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Conserved extracellular cysteines differentially regulate the inhibitory effect of ethanol in rat $P2X_4$ receptors

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ABSTRACT

Relatively little information is available about the molecular mechanism of ethanol inhibition of P2X receptors. Here, we investigated the possibility that 10 conserved cysteine residues in the extracellular loop of the rat $P2X_4$ receptor may regulate ethanol inhibition of the receptor using a series of individual cysteine to alanine point mutations. Each of the mutated receptors generated robust inward current in response to ATP and the mutations produced less than a sixfold change in the ATP EC_{50} value. For the C116A, C126A, C149A, and C165A mutants, 100 mM ethanol did not significantly affect the current activated by an EC_{40} concentration of ATP. By contrast, for the C261A and C270A mutants, ethanol inhibited ATP-activated current in a competitive manner similar to that for the wild-type receptor. Interestingly, for the C132A, C159A, C217A, and C227A mutants, ethanol inhibited ATP-activated current, but decreased the maximal response to ATP by 70–75% without significantly changing the EC_{50} value of ATP, thus exhibiting a noncompetitive-type inhibition. The results suggest that cysteines and disulfide bonds between cysteines are differentially involved in the inhibition of the rat $P2X_4$ receptor by ethanol.

P2X receptors are ligand-gated ion channels that are activated by extracellular ATP, and a growing body of evidence suggests that P2X receptors have important functions in the central and peripheral nervous systems. P2X receptors are widely distributed in the central nervous system, including cerebral cortex, hippocampus, thalamus, hypothalamus, midbrain, cerebellum, and spinal cord, and in sensory and autonomic ganglia in the peripheral nervous system [1–3]. Activation of postsynaptic P2X receptors elicits excitatory postsynaptic potentials in both central and peripheral neurons [2,3], and activation of presynaptic P2X receptors enhances release of neurotransmitters, including glutamate, γ-aminobutyric acid, noradrenaline, and acetylcholine [2,3]. To date, seven P2X receptor subunits, designated $P2X_1$ to $P2X_7$, have been cloned and these subunits have been found to be widely distributed in the nervous system [1–3]. Among the seven cloned P2X receptors, the $P2X_4$ subunit has been found to be the most abundant P2X receptor expressed in brain [1–3].

Previous studies have revealed that pharmacological concentrations of ethanol can differentially regulate the function of P2X receptors. For instance, ethanol inhibits ATP-activated current in bullfrog dorsal root ganglion neurons [4–6], freshly isolated adult rat hippocampal CA1 neurons [7], and rat recombinant $P2X_2$ [8] and $P2X_4$ receptors [8–11], and potentiates ATP-activated current mediated by recombinant rat $P2X_3$ receptors [10,12]. Only a few recent investigations have begun to explore the molecular basis for the differential regulation of P2X receptor function by ethanol. It has been found that in the rat $P2X_4$ receptor, mutations of histidines 140 and 286 do not significantly alter ethanol inhibition of ATP-activated current, but mutation of 241 alters the mechanism by which ethanol inhibits $P2X_4$ receptor function: from apparently competitive to noncompetitive [11]. In addition, a recent study using chimeric strategies found that ectodomain segments at TM interfaces played key roles in determining qualitative and quantitative responses to ethanol of $P2X_2$ and $P2X_3$ receptors [12]. However, more information is required to better understand how the molecular structure of P2X receptors determines the effect of ethanol on these receptor-channels. In the present study, we investigated whether conserved cysteine residues in the extracellular loop of rat $P2X_4$ receptors are involved in the regulation of ethanol on these receptors.

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Materials and methods

DNA site-directed mutagenesis. The rat $P2X_4$ receptor cDNA clone in the vector pcDNA3 was kindly provided by Dr. Gary Buell (Serono Pharmaceutical Research Institute, Geneva, Switzerland). Site-directed mutagenesis of C116, C126, C132, C149, C159, C165, C217, C227, C261, and C270 in the rat $P2X_4$ cDNA was performed using the Quikchange kit (Stratagene, Inc., La Jolla, CA, USA). Sixteen cycles of amplification by polymerase chain reaction catalyzed by Pfu DNA polymerase were performed with the following temperature protocol: strand separation 98 °C, primer annealing 50 °C and primer extension 68 °C for 20 min. Next the parental template was digested with DpnI, the mutated $P2X_4$ receptor construct transformed into competent host cells, and the transformed cells plated on ampicillin-containing agar plates. Individual clones were grown in Luria–Bertani medium; DNA was isolated and then sequenced to verify each mutation. Each mutant is referred to by the original amino acid (one letter code) followed by the residue number and the substituted amino acid (one letter code).

