

# Ethanol potentiation of GABA<sub>A</sub> receptors requires phosphorylation of the alternatively spliced variant of the $\gamma 2$ subunit

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The mammalian GABA<sub>A</sub> receptor is a multisubunit protein containing a variety of binding sites for psychotropic agents. One of the most widely used of these drugs, ethanol, enhances the function of GABA<sub>A</sub> receptors in certain circumstances but not others. Previous studies have demonstrated that alternative splicing of the  $\gamma 2L$  GABA subunit results in an ethanol sensitive and an ethanol-insensitive form, when combined with  $\alpha$  and  $\beta$  subunits. We have used *in vitro* mutagenesis and expression in *Xenopus* oocytes to show that the consensus site for phosphorylation by protein kinase C contained in the  $\gamma 2L$  insert is critical for modulation by ethanol but not benzodiazepines, and manipulation of the phosphorylating enzymes in oocytes containing  $\alpha 1\beta 1\gamma 2L$  can prevent ethanol enhancement. It is likely that phosphorylation or dephosphorylation of a specific site on the GABA<sub>A</sub> receptor protein can act as a control mechanism for neuronal responses to alcohol exposure.

GABA<sub>A</sub> receptor; Ethanol; Phosphorylation; Protein kinase C; Mutagenesis

## 1 INTRODUCTION

The GABA<sub>A</sub> receptor is a member of the ligand-gated ion channel family and probably exists in a variety of different forms in distinct regions of the nervous system. The GABA receptor complex is made up from a combination of  $\alpha$  [1–6],  $\beta$  [1–3] and  $\gamma$  [1–3] subunits [1–9].  $\delta$  and  $\rho$  subunits have also been identified [10,11]. Alternative splicing of subunits has added yet another layer of complexity to the possible forms of the GABA receptor [12–14] and the pharmacological consequences of this phenomenon remain relatively unexplored. The GABA receptor complex contains binding sites for a number of therapeutically active drugs, including benzodiazepines, barbiturates and steroids. Alcohol also exerts at least some of its neurological effects by influencing the activity of the GABA<sub>A</sub> receptor. Historically ethanol was believed to act via its actions on biological membranes, however, recent studies suggest that its effects are mediated by a more direct action on a number of different receptor proteins and ion channels; GABA, NMDA, voltage-sensitive calcium channels and 5HT<sub>3</sub> receptors are all influenced by intoxicating levels of alcohol [15]. The sedating properties of ethanol are almost certainly mediated via the GABA<sub>A</sub> receptor and studies have demonstrated an enhancement of GABA function in a number of different systems [16–22]. This enhancement is not universal throughout the nervous system and some regions seem particularly sensitive e.g.

cerebellum, whereas others e.g. hippocampus are relatively insensitive. Benzodiazepine potentiation of the GABA receptor is influenced by the presence of a  $\gamma 2$  subunit [2]. Ethanol potentiation is also dependent on the presence of a  $\gamma$  subunit, but further to this requires a specific alternatively spliced version of the  $\gamma 2$  subunit containing an extra eight amino acids in the region between M3 and M4, a proposed intracellular loop [23]. Interestingly this additional splice insert contains a consensus phosphorylation sequence for protein kinase C [12]. We have used *in vitro* mutagenesis techniques and manipulation of GABA receptor subunits expressed in *Xenopus* oocytes to explore the role of the consensus phosphorylation sequence in drug modulation of the GABA receptor complex.

## 2. MATERIALS AND METHODS

Frogs were maintained in aquarium tanks at room temperature with a 12/12 h light/dark cycle and fed a regular diet of chopped steak and crickets. To obtain oocytes, frogs were anaesthetised with 0.5% tricaine and a small piece of ovary was removed through an incision in the abdominal wall. Stage V and VI oocytes were isolated and the theca and epithelial cell layer were dissected away with fine forceps. Follicle cells were removed by an 8-min treatment in Sigma Type IA collagenase (0.5 mg/ml) dissolved in modified Barth's saline (MBS) (88 mM NaCl, 1 mM KCl, 10 mM HEPES, 0.82 mM MgSO<sub>4</sub>, 0.33 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.91 mM CaCl<sub>2</sub>, 2.4 mM NaHCO<sub>3</sub>, pH 7.5). Oocytes were injected with 50 nl of a solution containing mixtures of subunit cRNAs (1–2 mg/ml) using a 10  $\mu$ l micropipette with an internal diameter of 20  $\mu$ m. Oocytes were incubated in individual wells of a 24-well culture plate at 19°C for 2 days, in MBS supplemented with 2 mM sodium pyruvate, penicillin (100 U/ml), streptomycin (100 mg/ml) and gentamycin (50 mg/ml).

