British Journal of Pharmacology (1998) 125, 263–270
http://www.stockton-press.co.uk/bjp

Enhancement of glycine receptor function by ethanol: role of phosphorylation

[1,3]Maria Paola Mascia, [1,2]Marilee J. Wick, [1]Larry D. Martinez & [1,2]R. Adron Harris

[1]Department of Pharmacology, University of Colorado Health Sciences Center, Denver, Colorado 80262 and [2]Department of Veterans Affairs Medical Center, Denver, Colorado 80220, U.S.A.

1 The effects of several kinase inhibitors (staurosporine, GF 109203X, H89, KN62, genistein) and of the phosphatase inhibitor calyculin A were studied on the ethanol potentiation and on the function of homomeric α1 glycine receptor expressed in *Xenopus* oocytes using a two electrode voltage clamp recording technique.

2 The function of the homomeric α1 glycine receptor was not modified in *Xenopus* oocytes pretreated with kinase inhibitors or with the phosphatase inhibitor calyculin A.

3 The potentiation of the glycine receptor function induced by ethanol (10–200 mM) was significantly reduced in *Xenopus* oocytes pretreated with the PKC inhibitors staurosporine or GF 109203X.

4 No differences in propofol (2.5 μM) or halothane (250 μM) actions were found after exposure of *Xenopus* oocytes to staurosporine.

5 No differences in ethanol sensitivity were found after exposure of *Xenopus* oocytes expressing glycine α1 receptors to H89, KN62, genistein or to the phosphatase inhibitor calyculin A.

6 The mutant α1 (S391A), in which the PKC phosphorylation site at serine 391 was mutated to alanine, was less sensitive to the effects of ethanol than was the α1 wild type receptor. Moreover, the ethanol potentiation of the glycine receptor function was not affected by treatment with staurosporine in oocytes expressing α1 (S391A).

7 The splice variant of the α1 glycine receptor subunit, $\alpha1^{ins}$, containing eight additional amino acids and a potential phosphorylation site for PKA, did not differ from wild type for sensitivity to ethanol.

8 These results indicate that phosphorylation by PKC of the homomeric α1 glycine receptor subunit modulates ethanol potentiation, but not the function of the glycine receptor.

Keywords: Strychnine-sensitive glycine receptor; α1 subunit; *Xenopus* oocytes; ethanol; propofol; halothane; phosphorylation; protein kinase C

Introduction

The strychnine-sensitive glycine receptors are ligand-gated ion channel which mediate inhibitory neurotransmission in spinal cord, brain stem and several other regions of the central nervous system (Betz, 1991). Activation of the glycine receptors produce the opening of an integral chloride channel with subsequent membrane hyperpolarization. Most native glycine receptor appear to be composed of two different subunits, α and β, that associate in a pentameric stoichiometry of 3α and 2β (Betz, 1991). Four different α subunits isoforms (α1–4) have been cloned (Bechade *et al*., 1994; Matzenbach *et al*., 1994). Both α and β subunits of the glycine receptor, have a transmembrane topology comparable to that observed for other ligand-gated ion channels with four transmembrane domains and a large cytoplasmic region between transmembrane domain 3 (TM3) and transmembrane domain 4 (TM4) (Bechade *et al*., 1994).

Protein phosphorylation is a major mechanism for regulation of receptor function and synaptic transmission in the central nervous system. Several studies have demonstrated that ligand-gated ion channels, such as nicotinic-acetylcholine, glutamate and the $GABA_A$ receptors are phosphorylated by a large number of protein kinases (Swope *et al*., 1992). These protein kinases act at different phosphorylation sites, mainly located in the intracellular loop between TM3 and TM4 (Swope *et al*., 1992; Moss & Smart, 1996). Recent reports indicate that the glycine receptor is a target for phosphorylation by protein kinase A (PKA) and protein kinase C (PKC). These results may be summarized as follows: (1) Activation of PKA increased the glycine-induced Cl^- currents in cultured spinal trigeminal neurones (Song & Huang, 1990) and in *Xenopus* oocytes expressing mRNA from spinal cord (Vaello *et al*., 1994). Moreover, *in vitro* phosphorylation, by cyclic AMP dependent PKA, of the α subunit of the glycine receptor has been reported (Vaello *et al*., 1994); (2) Activation of PKC by phorbol esters, positively modulates glycine receptor function in hippocampal neurones (Schonrock & Bormann, 1995), but inhibits glycine-induced Cl^- currents in *Xenopus* oocytes (Uchiyama *et al*., 1994; Vaello *et al*., 1994). However, a potentiation of the glycine currents in *Xenopus* oocytes was also observed when PKC was endogenously stimulated (Nishizaki & Ikeuchi, 1995); (3) The purified α1 subunit of the glycine receptor is specifically phosphorylated by PKC in a region corresponding to the intracellular loop between TM3 and TM4, with serine 391 being the phosphorylated residue (Ruiz-Gomez *et al*., 1994) and (4) A splicing variant of the α1 glycine receptor subunit, expressed in postnatal rat spinal cord and brain stem ($\alpha1^{ins}$), has been recently reported. This $\alpha1^{ins}$ glycine receptor subunit contains an insertion of eight

[1]Author for correspondence at: Department of Pharmacology, Campus Box c236, 4200 E. Ninth Ave., Denver, Colorado 80262, U.S.A

additional amino acids located in the cytoplasmic loop between TM3 and TM4. These eight additional amino acids might result in a potential consensus sequence for a phosphorylation site (Malosio *et al*., 1994)