Expression of receptors in Xenopus oocytes and two-electrode voltage-clamp recording. The preparation of cRNA, expression of receptors in *Xenopus* oocytes and two-electrode voltage-clamp recording from *Xenopus* oocytes were performed as described previously [9,11]. Oocytes were placed in a recording chamber and impaled with two sharp microelectrodes filled with 3 M KCl (tip resistances: 0.5–1.5 MΩ). The oocytes were constantly superfused at the rate of ~2.5 ml/min with modified Ringer solution containing (in mM): 96 NaCl, 2 KCl, 1.8 $BaCl_2$, 1 $MgCl_2$, 5 Hepes (pH 7.4; Ba^{2+} replaced Ca^{2+} to prevent the activation of the endogenous calcium-activated chloride current in these cells). Agonist and other chemical solutions were prepared in the bathing solution. Solutions of ATP, added as the Na^+ salt, were prepared daily. Oocytes were recorded 2–5 day after RNA injection at room temperature using a Geneclamp Amplifier (Axon Instruments Inc., Foster City, CA, USA), and voltage-clamped at −70 mV.

Drugs and chemicals. The drugs and chemicals used in these experiments were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO).

Data analysis. Average values are expressed as means ± SEM, with *n* equal to the number of cells studied. Statistical significance of results was assessed using analysis of variance (ANOVA) followed by the Dunnett's test, as noted. Statistical analysis of concentration–response data was performed using the nonlinear curve-fitting program ALLFIT [13], which uses an ANOVA procedure. Values reported for concentration–response analysis are those obtained by fitting the data to the equation:

$$Y = E_{max}/[1 + (EC_{50}/X)_n$$

where *X* and *Y* are concentration and response, respectively, E_{max} is the maximal response, EC_{50} is the concentration yielding 50% of maximal effect (EC_{50} for activation, IC_{50} for inhibition), and n is the slope factor.

Table 1
Summary of data for wild-type and cysteine-mutated rat $P2X_4$ receptors.

Mutant	ATP EC_{50} (μM)	ATP E_{max}[a] (μA)	ATP slope factor
WT	8.2 ± 0.7	2.5 ± 0.2	1.18 ± 0.22
C116A	22.8 ± 1.2*	1.63 ± 0.1**	1.11 ± 0.12
C126A	28.5 ± 1.3*	1.87 ± 0.1*	1.28 ± 0.37
C132A	11.1 ± 0.9	1.9 ± 0.1*	1.27 ± 0.1
C149A	19.6 ± 0.9*	1.61 ± 0.1**	1.31 ± 0.4
C159A	10.2 ± 0.8	1.68 ± 0.1**	1.3 ± 0.15
C165A	47.2 ± 2.2**	1.53 ± 0.1**	1.2 ± 0.17
C217A	16.5 ± 1.1*	1.72 ± 0.1**	1.1 ± 0.1
C227A	22.5 ± 1.4*	1.51 ± 0.1**	1.2 ± 0.1
C261A	8.8 ± 0.6	1.42 ± 0.1**	1.0 ± 0.1
C270A	10.4 ± 0.5	1.3 ± 0.1**	1.26 ± 0.1

Values are expressed as mean peak current ± SEM (n = 6–10 oocytes). Significant differences from WT values are indicated as $^{**}p < 0.01$ and $^{*}p < 0.05$.

[a] ATP E_{max} corresponds to the current activated by 500 μM ATP.