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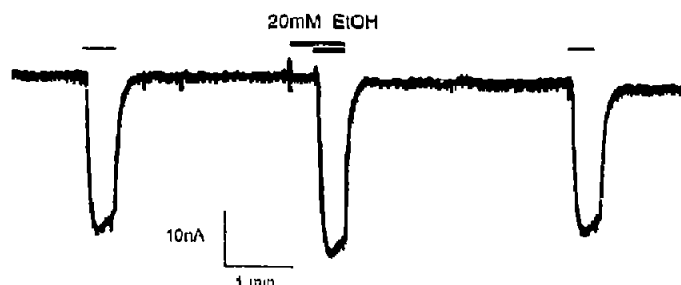
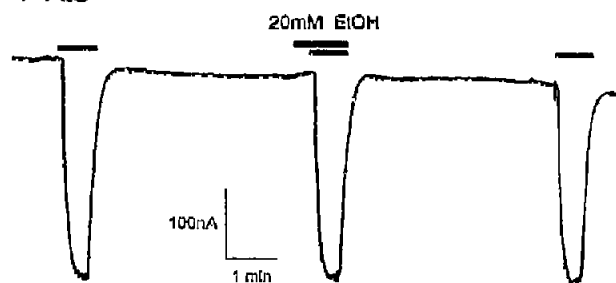
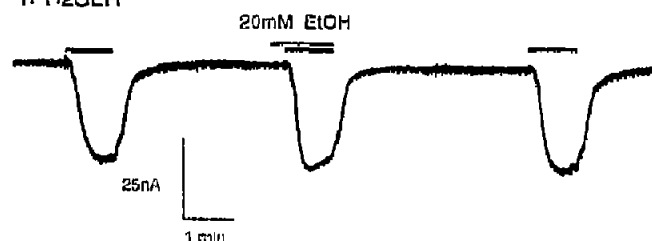
a)  $\alpha 1\beta 1\gamma 2L$ b)  $\alpha 1\beta 1\gamma 2S$ c)  $\alpha 1\beta 1\gamma 2SER$ 

Fig. 1 Typical examples of currents recorded in *Xenopus* oocytes expressing GABA<sub>A</sub> receptors, after injection of mRNA [29] made up from combining the following subunits (a)  $\alpha 1\beta 1\gamma 2L$ , (b)  $\alpha 1\beta 1\gamma 2S$  and (c)  $\alpha 1\beta 1\gamma 2SER$ . The effect of 20 mM ethanol on current responses to 30  $\mu$ M GABA is shown for each combination as indicated by the bars over each trace, and are typical of at least six different cells from three separate donor frogs.

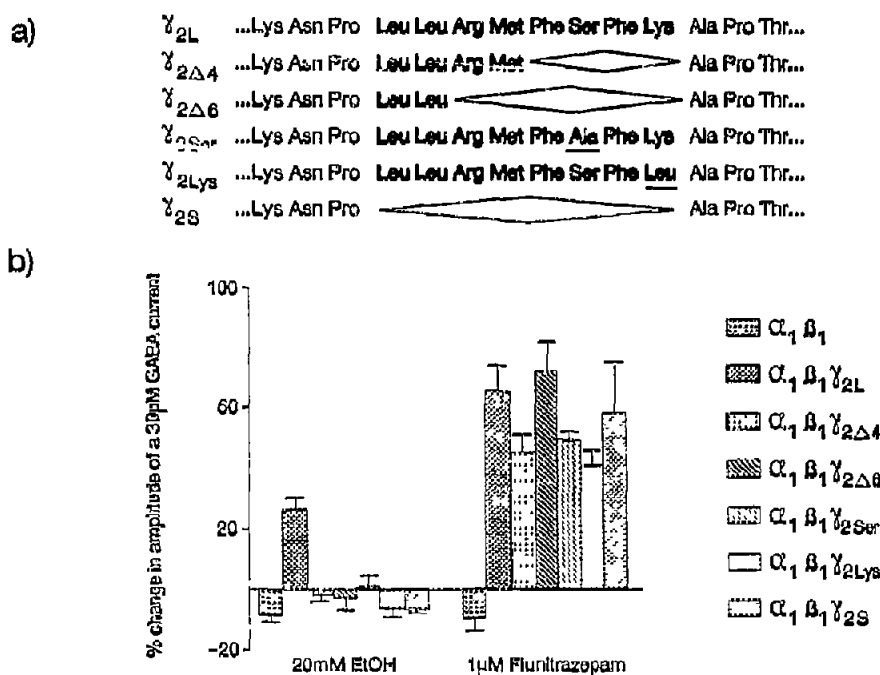
For recording, oocytes were placed in a 50  $\mu$ l bath and perfused with MBS at 10–13 ml/min. Cells were impaled with two 1–3 M electrodes containing 3 M KCl and voltage-clamped at  $-70$  mV. Drugs were applied in the perfusate and were preapplied for 30 s prior to exposure to GABA in combination with the drug. GABA was applied until the response reached a maximum, usually approximately 30 s.