Several laboratories have shown that ethanol enhances the function of the strychnine-sensitive glycine receptor (Celentano *et al*., 1988; Engblom *et al*., 1991; Aguayo & Pancetti, 1994). In particular, we demonstrated that ethanol, at pharmacological relevant concentrations, potentiated the function of homomeric $\alpha 1-2$ glycine receptors expressed in *Xenopus* oocytes (Mascia *et al*., 1996a,b). In addition, a recent study of transiently or stably transfected cells showed that the effect of ethanol on glycine receptors was less than observed in studies using *Xenopus* oocytes (Valenzuela *et al*., 1998). The interaction between ethanol and the strychnine-sensitive glycine receptor is supported by a recent *in vivo* investigation: intracerebroventricular administration of glycine, in mice, enhances the central depressant effects of ethanol measured as loss of righting reflex. This effect is blocked by the glycine receptor antagonist strychnine (Williams *et al*., 1995). Previous studies demonstrated that the ethanol modulation of the function of several receptors is regulated by phosphorylation. The inhibitory effect of ethanol on NMDA receptor is abolished by PKC inhibitors (Snell *et al*., 1994). Similarly, the ethanol inhibition of 5-HT_{2c} receptor is prevented by both the PKC inhibitor peptide and staurosporine (Sanna *et al*., 1994). Potentiation of the $GABA_A$ receptor function by low concentration of ethanol appears to require the presence of the γ_{2I} subunit in a phosphorylated state, although interpretation of these results is complicated by differences between expression systems (Wafford & Whiting, 1992; Harris *et al*., 1997). Since α glycine receptor subunits expressed in *Xenopus* oocytes assemble homomerically into functional glycine receptors with properties similar to those of the native receptors (Betz, 1991), we used the *Xenopus* oocytes expression system to examine the possible role of phosphorylation in ethanol modulation of the strychnine-sensitive glycine receptor. Specifically, we determined the effect of several kinase inhibitors: the nonspecific protein kinase inhibitor staurosporine (Huidobro-Toro *et al*., 1996), the PKC inhibitor GF109203X (Minami *et al*., 1998), the PKA inhibitor H89 (Waldegger *et al*., 1996), the CaM kinase inhibitor KN62 (Valenzuela *et al*., 1995b) and the tyrosine kinase inhibitor genistein (Valenzuela *et al*., 1995a) as well as the phosphatase inhibitor calyculin A (Huidobro-Toro *et al*., 1996) in *Xenopus* oocytes expressing homomeric $\alpha 1$ glycine receptors or the mutant $\alpha 1$ (S391A).

Methods

Construction of the $\alpha 1^{ins}$ glycine receptor subunit.

The glycine $\alpha 1^{ins}$ cDNA was prepared by insertion of a 24 bp (base pair) segment encoding for the amino acids (SerProMet LeuAsnLeuPheGln) into the glycine $\alpha 1$ cDNA at amino acid 353, using the Quik Change mutagenesis kit and complementary sense and antisense 69-mer oligonucleotides containing this 24-bp insertion. The sequence of the glycine $\alpha 1^{ins}$ cDNA was confirmed by double stranded DNA sequencing.

Microinjection into Xenopus *oocytes and electrophysiological recording*

Preparation of the oocytes and microinjection of cDNA were performed as described elsewhere (Lin *et al*., 1992). Stage V and VI *Xenopus laevis* oocytes were isolated and placed in MBS containing (mM): NaCl 88, KCl 1, HEPES 10, $MgSO_4$ 0.82, $NaHCO_3$ 2.4, $CaCl_2$ 0.91 and $Ca(NO_3)_2$ 0.33, adjusted to pH 7.5). Glycine receptor subunit cDNAs [wild type $\alpha 1$, mutant $\alpha 1$ (S391A), $\alpha 1^{ins}$, 0.4 ng/30 nl] were injected to the animal poles of the oocytes according to the blind method of Colman 1984). The injected oocytes were cultured at 15–19°C in sterile MBS supplemented with 10 mg l^{-1} of streptomycin, 10,000 units l^{-1} penicillin, 50 mg l^{-1} gentamicin, 90 mg l^{-1} and 220 mg l^{-1} pyruvate.

Oocytes were used for recording on days 1–4 after injection. Oocytes were placed in a chamber (~ 100 μl volume) and perfused (2 ml min^{-1}) with MBS with or without drugs *via* a roller pump (Cole-Parmer Instruments, Chicago, IL) through 18-gauge polyethylene tubing (Clay Adams Co., Parsippany, NJ, U.S.A.) that delivered the drug solutions to the recording chamber. The animal poles of the oocytes were impaled with two glass electrodes (0.5–10 MΩ) filled with 3 M KCl and voltage clamped at -50 mV using an Axoclamp 2A amplifier (Burlingame, CA, U.S.A.). Clamping currents were continuously plotted on a strip chart recorder. Glycine was dissolved in MBS and applied for 20 s. Oocytes were perfused with ethanol or halothane for 2 min, to allow for complete equilibration in the bath, before being coapplied with glycine for 20 s. A 5 min washout period was allowed between drugs applications. The solution of halothane was prepared immediately before use. The loss in concentration from vial to bath was approximately 50–60% (Dildy-Mayfield *et al*., 1996). Propofol was dissolved in DMSO, then diluted in MBS to a final DMSO concentration of 0.001%. The loss in concentration from vial to bath was 60% (Lin *et al*., 1992). Concentrations given in the figure represent the final bath concentrations. Staurosporine was dissolved in 10% DMSO then diluted in MBS to a final DMSO concentration of 0.03% DMSO. Oocytes were exposed to staurosporine for 4 h before recording. H89 and KN62 were dissolved in H_2O and microinjected into the oocyte. Genistein was dissolved in H_2O. Calyculin A was first dissolved in 10% DMSO to a 100 μM stock solution. Concentrations of microinjected drugs represent the intraoocyte final concentrations, calculated assuming an oocyte volume of 1 μl.

Materials

Adult female *Xenopus laevis* frogs were obtained from Xenopus I (Ann Arbor, MI, U.S.A); the human $\alpha 1$ and the mutant $\alpha 1$ (A391S) glycine receptor subunit, were subcloned into the mammalian expression vector pCIS 2 (Sontheimer *et al*., 1989), glycine was obtained by Bio-Rad Laboratories (Hercules, CA, U.S.A.); ethanol was obtained by Aaper Alcohol and Chemical Co. (Shelbyville, KY, U.S.A); Halothane was purchased from Halocarbons Laboratories (River Edge, NJ, U.S.A), propofol was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A). Staurosporine was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A). GF 109203X and H89 were purchased from Calbiochem Co. (La Jolla, CA, U.S.A). KN-62 was bought from RBI (Natick, MA, U.S.A). Genistein and calyculin A were purchased from LC Laboratories (Woburn, MA, U.S.A). All other reagents used were of reagent grade. The Quik Change site directed mutagenesis kit was purchased from Stratagene (La Jolla, CA, U.S.A.). Oligonucleotides were synthesized by the University of Colorado Health Sciences Center, Department of Pharmacology, Oligo Synthesis Laboratory.

Statistical analyses

Statistical analyses were performed on normalized data using either a *t*-test or two-way ANOVA and Fishers' *post hoc* test, using the SOLO program, BMPD statistical software, (Los Angeles, CA, U.S.A) running on an IBM compatible computer.