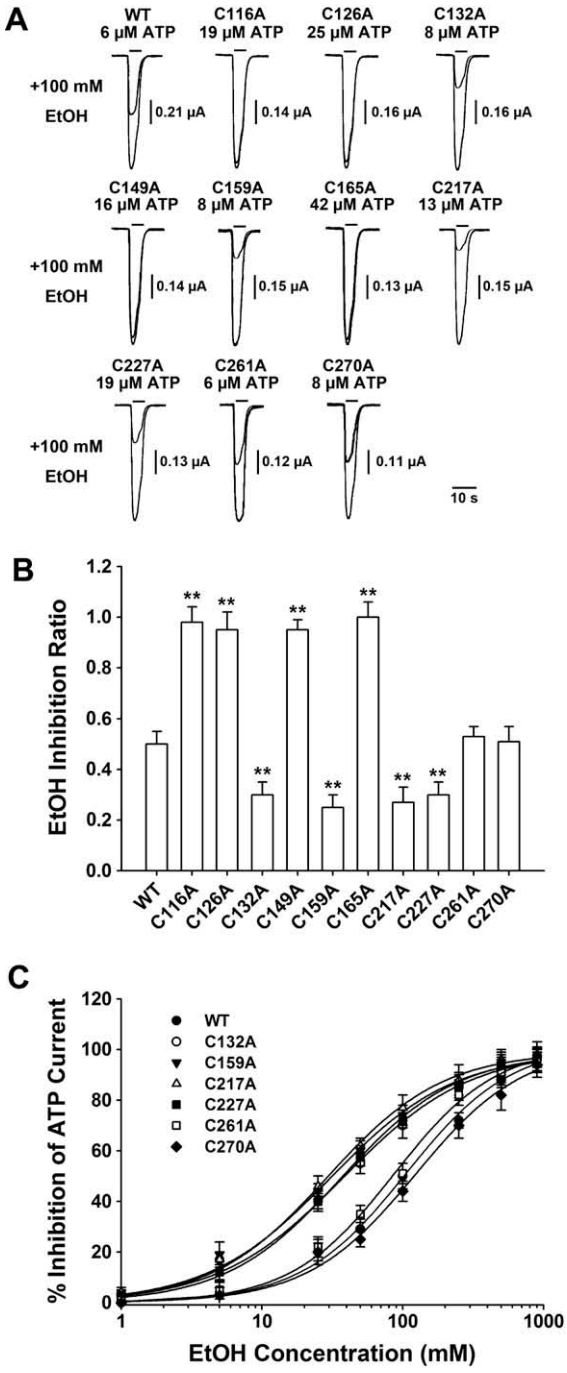

Fig. 1. Effect of cysteine mutation on ethanol inhibition of ATP-activated current in rat $P2X_4$ receptors. (A) Records showing currents activated by 6, 19, 25, 8, 16, 8, 42, 13, 19, 6, and 8 μM ATP in WT, C116A, C126A, C132A, C149A, C159A, C165A, C217A, C227A, C261A, and C270A rat $P2X_4$ receptors in the absence and in the presence of 100 mM EtOH. (B) Graph plotting mean EtOH inhibition ratio of 6–9 oocytes per construct. The EtOH inhibition ratio is the ratio of the current amplitude in the presence of ATP and 100 mM EtOH to the current amplitude of ATP alone. **Values differed significantly from WT (ANOVA and Dunnett's test; $p < 0.01$). (C) Graph plotting average percentage inhibition of ATP-activated current as a function of EtOH concentration for the mutants indicated. Each data point is the average of 6–10 cells; error bars not visible are smaller than the size of the symbols. In (B,C), as ATP sensitivity was different in WT and mutated receptors, an ATP concentration that was close to the EC_{40} value of each ATP concentration–response curve was used for each mutant which is indicated in (A).

Results

Effect of cysteine mutations on the properties of rat P2X$_4$ receptors

Similar to previous reports [9,11], extracellular ATP evoked a concentration-dependent inward current in oocytes injected with wild-type (WT) rat P2X$_4$ receptor cRNA with an EC_{50} value of 8.2 ± 0.7 μM (Table 1). The functional role of the 10 conserved cysteine residues in the extracellular loop was investigated using a series of individual cysteine to alanine point mutations. Like the WT receptor, each of the 10 mutated receptors generated robust inward current in response to ATP. ATP, at concentrations up to 500 μM, did not activate detectable current in uninjected *Xenopus* oocytes ($n = 6$, data not shown). Substitution at positions C132, C159, C261, and C270 had no effect on ATP potency (Table 1). For mutants C116, C126, C149, C217, and C227 there was a less than fourfold decrease in potency ($p < 0.05$), whereas a larger decrease (sixfold, $p < 0.01$) in potency was recorded for C165 (Table 1). The peak current amplitude evoked by a maximal concentration of ATP was significantly decreased by 24–48% in all of the mutants (Table 1). Cysteine to alanine substitutions did not significantly alter the ATP slope factor in any of the mutated receptors (Table 1).

