voltage-clamp analysis (Popova et al., 2010). It is noteworthy that these mutations did not seem to be involved in agonist action and did not significantly alter basic receptor function. Mutants were slightly more sensitive to ATP than the WT receptor with EC\(_50\) = 3.0 \( \mu \)M, Hill coefficient = 1.08 for D331A and EC\(_50\) = 5.5 \( \mu \)M, Hill coefficient = 0.86 for M336A (Fig. 6B) but they were much less sensitive to 100 mM ethanol at ATP concentrations 1 and 3 \( \mu \)M (Fig. 6, A and C). To gain insight into the biophysical changes that the mutations might cause, we tested whether residues D331 and M336 are important for channel operation using 3 \( \mu \)M ATP as the agonist. We found that substituting alanine at either position 331 or 336 did not significantly alter the activation rate of the...
P2X4R currents in the absence and presence of ethanol. Showing activation (D) and deactivation (E) kinetics of WT and mutant with ethanol effects in the WT P2X4R ANOVA. D and E, bar graphs of desensitization rates of WT, D331A, and M336A in the absence and presence of ethanol. For WT, D331A, and M336A residual deactivation rate occurring after ATP removal still did proceed at a slower rate (Fig. 7A, arrows). inclusion of ethanol in the experimental paradigm did not significantly alter channel desensitization (Fig. 7A). This latter finding suggests that ethanol does not inhibit P2X4R by increasing channel desensitization.

P2X4Rs Overexpressed in Rat Hippocampal Neurons Are Inhibited by Ethanol. To begin investigating the physiological consequences stemming from ethanol effects on P2XRs, we overexpressed P2X4R in cultured E18 rat hippocampal neurons using a recombinant lentiviral system. Pilot studies determined that cultured neurons at this stage (days in vitro 9–14) expressed negligible levels of functioning P2XRs. Application of 100 μM ATP did not evoke any measurable currents when tested in noninfected neurons (data not shown). Thus this system allows for testing of recombinant P2XRs in a neuronal environment without the complications of native P2XRs as would be found when testing adult, freshly cultured hippocampal neurons (Li et al., 2000; Cunha, 2000; Pankratov, 2009). Results from this experiment provide initial insight into the biophysical properties and susceptibility to ethanol of P2X4Rs when expressed in neurons. Application of 10 μM ATP induced a robust response in lentivirus-transduced neurons, being 78.9 ± 2.4% (n = 3) of maximal (100 μM) response (Fig. 8). Ethanol (100 μM ATP-activated currents. B, concentration-response curves for mutants: D331A, EC50 = 3.0 μM, Hill coefficient = 1.08; and M336A, EC50 = 5.5 μM, Hill coefficient = 0.86. C, bar graph showing the effect of ethanol on mutant P2X4R current amplitude. For D331A the residual current was 90.9 ± 1.9% (n = 5) at 1 μM ATP and 90.4 ± 3.1% (n = 9) at 3 μM ATP. For M336A the residual current was 98.1 ± 3.6% (n = 6) and 86.5 ± 1.9% (n = 12) at 1 and 3 μM ATP, respectively. P < 0.05 compared with ethanol effects in the WT P2X4R ANOVA. D and E, bar graphs showing activation (D) and deactivation (E) kinetics of WT and mutant P2X4R currents in the absence and presence of ethanol. P < 0.01 for τoff of both mutants versus WT (ANOVA, Dunnett’s post-test).

mutant receptors compared with WT (Fig. 6D) |τon for 331A, 143 ± 12 ms (n = 10); 336A, 165 ± 16 ms (n = 10); and WT, 174 ± 18 ms (n = 14)]. However, the current deactivation rate, i.e., kinetics of rapid channel closing upon agonist removal, was significantly altered in the mutant receptors compared with WT P2X4Rs (Fig. 6E) |τoff for 331A, 485 ± 52 ms (n = 10); 336A, 454 ± 65 ms (n = 10); and WT, 161 ± 14 ms (n = 14)]. Ethanol did not change the kinetic properties of activation and deactivation of the mutants compared with WT receptors (Fig. 6, D and E). In agreement with our findings in oocytes (Popova et al., 2010), current results support the hypothesis that positions 331 and 336 are important for the action of ethanol in P2X4R. Moreover, our new findings suggest that the amino acid residues at positions 331 and 336 play a role in channel functioning.