Results

The effects of several protein kinases inhibitors (the nonspecific PKC inhibitor staurosporine, the specific PKA inhibitor GF 109203X, the PKA inhibitor H-89, the CaM kinase inhibitor KN 62 and the tyrosine kinase inhibitor genistein), as well as the phosphatase inhibitor calyculin A, were examined in *Xenopus* oocytes expressing homomeric α1 glycine receptors. Concentration-response curves for glycine (10–1000 μM) were determined before and after exposure of *Xenopus* oocytes to the protein kinases inhibitors or to calyculin A. Treatment of oocytes with the protein kinases inhibitors or with calyculin A did not change the EC_{50} for glycine, nor the E_{max} or the Hill coefficient (Table 1).

To evaluate the role of PKC on the ethanol enhancement of the glycine receptor function, a complete ethanol (10–200 mM) concentration-response curve was performed in *Xenopus* oocytes expressing α1 glycine receptor subunit, before and after treatment of the oocytes with staurosporine (800 nM) or GF109203X (200 nM). In agreement with our previous reports (Mascia *et al*., 1996a,b), ethanol potentiated in a reversible and concentration-dependent manner the action of a concentration of glycine which produces a peak current that was 2% of the maximal current observed (EC_2). However, the ethanol potentiation of the glycine response was significantly lower when the oocytes were previously exposed to the PKC inhibitors staurosporine or GF109203X, suggesting that activation of PKC might be involved in the ethanol potentiation of the glycine receptor (Figure 1).

Because both staurosporine and GF109203X reduced the ethanol potentiation of the glycine response, the effects of staurosporine were also tested on the enhancement of the glycine receptor function by anaesthetics. The potentiation of the function of the glycine receptor induced by halothane (0.25 mM) or propofol (2.5 μM) was not affected by pretreatment of the oocytes with staurosporine (Figure 2).

Table 1 Kinase inhibitors and the phosphatase inhibitor calyculin A do not alter the glycine action

Treatment		E_{max} (nA)	EC_{50} (μM)	*Hill*
Staurosporine	control	3975±238	61±18	1.5
(800 μM)	treated	3710±479	73±21	1.5
GF 109203X	control	3250±167	90±27	2.2
(200 nM)	treated	3912±391	70±15	2.2
H-89	control	3175±380	57±14	1.9
(500 nM)	treated	3296±642	51±21	1.7
KN 62	control	3893± 38	43± 7	1.7
(15 μM)	treated	3529±446	48± 4	2.7
Genistein	control	4439±95	80±13	1.7
(100 μM)	treated	4408±125	60± 9	2.7
Calyculin A	control	3186±222	44± 4	1.9
(60 nM)	treated	3278±259	31± 5	2.1

E_{max}, EC_{50}, and Hill values for glycine concentration-response curves after staurosporine, GF109203X, H-89, KN62, genistein and calyculin A exposure in *Xenopus* oocytes expressing homomeric α1 glycine receptors. Oocytes were incubated for 4 h with staurosporine, for 30 min with GF109203X or genistein. H89, KN62 were microinjected and oocytes were used 5 min after the injection. Glycine was applied for 20 s. Values are the mean±s.e.m. of five to six oocytes.

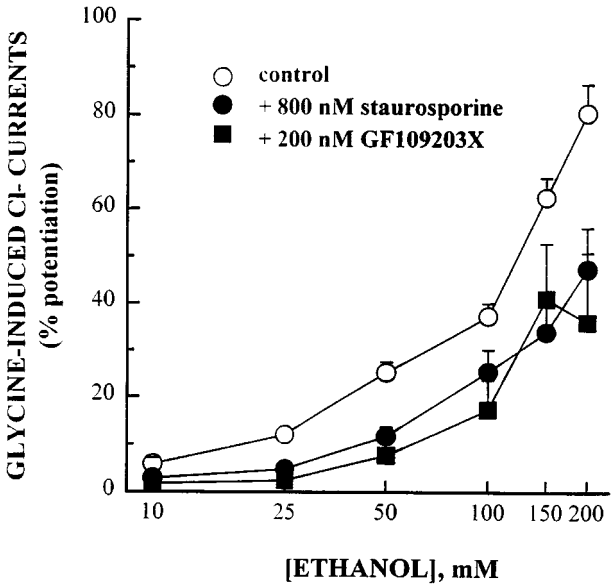

Figure 1 Staurosporine and GF103209X reduce the ethanol enhancement of currents evoked by glycine in *Xenopus* oocytes expressing homomeric α1 glycine receptor subunits. Control responses were determined and oocytes were then incubated for 4 h in 800 nM staurosporine or for 30 min in 200 nM GF 103209X and tested again. Ethanol (10–200 mM) was bath-applied for 2 min before being coapplied with an EC_2 concentration of glycine. Enhancement of glycine currents induced by ethanol was significantly lower after treatment with staurosporine or GF 103209X. Values are the mean±s.e.m. of 10 to 11 oocytes, $P<0.0001$ for controls vs 800 nM staurosporine or vs 200 nM GF 103209X.

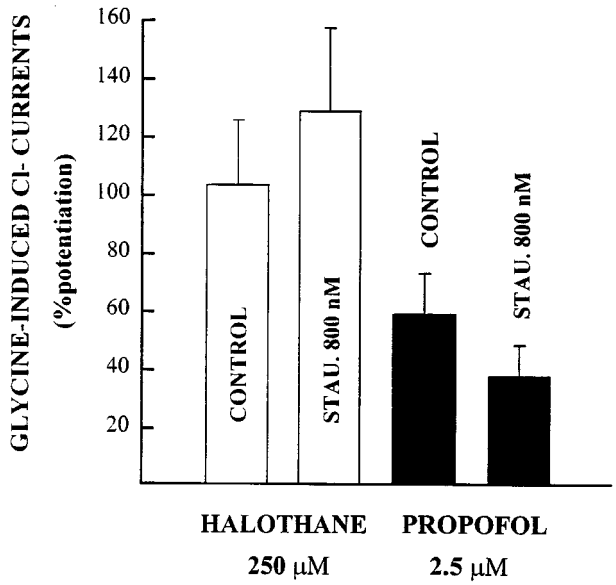

Figure 2 Staurosporine did not affect halothane (0.25 mM) or propofol (2.5 μM) potentiation of currents evoked by glycine in *Xenopus* oocytes expressing α1 glycine receptor subunits. Control responses were determined and oocytes were then incubated with 800 nM staurosporine for 4 h and tested again. Halothane or propofol were perfused for 2 min before being coapplied with an EC_2 concentration of glycine. Values are the mean±s.e.m. of five to seven oocytes.