Effect of cysteine mutations on the regulation of rat P2X$_4$ receptors by ethanol

To investigate whether cysteine residues in the extracellular loop might be involved in the regulation of P2X$_4$ receptor function by ethanol, effects of ethanol on the cysteine to alanine mutants were examined. Because ATP sensitivity was different in WT and mutated receptors and ethanol inhibition of ATP-activated current depends on ATP concentration [8,9,11], an ATP concentration that was close to the EC_{40} value of the ATP concentration–response curve was used in these experiments for the WT and each mutated receptor; the EC_{40} values were 6, 19, 25, 8, 16, 8, 42, 13, 19, 6, and 8 μM for WT, C116A, C126A, C132A, C149A, C159A, C165A, C217A, C227A, C261A, and C270A receptors, respectively. Similar to the WT receptor, 100 mM ethanol markedly inhibited ATP-activated current in C132A, C159A, C217A, C227A, C261A, and C270A receptors (Fig. 1A and B). By contrast, 100 mM ethanol did not produce any significant effects in C116A, C126A, C149A, and C165A receptors (Fig. 1A and B).

To study the inhibitory effect of ethanol in mutated rat P2X$_4$ receptors, we constructed ethanol concentration–response curves for inhibition of ATP-activated current for WT, C132A, C159A, C217A, C227A, C261A, and C270A receptors (Fig. 1C). As shown in Fig. 1C and Table 2, for the C261A and C270A mutants, the calculated values of IC_{50} for ethanol inhibition of ATP-activated current, the slope factor and the E_{max} are not significantly different from those in the WT receptor (ANOVA, $p > 0.05$). However, for the C132A, C159A, C217A, and C227A mutants, the IC_{50} values for ethanol inhibition of ATP-activated current are significantly lower than that of the WT receptor (ANOVA, $p < 0.01$), whereas the slope factors and E_{max} values do not differ significantly from those of the WT receptor ($p > 0.05$). Over the concentration range 1 to 900 mM, the application of ethanol alone did not activate detectable current in *Xenopus* oocytes expressing WT and each of the six mutated rat P2X$_4$ receptors ($n = 6$ for WT and each mutant, data not shown).

To investigate further the effect of cysteine mutation on ethanol inhibition of rat P2X$_4$ receptors, we examined whether the concentration of ATP affects ethanol inhibition in C132A, C159A, C217A, C227A, C261A, and C270A receptors. As shown in Fig. 2 and Table 2, 100 mM ethanol reduced the E_{max} value of the ATP concentration-response curve by 70–75%, without significantly changing the slope (ANOVA, $p > 0.05$) or the EC_{50} value of ATP concentration–response curve ($p > 0.05$) in C132A, C159A, C217A, and C227A receptors. By contrast, for the C261A and C270A mutants, like the WT receptor, 100 mM ethanol shifted the ATP concentration–response curve to the right, significantly increasing the EC_{50} value for the ATP concentration–response curve ($p < 0.01$), without significantly changing the slope ($p > 0.05$) or the E_{max} value of the ATP concentration-response curve ($p > 0.05$).

Discussion

Although previous studies have revealed that pharmacological concentrations of ethanol can differentially regulate the function of P2X receptors [8,10–12], the molecular basis for ethanol action on P2X receptors is poorly understood. A previous study reported that the mutation of histidine 241 in the rat P2X$_4$ receptor altered the mechanism of ethanol inhibition from apparently competitive to noncompetitive [11]. In addition, a recent study using chimeric strategies found that ectodomain segments at TM interfaces played key roles in determining qualitative and quantitative responses to ethanol of P2X$_2$ and P2X$_3$ receptors [12]. In the present study, we investigated the possible role of cysteine residues in the effect of ethanol on the rat P2X$_4$ receptor function by using a series of individual cysteine to alanine point mutations.

One feature of the family of P2X receptors is that the extracellular domain contains 10 cysteines conserved in all cloned P2X receptors. Previous studies suggest that these conserved cysteine residues play an important role in P2X receptor function. For instance, cysteine residues in the extracellular loop form disulfide bonds in human P2X$_1$ [14] and rat P2X$_2$ [15] receptors, and are involved in receptor trafficking to the cell surface in human P2X$_1$ receptors [14] and in zinc and proton modulation in rat P2X$_2$ receptors [15]. In the present study, each of the 10 mutated receptors generated robust inward current in response to ATP and the cysteine to alanine mutation produced less than a sixfold change in the EC_{50} value of the ATP concentration–response curve. These results suggest that individual cysteine residues and the disulfide bonds they form are not essential for the production of functional

Table 2
Summary of data on the effect of cysteine mutation on ethanol inhibition of ATP-activated current in rat P2X$_4$ receptors.

