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Commentary

Ethanol, γ-aminobutyrate type A receptors, and protein kinase C phosphorylation

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The search for the basis for the behavioral effects of ethanol has been prolonged and intensive. While many actions of ethanol have been reported, a specific mechanism of action of ethanol has been elusive. Proposed mechanisms of action have generally been divided into two classes: those which involve an alteration of membrane fluidity due to partitioning of ethanol into neuronal membrane lipids and those which involve an interaction between ethanol and specific neuronal membrane proteins. The latter hypothesis has held sway in recent years. Ethanol has been shown to interact with a wide variety of transmembrane voltage-gated ion channels and neurotransmitter receptor channels (1–3). However, recent emphasis has been on an interaction of ethanol with neurotransmitter receptor channels including the γ-aminobutyrate type A receptor (GABAR) channel (4, 5). Ethanol has been shown to enhance the uptake of γ-aminobutyrate (GABA)-induced Cl^- flux in a number of preparations (6–9) and to enhance GABAR currents in some (10–16), but not all (17–20), studies. The discrepancy in ethanol actions may be due to heterogeneity of GABARs in different brain regions, resulting in regionally specific effects of ethanol (6, 8, 21, 22). Behavioral data have also provided support for an action of ethanol on GABAR channels (23–26). The physiological basis for enhancement of GABAR currents and the basis for the variability among reports are unclear. The recent cloning of multiple GABAR subunits and subunit subtypes has provided new insights into possible mechanisms of ethanol interactions with GABARs. Ethanol has been shown to enhance recombinant GABAR currents when the receptors contain a splice-variant long form of the γ2 subunit (γ2L) (27), and this effect has been shown to be dependent upon phosphorylation of the subunit by Ca^{2+}/phospholipid-dependent protein kinase C (PKC) (28). This unusual observation leads to the conclusion that removal or inactivation of PKC should block the behavioral effects of ethanol. This hypothesis has been tested by Harris *et al.* (29) in this issue by examining the effects of ethanol in null mutant mice that lack the γ isoform of PKC. While the results appear to confirm the general hypothesis, they raise as many questions as they answer.

Pharmacology of GABARs. GABA is the major inhibitory neurotransmitter in the central nervous system. The GABAR is a macromolecular protein composed of a Cl^- channel with specific binding sites at least for GABA, picrotoxin, barbiturates, benzodiazepines, and neurosteroids (30), but no specific binding site for ethanol has been described. Barbiturates, benzodiazepines, and neurosteroids enhance GABAR current by binding to their specific allosteric regulatory sites. Ethanol enhancement of GABAR current does not appear to act by binding of ethanol to the barbiturate, benzodiazepine, or neurosteroid GABAR regulatory sites.

Molecular Biology of GABARs. The mammalian GABAR has structural features similar to other ligand-gated ion channels (30). At least five different subunit families (α, β, γ, δ, and ρ) have been isolated (Table 1) (31–34). Multiple cDNAs encoding various subtypes of these subunits have been isolated (35–39). Six α, four β, four γ, and two ρ subtypes and several splice variants have been described (Table 1). It is uncertain which GABAR isoforms are expressed *in vivo*, but the widespread and regionally specific distribution of GABAR subunit mRNAs identified by *in situ* hybridization (40) and the large number of subunit subtypes suggest that GABARs may exist *in vivo* in multiple isoforms.

Phosphorylation of GABARs. GABAR channel function may be modified by treatment with agents that increase protein phosphorylation. Several GABAR subunits contain consensus kinase substrate sequences in the cytoplasmic loop between the third and fourth transmembrane region for cAMP-dependent protein kinase (PKA), PKC, Ca^{2+}/calmodulin-dependent protein kinase II, and protein-tyrosine kinase (Table 1). Affinity-purified preparations of GABARs have been shown to be phosphorylated by both PKA and PKC (41, 42), suggesting that direct phosphorylation of GABARs modifies GABAR function.

All β subunit subtypes contain a conserved PKA phosphorylation site (β1, serine-409; β2, serine-410; β3, serine-408; β4′, serine-423) in a PKA consensus sequence for phosphorylation in the cytoplasmic loop between the third and fourth transmembrane regions. However, β subunits have been shown to be substrates *in vitro* for both PKA and PKC (42, 43). The phosphorylation by both kinases was completely prevented by preincubation with an antibody prepared against a synthetic peptide corresponding to the consensus substrate for PKA in the β subunits (44) and was blocked by a serine to alanine mutation (43, 45). Tryptic digestion and microsequencing revealed that the phosphorylated residue was in a fragment containing the cytoplasmic loop (44).