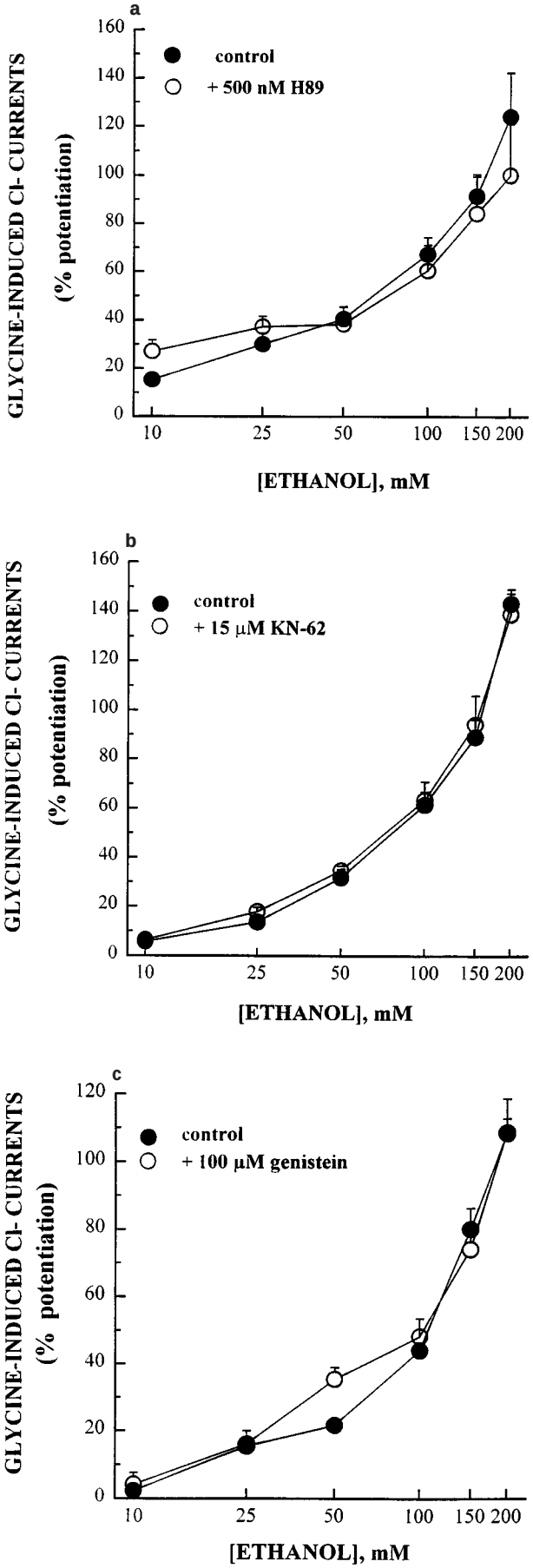

Figure 3 H-89 (A), KN 62 (B) and genistein (C) did not alter the ethanol action on *Xenopus* oocytes expressing α1 glycine receptor subunits. Control responses were determined and oocytes were microinjected with H-89 (500 nM) or KN 62 (15 μM) or preincubated with genistein (100 μM) for 30 min. Ethanol (10–200 mM) was perfused for 2 min before being coapplied with an EC_2 concentration of glycine for 20 s. Values are the mean±s.e.m. of five to seven oocytes.

To explore the role of other kinases on the ethanol potentiation of the glycine response, we examined the effects of several kinase inhibitors in *Xenopus* oocytes expressing the α1 glycine receptor subunit. Microinjection of the oocytes with the specific PKA inhibitor H89 or with the CaM kinase inhibitor KN62, as well as the pretreatment with the tyrosine kinase inhibitor genistein, induced no significant changes in the ethanol modulation of the glycine receptor function (Figure 3A, B and C).

Next, the effect of the serine-threonine phosphatase inhibitor calyculin A was examined. Ethanol concentration-response (10–200 mM) curves were performed in *Xenopus* oocytes expressing α1 glycine receptor subunit, before and after microinjection with calyculin A (60 nM). Ethanol potentiated in a concentration-dependent and reversible way the effect of glycine in both control and treated oocytes. No significant differences were found between the two conditions studied (Figure 4).

The effect of ethanol was studied in a mutant of the α1 glycine receptor subunit where an alanine replace the serine at the 391 residue [α1 (S391A)] with consequent elimination of the PKC phosphorylation site. Glycine (10–1000 μM) concentration response curves were determined and compared for oocytes expressing the α1 wild type subunit and oocytes expressing the mutant α1 (S391A). As shown in Table 2, no differences in the glycine EC_{50} values, in the E_{max} values or in the Hill coefficient were found when results from the wild type α1 glycine receptor were compared to results from the mutant α1 (S391A). A complete ethanol (10–200 mM) concentration

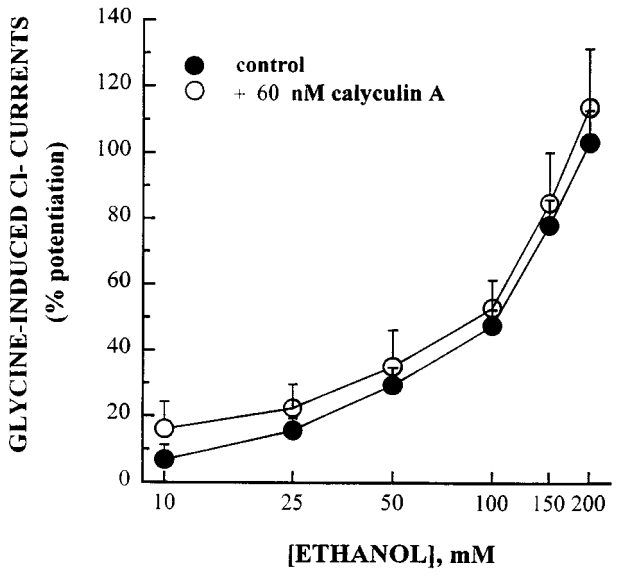

Figure 4 The protein phosphatase inhibitor calyculin A did not alter the ethanol action in *Xenopus* oocytes expressing α1 glycine receptor subunits. Control responses were determined and oocytes were microinjected with (60 nM) calyculin A. Ethanol (10–200 mM) was perfused for 2 min before being coapplied with an EC_2 concentration of glycine for 20 s. Values are the mean±s.e.m. of five to seven oocytes.

response curve was next performed (Figure 5). Ethanol potentiated the glycine receptor function in both homomeric type α1 glycine receptors and in the mutant α1 (S391A) receptors. However, the substitution of S391A in the α1 mutant significantly decreased its ethanol sensitivity when compared with α1 wild type ($P<0.001$). Moreover the PKC inhibitor staurosporine did not affect the ethanol (10–200 mM) potentiation of the glycine response in *Xenopus* oocytes expressing α1 (S391A) glycine receptors. To determine whether the effect of staurosporine on wild type glycine α1 receptors (as presented in Figure 1) is similar to the effect of mutation of Ser 391 to Ala, oocytes from the same batch were injected with cDNAs for either the wild type or mutated (S391A) glycine α1 subunits, and oocytes expressing the wild type glycine α1 receptor were treated with staurosporine. Under these conditions the effect of ethanol was reduced to the same extent in staurosporine-treated oocytes expressing α1 wild type and in untreated oocytes expressing α1 (S391A).