The bovine  $\alpha 1$ ,  $\beta 1$ ,  $\gamma 2L$  and  $\gamma 2S$  cDNAs have been described previously [1,12,24]. cRNA was prepared from  $\alpha 1$  and  $\beta 1$  cDNAs as previously described. cRNA was prepared from  $\gamma 2L$  cDNA (wild type and mutants) in Bluescript Sk<sup>−</sup> (Stratagene) by linearization with *Kpn*I and transcription with T3 polymerase. Site-directed mutagenesis was performed using the method of Kunkel et al. [25] using the oligonucleotides 5'-AACCCCTCTTCTTCGGATGGCCCTACAAATTGACATCCG-3' ( $\gamma 2L^{\Delta 4}$ ), 5'-GAAGAAAAACCCCTCTTCTTGCCCTACATTGACATCCG-3' ( $\gamma 2L^{\Delta 6}$ ), 5'-CTTCGGATGTTTGCCAAGGCC-3' ( $\gamma 2LSer \rightarrow Ala$ ) and 5'-GTTTCCTTCCTGGCCCTACAAATTG-3' ( $\gamma 2LLys \rightarrow Leu$ ). Single-stranded phagemid DNA prepared from  $\gamma 2L$  cDNA in Bluescript Sk<sup>−</sup> was used as template. Mutant cDNAs were screened by sequencing of individual clones.

### 3. RESULTS

GABA<sub>A</sub> receptor subunit mRNAs were made from the appropriate bovine cDNAs, and expressed in *Xenopus* oocytes. The combinations of  $\alpha 1\beta 1\gamma 2L$  and  $\alpha 1\beta 1\gamma 2S$ , both had equivalent responses to GABA with a concentration of 30  $\mu$ M eliciting approximately half-maximal amplitudes. The benzodiazepine flunitrazepam potentiated the current elicited by 30  $\mu$ M GABA with a maximal effect at a concentration of 1  $\mu$ M. The extent of potentiation was approximately 65% in both cases and there was no significant difference in the extent of potentiation. Ethanol potentiated only receptors containing the  $\gamma 2L$  subunit ( $25.5 \pm 3.8\%$  ( $n = 19$ )) and had no effect on those containing the  $\gamma 2S$  subunit ( $-6 \pm 0.8\%$  ( $n = 6$ )) (Figs. 1a,b and 2) or those consisting of only  $\alpha 1\beta 1$  ( $-8.5 \pm 2\%$  ( $n = 6$ )). As there are only eight extra amino acids in the  $\gamma 2L$  subunit, deletion mutants were made by selectively removing amino acids. GABA EC<sub>50</sub> was not affected by any of the mutations, and we used the same concentration of GABA to evaluate drug modulation. By removing either six or four amino acids we eliminated the potentiating effect of ethanol on the GABA response (Fig. 2). The eight amino acid insert contains a consensus site for protein kinase C (Ser-X-Lys, [26]). To determine its role in ethanol potentiation we made point mutations to alter this consensus sequence. Mutating Ser<sup>343</sup> to an alanine, which cannot be phosphorylated, or by changing Lys<sup>345</sup> (the C-terminal basic residue required for phosphorylation site consensus sequence), also abolished any ethanol potentiation (Fig. 1c). Benzodiazepine potentiation was not affected by any of the above mutations in the  $\gamma 2L$  subunit, demonstrating a special role for the 8 amino acid insert, and specifically the protein kinase C consensus phosphorylation site, for ethanol potentiation (Fig. 2).