The γ2 subunit has been shown to be phosphorylated *in vitro* by purified PKC and Ca^{2+}/calmodulin-dependent protein kinase II, but not by PKA, at serine-327 in the cytoplasmic loop between the third and fourth transmembrane regions (42, 43, 46). The γ2 subunit subtype has an mRNA splice variant, γ2L, which contains an 8-amino acid insert with a consensus substrate sequence for PKC phosphorylation at serine-343 (47). In addition, there is a tyrosine kinase consensus sequence in the γ2 subunit outside of the spliced insert.

There are also possible PKC phosphorylation sites in α4, α5, α6, and δ subunits.

Table 1. GABAR subunit subtypes and consensus sites for phosphorylation

Subunits	No. of subtypes	No. of splice variants	Consensus sites	
			Subtype	Kinase
α	6	1	α4, α6	PKC
β	4	3	β1–β4	PKA, PKC
γ	4	1	γ1, γ3	PTK
			γ2S/L	PTK, PKC
δ	1	0	δ	PKC
ρ	2	0	ρ1, ρ2	PKC

PKA, cAMP-dependent protein kinase; PKC, Ca^{2+}/phospholipid-dependent protein kinase; PTK, protein-tyrosine kinase.

Despite the abundance of potential phosphorylation sites on GABAR subunits, the functional effects of GABAR phosphorylation by PKA or PKC remain controversial.

Regulation of GABARs by PKA. Increases in cAMP decreased GABAR function in rat cortical synaptoneurosomes (48) and in cultured chick cortical neurons (49). Injection of the catalytic subunit of PKA into mouse spinal cord neurons resulted in a reduction in GABAR currents (50). The reduction in current was due to a decrease in opening frequency. The PKA catalytic subunit has also been shown to reduce GABAR Cl^- flux into lysed and resealed rat synaptoneurosomes and to increase phosphorylation of a polypeptide that could be immunoprecipitated with antibodies specific for GABARs (51). Currents through recombinant receptor channels expressed in human embryonic kidney 293 cells with $\alpha1\beta1$ and $\alpha1\beta1\gamma2$ subunit combinations were also reduced by cAMP, and this functional modulation was prevented by site-directed mutagenesis of residue serine-409 on the $\beta1$ subunit, indicating that acute phosphorylation of this residue was responsible for the reduction of GABAR current (52).

In contrast to the above studies, in cerebellar Purkinje cells application of 8-bromo-cAMP enhanced GABAR current, mimicking the physiological effect of norepinephrine innervation, known to be mediated by β-adrenergic receptors coupled to PKA (53). Furthermore, expression of $\alpha1\beta1\gamma2$ subunit combinations in mouse fibroblast L929 cells stably transfected with cDNA encoding the catalytic subunit of PKA resulted in enhanced GABAR currents when compared with expression of $\alpha1\beta1\gamma2$ subunit combinations in the parent L929 cell line (54). The enhancement in GABAR current was abolished by mutation of the PKA phosphorylation site on the β subunit. These experiments suggested that chronic PKA phosphorylation also enhanced GABAR currents.

Alteration of GABARs by PKC. PKC was found to phosphorylate recombinant $\beta1$ and $\gamma2S$ as well as $\gamma2L$ subunits on intracellular serine residues as shown by site-directed mutagenesis; however, no functional consequences were determined in this study (43). Recently, Krishek *et al.* (45) reported that site-directed mutagenesis of the serine residues differentially reduced the effects of phorbol esters on GABAR currents expressed in human embryonic kidney cells and *Xenopus* oocytes. The functional significance and mechanisms of phosphorylation of GABARs by direct PKC treatment, however, remain uncertain. In oocytes injected with brain or GABAR subunit mRNA, phorbol esters reduced GABAR currents via activation of PKC; site-directed mutagenesis of serine-410 in the $\beta2$ subunit and serine-327 in the $\gamma2S$ subunit demonstrated that phosphorylation of these residues in the GABAR by phorbol ester-stimulated kinase was responsible for the inhibition of function (55). The phorbol ester inhibition of GABAR function expressed in oocytes also could be shown in brain microsacs, where PKC activation appeared to inhibit selectively the fraction of GABAR flux which was not rapidly desensitized by prolonged (several seconds) exposure to agonist (56).