Parameter	WT	C132A	C159A	C217A	C227A	C261A	C270A
EtOH IC_{50} (mM)[a]	104.3 ± 7.1	38.2 ± 3.1**	30 ± 2.2**	29.2 ± 1.6**	36.6 ± 1.7**	93 ± 6.7	111 ± 12
EtOH slope factor	1.1 ± 0.1	0.9 ± 0.2	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.2 ± 0.2	1.1 ± 0.1
EtOH E_{max}	98 ± 2%	99 ± 3%	99 ± 2%	98 ± 2%	97 ± 5%	99 ± 3%	96 ± 6%
ATP EC_{50} with EtOH (μM)[b]	2.5 ± 2$^{\#\#}$	11.4 ± 1	9.5 ± 0.7	15.6 ± 1.2	21 ± 1.3	17 ± 1.6%$^{\#\#}$	18 ± 1.7%$^{\#\#}$
ATP slope factor with EtOH[b]	0.98 ± 0.2	1.1 ± 0.1	1.0 ± 0.1	1.2 ± 0.2	1.3 ± 0.3	1.1 ± 0.2%	1.1 ± 0.3
ATP E_{max} ratio with EtOH[c]	1.0 ± 0.02	0.3 ± 0.04**	0.25 ± 0.02**	0.27 ± 0.03**	0.3 ± 0.05**	0.98 ± 0.04	0.95 ± 0.05

$^{**}p < 0.01$ vs WT and $^{\#\#}p < 0.01$ vs ATP EC_{50} without EtOH in the same receptor (see Table 1). Values are expressed as mean peak current ± SEM ($n = 6$–10 oocytes).
[a] An ATP concentration that was close to the EC_{40} value of each ATP concentration–response curve was used for the WT and each mutant receptor.
[b] 100 mM EtOH was used.
[c] The ratio of the current amplitude in the presence of 500 μM ATP and 100 mM EtOH to the current amplitude of ATP alone.