We continued this line of investigation to determine whether mutations at positions 331 and 336 and ethanol have an effect on channel desensitization, i.e., channel closing occurring during prolonged exposure to agonist. ATP (3 μM) was applied for 15 s, and decaying currents fit monoexponentially in the presence and absence of ethanol. As shown, there was not a significant difference in the rate of desensitization between WT and 331A or 336A mutant P2X4Rs |τdes 2586 ± 246% (n = 7), 2992 ± 569% (n = 6), and 1810 ± 177% (n = 6), respectively (Fig. 7B)]. This finding suggests that these mutations do not prevent the channel from proceeding normally into the desensitized state (occurring in the continuous presence of agonist). That is, substitution of alanine at either position 331 or 336 did not seem to alter the mechanism of channel desensitization compared with WT P2X4Rs. As expected, we found that the residual deactivation rate occurring after ATP removal still did proceed at a slower rate (Fig. 7A, arrows). Inclusion of ethanol in the experimental paradigm did not significantly alter channel desensitization (Fig. 7A). This latter finding suggests that ethanol does not inhibit P2X4R by increasing channel desensitization.

Fig. 6. P2X4R mutants D331A and M336A reveal altered gating and decreased ethanol effect. A, representative traces showing ethanol effects on 3 μM ATP-activated currents. B, concentration-response curves for mutants: D331A, EC50 = 3.0 μM, Hill coefficient = 1.08; and M336A, EC50 = 5.5 μM, Hill coefficient = 0.86. C, bar graph showing the effect of ethanol on mutant P2X4R current amplitude. For D331A the residual current was 90.9 ± 1.9% (n = 5) at 1 μM ATP and 90.4 ± 3.1% (n = 9) at 3 μM ATP. For M336A the residual current was 98.1 ± 3.6% (n = 6) and 86.5 ± 1.9% (n = 12) at 1 and 3 μM ATP, respectively. P < 0.05 compared with ethanol effects in the WT P2X4R ANOVA. D and E, bar graphs showing activation (D) and deactivation (E) kinetics of WT and mutant P2X4R currents in the absence and presence of ethanol. P < 0.01 for τoff of both mutants versus WT (ANOVA, Dunnett’s post-test).

Fig. 7. Ethanol does not change the rate of desensitization of P2X4Rs. A, P2X currents normalized to peak value in the presence and absence of 100 mM ethanol upon 15-s ATP application in WT (left) and D331A mutant (right) P2X4Rs. Arrows mark the course of slow deactivation. B, bar graph of desensitization rates of WT, D331A, and M336A in the absence and presence of ethanol. For WT, D331A, and M336A τdes was 2586 ± 246% (n = 7), 2992 ± 569% (n = 6), and 1810 ± 177% (n = 6), respectively (P > 0.05, ANOVA, Dunnett’s post-test).
mM) significantly reduced ATP-gated currents [residual current was 71.3 ± 5.4% (n = 4)]. A prominent rebound to 94.6 ± 5.6% of control was apparent after ethanol removal. Overall, the findings in E18 neurons transduced with P2X4Rs resulted in a similar degree and pattern of ethanol inhibition compared with those of transfected HEK293 cells.

Discussion

The current study represents the first comprehensive investigation focusing on the mechanism of ethanol action on P2X4Rs expressed/overexpressed in a mammalian cell system using whole-cell patch-clamp analysis. It is noteworthy that findings from the current investigation and previous studies demonstrate a similar degree of ethanol inhibition in both oocyte and mammalian expression systems (Xiong et al., 1999, 2000, 2005; Davies et al., 2002, 2005; Popova et al., 2010). Our data also support the previous evidence that ethanol directly interacts with P2XRs (Li et al., 1994). Furthermore, this interaction occurs at concentrations similar to those that modulate other ligand-gated ion channels that are known targets of alcohol action (Yu et al., 1996; Mihic et al., 1997; Peoples et al., 1997).

On the other hand, it does not seem that the action of ethanol can be explained by changes in membrane fluidity. Evidence has been presented over the past 30 years that the effects of alcohols on membrane lipid order are unable to account for ion channel modulation (e.g., equivalent to a <1°C change in temperature; reviewed in Franks and Lieb, 1994), and a number of studies support a direct interaction of alcohols with ion channels. For example, alcohol "cutoffs" for P2X or NMDA receptor modulation differ markedly (Li et al., 1994; Peoples and Weight, 1995; Asatryan et al., 2010). Moreover, mutations in alcohol-sensitive sites of GABA<sub>A</sub>, glycine, NMDA, nicotinic acetylcholine, and P2XRs alter ethanol sensitivity in a manner that is inconsistent with lipid disordering (e.g., Mihic et al., 1997; Ren et al., 2003; Popova et al., 2010). Finally, sulfhydryl-containing alcohol analogs directly bind to, and persistently modify, putative sites of action on GABA<sub>A</sub> and glycine receptors (e.g., Mascia et al., 2000; Crawford et al., 2007).