Previous studies have reported the presence of a splicing variant of the α1 glycine receptor subunit, $\alpha1^{ins}$, with eight additional amino acid in the cytoplasmic loop between TM3 and TM4. This insert of eight additional amino acids may result in a consensus sequence for a phosphorylation site by PKA (Malosio *et al*., 1994). To determine the importance of this insert in the glycine receptor function and in the ethanol potentiation of the glycine receptor, oocytes expressing the α1 wild type glycine receptor subunit were compared to oocytes expressing $\alpha1^{ins}$ glycine receptor subunit. Glycine (10–1000 μM) concentration-response curve were determined. No differences were found in the glycine EC_{50} values, E_{max} or Hill coefficient (Table 2). Ethanol (10–200 mM) concentration-response curve were next examined in *Xenopus* oocytes expressing α1 or $\alpha1^{ins}$ glycine receptor subunit (Figure 6).

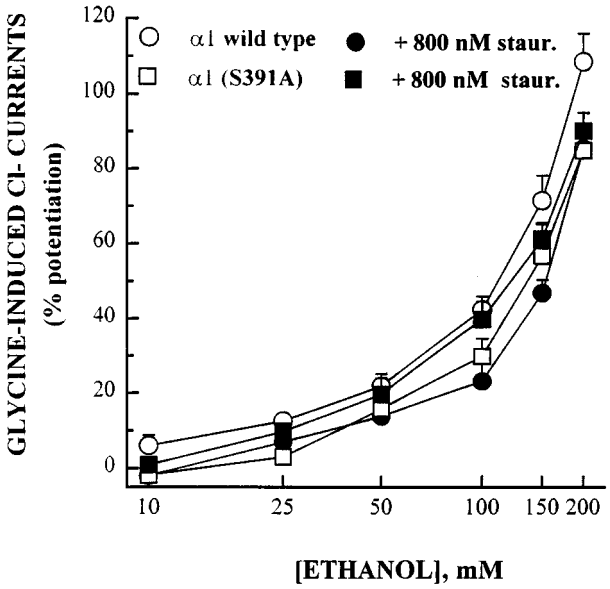

Figure 5 Ethanol potentiation of the currents evoked by glycine was reduced in oocytes expressing the mutant α1(S391A) when compared with the α1 wild type. Staurosporine does not alter the ethanol effects in oocytes expressing the mutant α_1 (S391A), but reduces the ethanol potentiation to the same level of the α1 (S391A) in oocytes expressing the α1 wild type glycine receptor subunit. Control responses were determined and oocytes expressing α1 wild type or α1 (S391A) were incubated for 4 h with 800 nM staurosporine. Ethanol (10–200 mM) was bath-applied for 2 min before being coapplied with an EC_2 concentration of glycine. Values are the mean ± s.e.m. of four to eight oocytes, $P<0.001$ for α1 wild type vs α1 (S391A) and $P<0.0001$ for α1 wild type vs staurosprine-treated α1 wild type glycine receptors.

Table 2 Glycine action did not differ in *Xenopus* oocytes expressing α1 wild type, the mutant α1(S391A) or the $\alpha1^{ins}$ glycine receptor subunit

Clones	E_{max} (nA)	EC_{50} (μM)	*Hill*
α1 wild type	2648 ± 385	71 ± 5	3.8
α1 (S391A)	2437 ± 213	71 ± 9	2.6
α1 wild type	3126 ± 465	129 ± 11	2.8
$\alpha1^{ins}$	3131 ± 392	122 ± 6	2.2

Glycine concentration-response curve were performed comparing α1 (S391A) or $\alpha1^{ins}$ glycine receptors to α1wild type glycine receptors expressed into the same batch of *Xenopus* oocytes. Glycine was applied for 20 s. Values are mean ± s.e.m. of six oocytes.

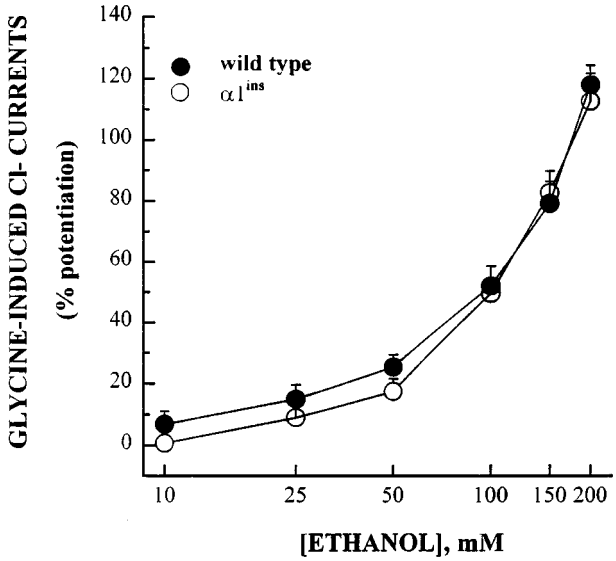

Figure 6 Ethanol potentiation of the currents evoked by glycine is not altered in oocytes expressing homomeric α1 or $\alpha1^{ins}$ glycine receptor subunit. Ethanol was bath applied for 2 min before an EC_2 concentration of glycine was coapplied for 20 s. Values are the mean ± s.e.m. of six different oocytes.

Ethanol potentiated in a reversible and concentration-dependent manner the glycine receptor function to the same extent in both the glycine receptors.