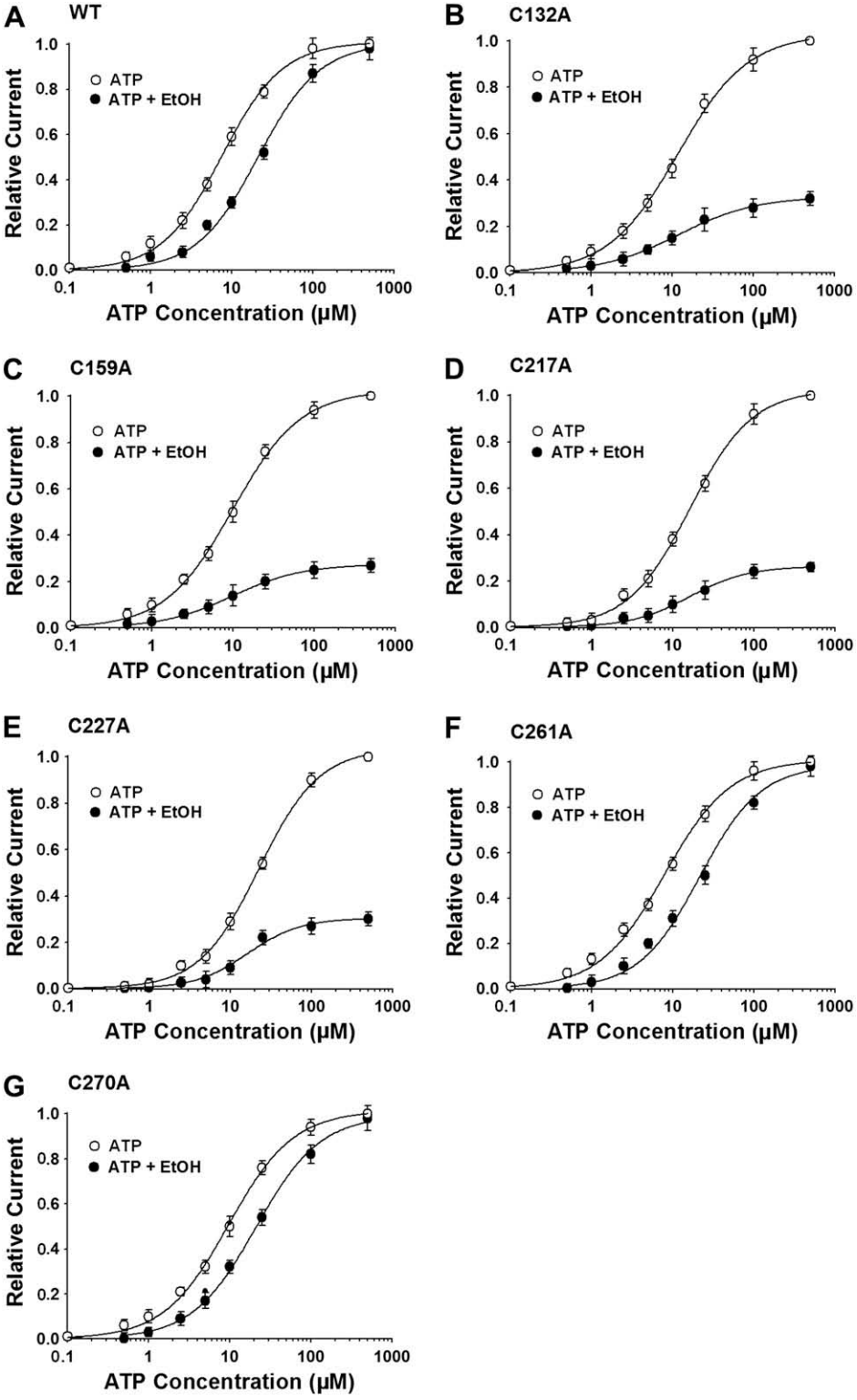

Fig. 2. Effect of ATP concentration on ethanol inhibition of ATP-activated current in the cysteine-mutated rat $P2X_4$ receptors. (A–G) Graphs plotting the amplitude of ATP-activated current in the presence of 100 mM EtOH relative to the amplitude of ATP-activated current in the absence of EtOH, as a function of ATP concentration in oocytes expressing WT, C132A, C159A, C217A, C227A, C261A, and C270A receptors. Each data point is the average current from 6–10 cells; error bars not visible are smaller than the size of the symbols.

rat $P2X_4$ receptors. However, the present study also indicates that the cysteine residues in the extracellular loop of the rat $P2X_4$ receptor play different roles in the inhibition of this receptor by ethanol. For the C116A, C126A, C149A, and C165A mutants, unlike the WT receptor, 100 mM ethanol did not significantly affect the current activated by an EC_{40} concentration of ATP, suggesting that these four cysteine residues are essential for ethanol to produce its inhibition in rat $P2X_4$ receptors. By contrast, for C261A and C270A mutants, ethanol inhibited ATP-activated current with an IC_{50} value similar to that for the WT receptor. In addition, 100 mM

ethanol shifted the ATP concentration-response curve to the right in a parallel manner for both the C261A and C270A mutants and the magnitudes of those shifts were similar to that of the WT receptor. Thus, these results suggest that mutation of cysteine 261 to alanine or cysteine 270 to alanine did not significantly alter the ethanol sensitivity of the rat $P2X_4$ receptor and the underlying mechanism by which ethanol inhibits receptor function. Interestingly, for the C132A, C159A, C217A, and C227A mutants, like the WT receptor, ethanol inhibited ATP-activated current, but decreased the maximal response to ATP by 70–75% without significantly changing the EC_{50} value of the concentration–response curve, thus exhibiting a noncompetitive-type inhibition. The results suggest that cysteines 132, 159, 217, and 227 are involved in determining the mechanism by which ethanol inhibits the $P2X_4$ receptor function.