Taken together, the findings suggest that the mechanism of ethanol effect on P2X4Rs is similar in different mammalian systems in vitro and may be relevant in vivo. Taken together, results from the current investigation provide new insights into the biophysical properties of P2X4Rs and mechanism of ethanol action on these receptors.

Ethanol Is a Rapid Inhibitor of P2X4 Channel in Open State. We explored different possibilities that could explain inhibitory ethanol action on P2X4Rs: 1) decrease in affinity or efficacy of agonist, i.e., ATP binding or channel gating; 2) stabilization of desensitized state; 3) change in ion permeability; and 4) open-channel block.

An interesting first outcome of the present study was that the magnitude of ethanol inhibition did not depend on the concentration of ATP tested being similar across a broad range of ATP concentrations (1–100 μM). In contrast, in previous investigations using oocytes and ATP-sensitive neurons, the action of ethanol was prominent at low ATP concentrations and insignificant at high saturating concentrations of ATP (Xiong et al., 1999, 2000, 2005; Davies et al., 2002, 2005; Popova et al., 2010). This scenario contradicts earlier suggestions that the mechanism of ethanol action is a result of an allosteric decrease in ATP affinity, i.e., that interaction with ethanol changes the conformation of the receptor, reshaping the ATP binding site (Li et al., 1998). Another fact that speaks against the mechanism of a decrease in ATP affinity is that, in this case, the channel deactivation would be accelerated. However, in our experiments τ<sub>off</sub> (as well as τ<sub>on</sub>) did not change under ethanol exposure (Fig. 4).

Next, the experiments with different application protocols confirmed the idea of a rapid rate of ethanol association/dissociation with its interaction locus. The speed of onset and offset of ethanol effect was comparable with the limits of our perfusion system, i.e., in the millisecond range.

Different offset times of ethanol inhibition at high (100 μM) and low (3 μM) ATP concentrations may suggest that action of ethanol is use-dependent. This serves as evidence that ethanol shares some properties of an open-channel blocker. On the other hand, ethanol apparently did not stabilize the desensitized state (Fig. 7) and did not change the ion permeability ratio (Fig. 5). Thus, it is plausible to suggest that the open state of the P2X4 channel plays the predominant role for ethanol effects, whereas resting and desensitized states do not support ethanol interaction with the channel.

Such a mode of action is expected from a low-affinity modulator with easy access to an interaction locus, possibly through the ion channel pore or the lipid bilayer. We would like to use the data from our rapid ethanol applications to patch-clamped preparations as a means to discriminate between pathways for ethanol entry into P2X4 binding sites from the ion pore versus the lipid bilayer. However, even though we have achieved very rapid kinetics, the equilibration of a small molecule, such as ethanol, with a lipid bilayer is likely to be even faster, in a microsecond range (Trudell and Hubbell, 1976; Xu et al., 1995; Cheng et al., 2008). Indeed, the consensus is that there are multiple pathways for entry of ethanol into binding sites in P2X4R and that what we refer to as “binding” is actually a series of rapid bounces of individual ethanol molecules on and off of putative binding sites or “interaction pockets.”

D331 and M336 Residues Participate in P2X4 Channel Gating and Ethanol Blocking Effect. The current study provided new information regarding changes in recep-
tor function caused by mutations that resulted in a decrease of ethanol effectiveness on P2X4Rs expressed in oocytes (Popova et al., 2010). In agreement with the oocyte data, the mutations did not significantly alter basic receptor function, namely, current amplitudes and dose-response relationship characteristics. On the other hand, both M336A and D331A mutants revealed significantly slower deactivation course. The significant change in gating properties of the mutants along with presumed location of the residues 331 and 336 at the ectodomain-TM2 domain interface suggests that these sites may be located near the P2X4R gate. This notion is strongly supported by crystallographic investigations and modeling data (Kawate et al., 2005; Asatryan et al., 2010). Aligning the sequence of zebrafish P2X4 with rat P2X4 suggests that rat M336 (zebrafish L339-L340) is a welcome door of the gate in the rat orthologue with its large hydrophobic side chain capable of occluding the pore and thus serving a putative function of L340 in zebrafish P2X4. Residue D331 (zN334) may serve a similar function of regulating ion flow at the entrance of a channel pathway. Structural data suggest there is an “extracellular vestibule” at the ectodomain-TM2 interface that may concentrate and direct ions into the pore. It is noteworthy that a similar mechanism was proposed for acid-sensing ion channels (Jasti et al., 2007). Residue D331 is apparently located at the very beginning of the TM2 domain, thus it is indeed positioned at the ectodomain-TM2 interface, whereas M336 is rather positioned at the TM2 vestibule-gate interface.