Discussion

In previous studies (Mascia *et al*., 1996a,b), we demonstrated that ethanol and anaesthetics potentiated the action of glycine in *Xenopus* oocytes expressing human homomeric α1 or α2 glycine receptor. In the present study we examined the possibility that phosphorylation of the glycine receptor could be involved in the ethanol potentiation of glycine-evoked Cl^- currents. Our results indicate that treatment of oocytes with protein kinase C inhibitors (staurosporine or GF 109203X) produced a partial reduction of the potentiation induced by ethanol. Moreover, the ethanol potentiation of the glycine receptor was significantly reduced after mutagenesis of a specific PKC consensus site, at serine 391, of the α1 glycine subunit. This reduction was almost complete at low concentrations of ethanol (10–25 mM), however was reduced to 22% when higher (150–200 mM) concentrations were tested. Furthermore, the effect of ethanol in this mutant was unaltered by preincubation of *Xenopus* oocytes with the PKC

inhibitor staurosporine. These results suggest that phosphorylation by PKC of the α subunit of the glycine receptor is important for ethanol potentiation of the glycine receptor function and are consistent with the evidence that the α glycine receptor subunit is phosphorylated by PKC at the serine 391 residue (Ruiz-Gomez *et al*., 1994). Similar findings were reported for the $GABA_A$ receptor ($\alpha1\beta1\gamma2L$): PKC inhibitors as well as mutagenesis of a PKC phoshorylation site were able to completely block the ethanol effect (Wafford & Whiting, 1992). However, in the case of the glycine receptor, phosphorylation by PKC of the α subunit is not sufficient to completely explain the ethanol potentiation because PKC inhibitors, as well as mutagenesis of the phosphorylated residue, were not able to completely block the ethanol potentiation of the glycine action. In addition, we recently identified an amino-acid residue, S267, which is essential for the ethanol potentiation of the glycine receptor function, but is not a phosphorylation site (Mihic *et al*., 1997). Moreover, a series of amino acids mutations at residue S267, demonstrate that the effect of ethanol depends upon the molecular volume of the substituents in position 267: replacement of the S267 residue with small volume amino acids resulted in potentiation of the ethanol action, and mutation of the S267 residue with large residues resulted in inhibition (Ye *et al*., 1998). As discussed below, these results suggest that there is an alcohol binding site on the α glycine receptor subunit that is formed in part by the 267 residue.

When the effect of staurosporine was tested at the same time and on the same batch of oocytes expressing either the 391 mutant or the α1 wild type, we did not find any difference in the reduction of the ethanol potentiation between staurosporine-treated oocytes expressing the wild-type α1 subunit and untreated oocytes expressing the mutant α1 (S391A). This suggests that phosphorylation of S391 can account for all the effects of PKC inhibitors. The effects of the PKC inhibitors were specific for the action of ethanol. In fact, treatment of the oocytes with staurosporine, did not change the potentiation of the function of the glycine receptor induced by the volatile anaesthetic halothane or by the intravenous anaesthetic propofol. This latter finding rules out the possibility that the strychnine-sensitive glycine receptor could be involved in the decrease of the EC_{50} for anaesthesia induced by staurosporine in tadpoles (Firestone *et al*., 1993).

Because the α subunit of the glycine receptor is phosphorylated *in vitro* by cyclic AMP dependent protein kinase A (Vaello *et al*., 1994), we examined the possibility that, in addition to PKC, other kinases (i.e., PKA, CaM kinase and tyrosine kinase) might be involved in the ethanol modulation of the glycine receptor function. In our hands, none of the inhibitors of PKA, CaM kinase or tyrosine kinase were able to modify the effects of ethanol at the glycine receptor. Moreover, forskolin, an activator of adenylate cyclase, was not able to modify the ethanol effect on the glycine α1 receptor expressed in *Xenopus* oocytes (data not shown). In transiently transfected HEK 293 cells activation of PKA with forskolin did not alter the ethanol effects on glycine α1 receptors (Valenzuela, unpublished data). Part of these results are in agreement with previous electrophysiological findings from Aguayo *et al*., (1996) demonstrating that, the potentiating effects of ethanol on the glycine currents, measured in cultured spinal neurones, were unmodified by treatment with the PKA inhibitors H7 or PKI peptide inhibitor or after dialysis of neurones with internal solution lacking ATP. Together, these results suggest that protein kinase A is not involved in the ethanol modulation of the glycine receptor function. The alternatively spliced form of the α1 glycine receptor subunit, $\alpha1^{ins}$ (Malosio *et al*., 1994), which in adult spinal cord represents ~30% of the total α1 subunit, contains in the eight additional amino acids, a potential consensus site for phosphorylation by PKA. A similar splicing variant, bearing eight amino acids and an additional consensus sequence for PKC, has been reported for the γ_2 subunit of the $GABA_A$ receptor (γ_2L) (Whiting *et al*., 1990). These eight amino acids in the alternatively spliced form of the γ2 $GABA_A$ subunit, are important for the low dose ethanol potentiation of the $GABA_A$ receptor; furthermore, at least one of these eight amino acids must be in a phosphorylated state for the low dose ethanol potentiation to be observed (Wafford & Whiting, 1992; Harris *et al*., 1997). Because of these findings, we compared the effects of ethanol in oocytes expressing the α1 glycine receptor subunit or the splice variant $\alpha1^{ins}$. Our results indicate that the $\alpha1^{ins}$ glycine receptor is as sensitive to the effects of ethanol as the α1 glycine receptor, and suggest that, if phosphorylation by PKA occurs in the splice variant, it does not affect the ethanol potentiation of the glycine receptor function.

Treatment of oocytes with the phosphatase inhibitor calyculin A did not modify the ethanol effect on glycine receptor function. A possible explanation could be the high level of endogenous phosphorylation inside the *Xenopus* oocytes (Woodland, 1979), so that PKC inhibitors could induce a reduction in the ethanol modulation of the function of the glycine receptor, but the effect of the phosphatase inhibitor could have been masked by the high phosphorylation state. A similar result was described by Wafford & Whiting (1992) for the $GABA_A$ receptor expressed in oocytes: kinase inhibitors were able to block the ethanol potentiation, but PKC activators were unable to modify the ethanol effect.

In this study we also investigated the role of kinases inhibitors, as well as the phosphatase inhibitor calyculin A, on the function of the glycine receptor. Several publications suggest that the function of the glycine receptor can be modulated by activation of PKC or PKA, although the results are contradictory. In spinal neurons (Song & Huang, 1990) and in *Xenopus* oocytes (Vaello *et al*., 1994) activation of PKA enhances the function of the glycine receptor. On the contrary, activation of PKC decreases the glycine receptor function in *Xenopus* oocytes (Vaello *et al*., 1994; Uchiyama *et al*., 1994), but potentiates the glycine response in hippocampal neurones (Schonrock & Bormann, 1995). In contrast to these reports, we found that the kinases inhibitors and calyculin A were not able to modulate the glycine response. In particular, the PKC inhibitors staurosporine and GF 109203X, under conditions that reduced the ethanol effects, did not alter the glycine response. Our results raise the question of why PKC inhibitors can modulate the ethanol effect, but not the action of glycine at the glycine receptor. One possibility is that ethanol binds a 'pocket' between transmembrane regions 2 and 3 of the glycine α1 subunit, as proposed by Mihic *et al*., (1997), and that receptor phosphorylation increases the affinity or efficacy of this interaction. For this explanation to be plausible, it would be necessary for ethanol to bind to different site on the glycine receptor than does halothane or propofol, or to bind to this site in a different manner than other anaesthetics. Indeed, mutations of amino acids in the TM2 and three regions have distinct effects on actions of ethanol, halothane and propofol (Mihic *et al*., 1997 and unpublished data).