In summary, previous studies have suggested that all 10 cysteine residues in the extracellular loop of P2X receptors are disulfide-bonded [14,15], and these disulfide bonds are involved in receptor trafficking [14] and receptor regulation by zinc and protons [15]. In the present study, we found that among 10 cysteine residues of the rat $P2X_4$ receptor only cysteine 261 and 270 mutations did not significantly affect ethanol inhibition, suggesting that C261–C270 normally forms a disulfide bond which is not crucial in determining the sensitivity and mechanism of ethanol inhibition. Although the results of the present study do not provide a sufficient basis for us to propose precise disulfide bond pairings in the rest of eight cysteines, two disulfide bonds could be formed among C116, C126, C149, and C165 and these disulfide bonds are essential for ethanol to produce its inhibition in rat P2X4 receptors. An additional two disulfide bonds could be formed among C132, C159, C217, and C227 and these disulfide bonds are apparently involved in determining the mechanism of ethanol inhibition in a manner similar to that of a previous report in which mutation of histidine 241 to alanine in the rat $P2X_4$ receptor changed the mechanism of ethanol inhibition from apparently competitive to noncompetitive [11]. Nevertheless, the results presented in the present study indicate that cysteines and disulfide bonds between cysteines are differentially involved in the inhibition of the rat $P2X_4$ receptor by ethanol.

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References

[1] G. Collo, R.A. North, E. Kawashima, E. Merlo-Pich, S. Neidhart, A. Surprenant, G. Buell, Cloning of $P2X_5$ and $P2X_6$ receptors and the distribution and properties of an extended family of ATP-gated ion channels, J. Neurosci. 16 (1996) 2495–2507.
[2] B.S. Khakh, R.A. North, P2X receptors as cell-surface ATP sensors in health and disease, Nature 442 (2006) 527–532.
[3] L. Koles, S. Furst, P. Illes, Purine ionotropic (P2X) receptors, Curr. Pharm. Des. 13 (2007) 2368–2384.
[4] C. Li, L. Aguayo, R.W. Peoples, F.F. Weight, Ethanol inhibits a neuronal ATP-gated ion channel, Mol. Pharmacol. 44 (1993) 871–875.
[5] C. Li, R.W. Peoples, F.F. Weight, Alcohol action on a neuronal membrane receptor: evidence for a direct interaction with the receptor protein, Proc. Natl. Acad. Sci. USA 91 (1994) 8200–8204.
[6] C. Li, R.W. Peoples, F.F. Weight, Ethanol-induced inhibition of a neuronal P2X purinoceptor by an allosteric mechanism, Br. J. Pharmacol. 123 (1998) 1–3.
[7] C. Li, K. Xiong, F.F. Weight, Ethanol inhibition of adenosine 5′-triphosphate-activated current in freshly isolated adult rat hippocampal CA1 neurons, Neurosci. Lett. 295 (2000) 77–80.
[8] D.L. Davies, T.K. Machu, Y. Guo, R.L. Alkana, Ethanol sensitivity in ATP-gated P2X receptors is subunit dependent, Alcohol. Clin. Exp. Res. 26 (2002) 773–778.
[9] K. Xiong, C. Li, F.F. Weight, Inhibition of ethanol of $P2X_4$ receptors expressed in *Xenopus* oocytes, Br. J. Pharmacol. 130 (2000) 1394–1398.
[10] D.L. Davies, A.A. Kochegarov, S.T. Kuo, A.A. Kulkarni, J.J. Woodward, B.F. King, R.L. Alkana, Ethanol differentially affects ATP-gated $P2X_3$ and $P2X_4$ receptor subtypes expressed in *Xenopus* oocytes, Neuropharmacology 49 (2005) 243–253.
[11] K. Xiong, X. Hu, R.R. Stewart, F.F. Weight, C. Li, The mechanism by which ethanol inhibits rat $P2X_4$ receptors is altered by mutation of histidine 241, Br. J. Pharmacol. 145 (2005) 576–586.
[12] L. Asatryan, M. Popova, J.J. Woodward, B.F. King, R.L. Alkana, D.L. Davies, Roles of ectodomain and transmembrane regions in ethanol and agonist action in purinergic P2X2 and P2X3 receptors, Neuropharmacology 55 (2008) 835–843.
[13] A. DeLean, P.J. Munson, D. Rodbard, Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves, Am. J. Physiol. 235 (1978) E97–E102.
[14] S.J. Ennion, R.J. Evans, Conserved cysteine residues in the extracellular loop of the human $P2X_1$ receptor form disulfide bonds and are involved in receptor trafficking to the cell surface, Mol. Pharmacol. 61 (2002) 303–311.
[15] J.D. Clyne, L.-F. Wang, R.I. Hume, Mutational analysis of the conserved cysteines of the rat $P2X_2$ purinoceptor, J. Neurosci. 22 (2002) 3873–3880.