The data generated by mutagenesis suggested that phosphorylation by protein kinase C was important for the potentiation of GABA receptors by ethanol. We attempted to show that this site was important in functional receptors containing the full  $\gamma 2L$  subunit by altering the state of phosphorylation. We allowed the oocytes to express GABA<sub>A</sub> receptors for two days in normal supplemented MBS medium, incubated them overnight in a medium containing 0.2 mM of the protein kinase inhibitor isoquinolinesulphonyl-2-methyl piperazine dihydrochloride (H-7), and then examined the potentiation of GABA currents by flunitrazepam and ethanol. Flunitrazepam enhanced currents in both control and H-7 treated oocytes to the same extent. Ethanol, however, was unable to potentiate GABA currents of oocytes treated with H-7 (Fig. 3). Phorbol esters stimulate protein kinase C and have been shown to reduce GABA<sub>A</sub> currents in *Xenopus* oocytes [27,28]. After 20 min treatment with 10 nM PMA the GABA response was inhibited by approximately 50%; however, the ethanol potentiation was unaffected (data not



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Fig. 2. Expression of different combinations of GABA<sub>A</sub> subunits in *Xenopus* oocytes and modulation of currents elicited by 30  $\mu$ M GABA by 20 mM ethanol and 1  $\mu$ M flunitrazepam respectively.  $\alpha_1$  and  $\beta_1$  subunits were expressed together with the  $\gamma$  subunit variants listed in (a) which illustrates the eight amino acid insert of  $\gamma_{2L}$  in bold. The amino acid deletion mutants made are indicated  $\gamma_{2\Delta 4}$  and  $\gamma_{2\Delta 6}$ , and the site-directed mutants are shown to change the Ser<sup>145</sup> to alanine ( $\gamma_{2Ser}$ ) and the Lys<sup>145</sup> to leucine ( $\gamma_{2Lys}$ ). (b) Modulation of all combinations tested by 20 mM ethanol and 1  $\mu$ M flunitrazepam. As the  $\alpha_1\beta_1$  combination had a slightly lower GABA EC<sub>50</sub>, 10  $\mu$ M GABA was used. Each bar is the mean  $\pm$  S.E.M. of six different observations on oocytes from at least 3 separate donor frogs.

shown) suggesting that the protein kinase C site on the  $\gamma_{2L}$  insert is already fully phosphorylated. We also studied the effect of cAMP dependent kinase by co-expressing the  $\beta$ -adrenergic receptor together with the GABA receptor. By activating the  $\beta$ -adrenergic receptor with 100  $\mu$ M isoproterenol which stimulates the cAMP dependent kinase or protein kinase A, again the GABA response was reduced, this time by  $21 \pm 2.7\%$  but the ethanol potentiation was not affected, with mean potentiation before exposure to isoproterenol of  $20.3 \pm 2.6\%$  ( $n = 3$ ) compared to  $22.8 \pm 4\%$  ( $n = 3$ ) after.

#### 4. DISCUSSION

By preventing phosphorylation of the receptor by either site-directed mutagenesis to remove this phosphorylation site, or by incubating *Xenopus* oocytes expressing receptors with the kinase inhibitor H-7, enhancement of the GABA current by ethanol no longer occurs, suggesting that phosphorylation of this site is necessary for ethanol potentiation. Using antisense hybridization to selectively eliminate subunit expression in *Xenopus* oocytes it has been demonstrated that the presence of the  $\gamma_{2L}$  subunit is necessary for the action of ethanol [23], and recent in vitro hybridization studies, measuring the distribution of  $\gamma_{2S}$  and  $\gamma_{2L}$  mRNAs show higher proportions of  $\gamma_{2L}$  expression in regions of the brain in which GABA<sub>A</sub> receptors have been