Finally, we can speculate about which receptor system, *in vivo*, could be involved in the ethanol modulation of the glycine receptor through phosphorylation by PKC. A possible candidate is the $5\text{-}HT_2$ receptor. Recent data suggest that the function of the glycine receptor, measured in neurones from the sacral dorsal commisural nucleus, is potentiated through

activation of the 5-HT_2 receptor (Xu *et al*., 1996). In fact, serotonin binding to the 5-HT_2 receptor activate phospholipase C with consequent increase in the level of diacylglycerol, the main endogenous activator of PKC.

We thank Dr Peter Schofield for kindly providing the α1 and α1(S391A) glycine clones and Drs Fernando Valenzuela and John Mihic for many helpful discussions. Supported by NIH grants AA06399 and AA05327 and by the Department of Veteran Affairs.

References

AGUAYO, L.G. & PANCETTI, F. (1994). Ethanol modulation of the γ-aminobutyric $acid_A$- and glycine-activated Cl^- currents in cultured mouse neurons. *J. Pharmacol. Exp. Ther.,* **270,** 61–69.

AGUAYO, L.G., TAPIA, J.C. & PANCETTI, F. (1996). Potentiation of the glycine-activated Cl^- current by ethanol in cultured mouse spinal neurons. *J. Pharmacol. Exp. Ther.,* **279,** 1116–1122.

BECHADE, C., SUR, C. & TRILLER, A. (1994). The inhibitory neuronal glycine receptor. *BioEssays.,* **16,** 735–744.

BETZ, H. (1991). Glycine receptors: heterogeneous and widespread in the mammalian brain. *Trends Neurosci.,* **14,** 458–461.

CELENTANO, J.J., GIBBS, T.T. & FARB, D.H. (1988). Ethanol potentiates GABA and glycine induced currents in chick spinal cord neurons. *Brain. Res.,* **455,** 377–380.

COLMAN, A. (1984). Expression of exogenous DNA in *Xenopus* oocytes. In *Transcription and Translation: A Practical Approach* ed. Hames, B.D. and Higgins, S.J., pp. 49–59. Washington D.C U.S.A.: Oxford Press.

DILDY-MAYFIELD, J.E., MIHIC, S.J., LIU. Y., DEITRICH, R.A. & HARRIS, R.A. (1996). Actions of long chain alcohols on $GABA_A$ and glutamate receptors: relation to *in vivo* effects. *Br. J. Pharmacol.,* **118,** 378–384.

ENGBLOM, A.C. & AKERMAN, K.E.O. (1991). Effect of ethanol on γ-aminobutyric acid and glycine receptor-coupled Cl^- fluxes in rat brain synaptoneurosomes. *J. Neurochem.,* **57,** 384–390.

FIRESTONE, S., FIRESTONE, L.L., FERGUSON, C. & BLANK, D. (1993). Staurosporine, a protein kinase C inhibitor, decreases the general anesthetic requirement in *Rana pipiens* tadpoles. *Anesth. Analg.,* **77,** 1026–1030.

HARRIS, R.A., MIHIC, S.J., BROZOWSKI, S., HADINGHAM, K. & WHITING, P.J. (1997). Ethanol, flunitrazepam, and pentobarbital modulation of $GABA_A$ receptors expressed in mammalian cells and *Xenopus* oocytes. *Alcohol Clin. Exp. Res.,* **21,** 444–451.

HUIDOBRO-TORO, J.P, VALENZUELA, C.F. & HARRIS, R.A. (1996). Modulation of the $GABA_A$ receptor function by G protein-coupled 5-HT_{2c} receptors. *Neuropharmacol.,* **35,** 1355–1363.

LIN, L-H., CHEN, L.L., ZIRROLLI, J.A. & HARRIS, R.A. (1992). General anesthetics potentiate γ-aminobutyric $acid_A$ receptors by *Xenopus* oocytes: lack of involvement of intracellular calcium. *J. Pharmacol. Exp. Ther.,* **263,** 569–578.

MALOSIO, M.L., GRENNINGLOH, G., KUHSE, J., SCHMIEDEN, V., SCHMITT, P.P. & BETZ, H. (1994). Alternative splicing generates two variants of the α_1 inhibitory glycine receptor. *J. Biol. Chem.,* **266,** 2048–2053.

MASCIA, M.P., MACHU, T.K. & HARRIS, R.A. (1996a). Enhancement of homomeric glycine receptor function by long-chain alcohols and anaesthetics. *Br. J. Pharmacol.,* **119,** 1331–1336.

MASCIA, M.P., MIHIC, S.J., VALENZUELA, C.F., SCHOFIELD, P.R. & HARRIS, R.A. (1996b). A single amino acid determines differences in ethanol actions on strychnine-sensitive glycine receptors. *Mol. Pharmacol.,* **50,** 402–406.

MATZENBACH, B., MAULET, Y., SEFTON, L., COURTIER, B., AVNER, P., GUENET, J-L. & BETZ, H. (1994). Structural analysis of mouse glycine receptor α subunit genes. *J. Biol. Chem.,* **269,** 2607–2612.

MIHIC, S.J., YE, Q., WICK, M.J., KOLTCHINE, V.V., KRASOWSKI, M.D., FINN, S.E., MASCIA, M.P., VALENZUELA, C.F., HANSON, K.K., GREENBLATT, E.P., HARRIS, R.A. & HARRISON, N.L. (1997). Sites of alcohol and volatile anaesthetic action on $GABA_A$ and glycine receptors. *Nature,* **389,** 385–389.

MINAMI, K., GEREAU, R.W., MINAMI, M., HEINEMANN, S.F. & HARRIS, R.A. (1998). The effects of ethanol and anesthetics on type 1 and 5 metabotropic glutamate receptors expressed in *Xenopus* oocytes. *Mol. Pharmacol.,* **53,** 148–156.