Structural and electrophysiology data could be interpreted as evidence of participation of mutated residues in channel gating or as a consequence of significant difference in sensitivity to agonist. In the case of mutations it is often not possible to distinguish between the changes of affinity (i.e., binding) and efficacy (i.e., gating) without single-channel recordings (Colquhoun, 1998). A change in deactivation would generally indicate a change in agonist binding versus gating, although both entities are intrinsically interdependent (Li et al., 1997a,b; Weight et al., 1999). However, altered affinity could also be secondary to changes in gating based on the facts that 1) D331 and M336 are not conserved residues that would be expected for the ATP binding site; 2) dose-response curves normalized to the peak current do not show a dramatic change in EC\textsubscript{50} although they are slightly shifted to the left; and 3) the presumed position of these residues in the channel pore, as based on modeling data, is more consistent with the regulation of gating.

Based on the findings from the current investigation, it is plausible to propose that ethanol affects P2X4 channel functioning through interaction with residues 331 and 336. Our modeling data also show that it is possible for these residues to be part of the same putative alcohol interaction pocket (Asatryan et al., 2010). However, we cannot distinguish between the possibilities of ethanol direct interaction with D331/M336 residues and ethanol interaction with separate loci, which change channel conformation upon mutations in these positions and render the channel invulnerable to ethanol.

**Multiple Interaction Sites of Ethanol in P2XRs.** Collectively, our data hint that ethanol may be acting as an open-channel blocker for P2X4Rs. Additional support for this hypothesis comes from evidence that EtOH action is highly impaired by mutations at positions 331 and 336, which apparently constitute an important part of the channel pore. We cannot resolve the question of mechanism completely without single-channel experiments that lie beyond the scope of the present work; however, comparison with the well-investigated ethanol action on NMDA receptors also supports the hypothesis of open-channel block. Similar to our findings, rapid ethanol action on NMDA receptors was characterized by a noncompetitive type of concentration-response curve and lack of effect on deactivation and desensitization (Weight et al., 1991; Lovinger, 1995; Peoples et al., 1997; Wirkrner et al., 2000). However, the effect of ethanol on peak and plateau phases of the current as well as during different application protocols was not in agreement with open-channel block and this was confirmed by single-channel experiments (Weight et al., 1996).

There is also building evidence that amino acids in the extracellular domain may differentially regulate action of ethanol, suggesting that ethanol may also associate with pockets within the extracellular loop (Xiong et al., 2005; Yi et al., 2009). Each of the P2X subunits and assembled channels possess a unique structure of both an extracellular loop and a pore with the help of nonconserved residues. This structural tuning leads to unique gating properties. Thus, it is reasonable to assume that ethanol may have at least two interaction sites or regions in P2XRs. One would be located in the extracellular loop and implicate the allosteric nature of ethanol’s effect on the ATP binding site that is also located in the ectodomain. A second site could be located close to a gating region. Superposition of native differences in structure of loci and affinity of ethanol for these loci may explain the difference in apparent mechanism of ethanol action on different subunits, their orthologues, and native P2X channels.

**Acknowledgments**

We thank Miriam Fine for conducting the molecular biology work and Jim Trudell for helpful scientific discussions.

**Authorship Contributions**

**Participated in research design:** Ostrovskaya, Asatryan, Alkana, and Davies.

**Conducted experiments:** Ostrovskaya, Asatryan, Wyatt, and Li.

**Performed data analysis:** Ostrovskaya.

**Wrote or contributed to the writing of the manuscript:** Ostrovskaya, Asatryan, Wyatt, Popova, Li, Alkana, Peoples, and Davies.

**Other:** Asatryan, Wyatt, Popova, Alkana, and Davies acquired funding for the research.

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