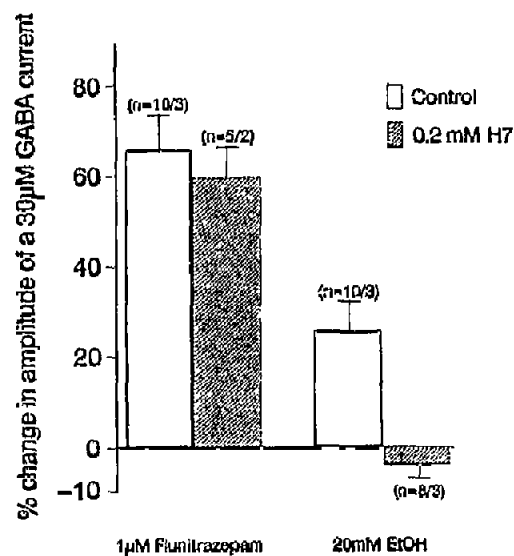


Fig. 3. Modulation of GABA currents in oocytes expressing the GABA<sub>A</sub> receptor combination of  $\alpha_1\beta_1\gamma_{2L}$ . Potentiation of a 30  $\mu$ M GABA response by 1  $\mu$ M flunitrazepam and 20 mM ethanol is shown either in untreated oocytes or those which have been incubated for 24 h in 0.2 mM isoquinolinesulphonyl-2-methyl piperazine dihydrochloride (H-7) the protein kinase inhibitor. Each bar represents the mean  $\pm$  S.E.M. of the number of oocytes tested as indicated above the bars together with the number of different donor frogs.

shown sensitive to ethanol, such as cerebellum [29,30]. However, in the hippocampus where  $\gamma 2S$  is more abundant than  $\gamma 2L$ , a number of cells are sensitive to ethanol [22], while others are not. Insensitivity to ethanol may be explained by expression of  $\gamma 2S$  rather than  $\gamma 2L$  or alternatively the level of phosphorylation/activity of kinases in these cells may be influencing the sensitivity of receptors to ethanol. *Xenopus* oocytes contain a large pool of endogenous phosphate [31] and it is possible that there may be a high level of phosphorylation when receptors are expressed in these cells. This might account for the observation that kinase inhibitors reduce the potentiation of GABA<sub>A</sub> receptors by ethanol but increasing the activity of protein kinase C with PMA does not affect the extent of enhancement. However, the fact that both PMA and cAMP dependent kinase can inhibit the GABA response itself suggests that other phosphorylation sites on the receptor are phosphorylated to a lesser degree. Other studies have also demonstrated that activation of protein kinase C [27,32] and cAMP dependent kinase can inhibit native GABA responses [33,34]. Recent studies with different subunits of the GABA<sub>A</sub> receptor show that all receptors made up of subunits which contain putative phosphorylation sites are inhibited equally by PMA [28]. Other evidence that phosphorylation may modulate ethanol sensitivity of GABA<sub>A</sub> receptors comes from studies of ethanol potentiation in mouse brain cortical microsomes where ethanol modulation is reduced with longer tissue incubation times whereas benzodiazepine and barbiturate potentiation are unaffected [36]. Important enzymes are probably lost over longer incubation times which can affect the phosphorylation state of the receptor. Ethanol modulation has also recently been shown to be highly sensitive to temperature, again suggesting some post-translational modification of the receptor is involved [30]. Further evidence which indicates that an enzyme may be involved comes from mouse lines selected for ethanol sensitivity. Genetically selected mice which are sensitive (LS) or insensitive (SS) to ethanol sedation have GABA receptors which are correspondingly sensitive (LS) or insensitive (SS) to potentiation by ethanol when whole brain mRNA is expressed in *Xenopus* oocytes [38]. Studies expressing different ratios of LS/SS brain mRNA in *Xenopus* oocytes show that SS sensitivity dominates, and that a simple mixing of different subunits cannot explain this behaviour [38]. Further, quantitation of subunit mRNA in the two strains shows no differences in the amounts or distribution of  $\gamma 2L$  or  $\gamma 2S$  [30].

The molecular mechanism by which ethanol potentiates GABA currents remains unclear. The rapid response makes it unlikely that ethanol itself modulates phosphorylation of the receptor, and recent *in vitro* experiments have shown that ethanol does not directly affect the phosphorylation of GABA<sub>A</sub> receptor subunits [39]. More likely is that phosphorylation of receptors at

Ser<sup>343</sup> alters the conformation of the receptor. This may then allow ethanol to cause its modulatory effect, perhaps through a specific binding site which could be extracellular, in the membrane spanning region, or intracellular.

Protein phosphorylation is an extremely important mechanism of receptor modulation and plays a major role in cell excitability and neuronal function (reviewed in [40]). These processes may be involved in many clinical disorders affecting signal transduction and brain function. We have demonstrated a new role for phosphorylation in the mechanism by which alcohol affects the nervous system. Individual differences in enzymes which can determine the phosphorylation state of receptors may explain differences in behavioural sensitivity to alcohol, and identification of these enzymes might provide new tools to aid in our understanding of the genetic basis of alcohol susceptibility and alcoholism.

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