MOSS, S.J. & SMART, T.G. (1996). Modulation of amino acid-gated ion channels by protein phosphorylation. In *International Review of Neurobiology*. eds Bradley, R.J., Harris, R.A. & Jenner, P. **39,** pp. 1–52. New York: Academic Press.

NISHIZAKI, T. & IKEUCHI, Y. (1995). Activation of endogenous protein kinase C enhances currents through α1 and α2 glycine receptor channels. *Brain Res.,* **687,** 214–216.

RUIZ-GOMEZ, A., VAELLO, M.L., VALDIVIESO, F. & MAYOR, F. Jr. (1994). Phosphorylation of the 48-kDa subunit of the glycine receptor by protein kinase C. *J. Biol. Chem.,* **266,** 559–566.

SANNA, E., DILDY-MAYFIELD, J.E. & HARRIS, R.A. (1994). Ethanol inhibits the function of 5-hydroxytryptamine type 1c and muscarinic M_1G protein-linked receptors in *Xenopus* oocytes expressing brain mRNA: role of protein kinase C. *Mol. Pharmacol.,* **45,** 1004–1012.

SCHONROCK, B. & BORMANN, J. (1995). Modulation of hippocampal glycine receptor channels by protein kinase C. *NeuroReport,* **6,** 301–304.

SNELL, L.D., TABAKOFF, B. & HOFFMANN, P.L. (1994). Involvement of protein kinase C in ethanol-induced inhibition of NMDA receptor function in cerebellar granule cells. *Alcohol. Clin. Exp. Res.,* **18,** 81–85.

SONG, Y. & HUANG, L-Y.M. (1990). Modulation of glycine receptor chloride channel by cyclic AMP-dependent protein kinase in spinal trigeminal neurons. *Nature,* **348,** 242–245.

SONTHEIMER, H., BECKER, C-M., PRITCHETT, D.B., SCHOFIELD, P.R., GRENNINGLOH, G., KETTENMAN, H., BETZ, H. & SEEBURG, P.H. (1989). Functional chloride channels by mammalian cell expression of rat glycine receptor subunit. *Neuron.,* **2,** 1491–1497.

SWOPE, S.L., MOSS, S.J., BLACKSTONE, C.D. & HUGANIR, R.L. (1992). Phosphorylation of ligand-gated ion channels: a possible mode of synaptic plasticity. *FASEB. J.,* **6,** 2514–2523.

UCHIYAMA, M., HIRAI, K., HISHINUMA, F. & AKAGI, H. (1994). Down-regulation of glycine receptor channels by protein kinase C in *Xenopus* oocytes injected with synthetic RNA. *Mol. Brain. Res.,* **24,** 295–300.

VAELLO, M.L., RUIZ-GOMEZ, A., LERMA, J. & MAYOR, F., Jr. (1994). Modulation of inhibitory glycine receptors by phosphorylation by protein kinase C and cyclic AMP dependent protein kinase. *J. Biol. Chem.,* **269,** 2002–2008.

VALENZUELA, C.F., CARDOSO, R.A., WICK, M.J., WEINER, J.L, DUNWIDDIE, T.V. & HARRIS, R.A. (1998). Effects of ethanol on recombinant glycine receptors expressed in mammalian cell lines. *Alcohol. Clin. Exp. Res.* (In press).

VALENZUELA, C.F., KAUZLAUSKAS, A., BROZOWSKY, S.J., WEINER, J.L., DEMALI, K.A., McDONALD, B.J., MOSS, S.J., DUNWIDDIE, T.V. & HARRIS, R.A. (1995a). Plateled-derived growth factor receptor is a novel modulator of type A γ-aminobutyric acid-gated ion channels. *Mol. Pharmacol.,* **48,** 1099–1107.

VALENZUELA, C.F., MACHU, T.K., McKERNAN, R.M., WHIYING, P., VANRENTERGHEM, B.B., McMANAMAN, J.L., BROZOWSKI, S.J., SMITH, G.B., OLSEN, R.W. & HARRIS, R.A. (1995b). Tyrosine kinase phosphorylation of the $GABA_A$ receptors. *Mol. Brain. Res.,* **31,** 165–172.

WAFFORD, K.A., BURNETT, D.M., LEIDENHEIMER, N.J., BURT, D.R., WANG, J.B., KOFUJI, P., DUNWIDDIE, T.V., HARRIS, R.A. & SIKELA, J.M. (1991). Ethanol sensitivity of the $GABA_A$ receptor expressed in *Xenopus* oocytes requires eight amino acids contained in the γ2L subunit. *Neuron.,* **7,** 27–33.

WAFFORD, K.A. & WHITING, P.J. (1992). Ethanol potentiation of $GABA_A$ receptors requires phosphorylation of the alternatively spliced variant of the γ2 subunit. *FEBS.,* **313,** 113–117.

WALDEGGER, S., RABER, G., SUSSBRICH, H., RUPPERSBERG, J.P., FAKLER, B., MURER, H., LANG, F. & BUSCH, A.E. (1996). Coexpression and stimulation of parathyroid hormone receptor positively regulates slowly activating IsK channels expressed in *Xenopus* oocytes. *Kidney Intern.,* **49,** 112–116.

WHITING, P.J., MCKERNAN, R.M. & INVERSEN, L.L. (1990). Another mechanism for creating diversity in γ-aminobutyrate type A receptors: RNA splicing directs expression of two forms of γ2 subunit, one of which contains a protein kinase C phosphorylation site. *Proc. Natl. Acad. Sci. U.S.A.,* **87,** 9966–9970.

WILLIAMS, K.L., FERKO, A.P., BARBIERI, A.J. & DI GREGORIO, G.J. (1995). Glycine enhances the central depressant properties of ethanol in mice. *Pharmacol. Biochem. Behav.,* **50,** 199–205.

WOODLAND, H.R. (1979). The modification of stored histones H3 and H4 during the oogenesis and early development of *Xenopus laevis*. *Dev. Biol.,* **68,** 360–370.

XU, T-L., NABEKURA, J. & AKAIKE, N. (1996). Protein kinase C-mediated enhancement of glycine response in rat sacral dorsal commissural neurones by serotonin. *J. Phisiol.,* **496.2,** 491–501.

YE, Q., KOLTCHINE, V.V., MIHICH, S.J., MASCIA, M.P., WICK, M.J., FINN, S.E., HARRISON, N.L. & HARRIS, R.A. (1998). Enhancement of glycine receptor function by ethanol is inversely correlated with molecular volume at position α267. *J. Biol. Chem.,* **273,** 3314–3319.

(Received November 17, 1997
Revised May 29, 1998
Accepted June 15, 1998)