In contrast to these studies, constitutively active PKC (PKM) has been shown to enhance recombinant $\alpha1\beta1\gamma2L$ GABAR currents recorded from transiently transfected L929 cells (57). This enhancement was blocked by mutation of the $\beta1$ and $\gamma2L$ phosphorylation sites and, thus, appeared to be due to phosphorylation of both subunits.

Alteration of GABARs by Unknown Kinases. In hippocampal neurons, GABAR currents appear to be "maintained" by phosphorylation (involving an unknown kinase and unknown substrate): the activity "runs down" in some cells when cytoplasmic contents are dialyzed during whole cell recording and can be maintained by addition of ATP and Mg^{2+} (58).

Ethanol, GABARs, and PKC Phosphorylation. The characterization of multiple subunits of GABARs, the finding that ethanol enhancement of GABAR currents was regionally specific, and the demonstration that GABAR subunits were heterogeneously distributed suggested that specific GABAR subunits were required for the enhancement of GABAR current by ethanol. To examine this question, Wafford *et al.* (27) expressed GABARs in *Xenopus* oocytes by injecting mouse brain mRNA or $\alpha1\beta1\gamma2S$ and $\alpha1\beta1\gamma2L$ cRNA and determined their ethanol sensitivity. They demonstrated that GABAR currents obtained from injecting mouse brain mRNA were enhanced by ethanol. However, when specific subunit expression was blocked by preincubation with a specific antisense oligonucleotide, ethanol sensitivity was absent when $\gamma2L$ but not $\gamma2S$ antisense oligonucleotide was injected. Furthermore, recombinant GABARs expressed with $\gamma2L$, but not with $\gamma2S$, were enhanced by ethanol. This led to the conclusion that the alternatively spliced region of $\gamma2L$ was required for ethanol sensitivity and, thus, that ethanol response heterogeneity may be based in part on regional expression of GABAR isoforms which express $\gamma2L$ and $\gamma2S$. This conclusion was confirmed by Wafford and Whiting (28), who expressed $\alpha1\beta1\gamma2L$ and $\alpha1\beta1\gamma2S$ mRNAs in *Xenopus* oocytes and showed that only GABAR currents obtained from receptors containing the $\gamma2L$ subtype were enhanced by ethanol. However, since the $\gamma2L$ insert introduced a PKC site, the question was raised whether or not PKC phosphorylation of the $\gamma2L$ serine-327 influenced ethanol enhancement of GABAR current. Wafford and Whiting (28) demonstrated that the effect of ethanol was blocked by mutating the $\gamma2L$ subtype to remove the serine in the PKC consensus in the splice insert. These results suggested another mechanism for heterogeneity: ethanol sensitivity might also depend on the presence of a phosphorylated $\gamma2L$ subunit. However, this conclusion has not had universal acceptance. Enhancement of recombinant GABAR currents expressed in human embryonic kidney 293 cells (59) or *Xenopus* oocytes (60) did not require the presence of the $\gamma2L$ splice variant. Nonetheless, these observations raise an interesting question: Can selective intracellular phosphorylation of a ligand gated receptor channel determine the regulation of the receptor channel? If so, the level of inhibition and response to exogenous (and endogenous?) regulatory compounds may be highly dependent on the phosphorylation state of the receptor. A similar statement concerning excitation may be made if similar mechanisms are confirmed for excitatory receptors such as the *N*-methyl-D-aspartate receptor (61).

The work reported by Harris *et al.* (29) in this issue of the *Proceedings* provides an important link among these many studies but raises a number of new questions. Harris *et al.* (29) have examined the GABA-induced Cl^- flux and the ability of ethanol, the benzodiazepine flunitrazepam and the barbiturate pentobarbital to enhance Cl^- flux in cortical and cerebellar synaptosomes and to cause the loss of righting reflex and reduction of body temperature in wild-type and PKCγ null mutant mice. They demonstrate that the mutant mice have no alteration in GABA-induced Cl^- flux but have reduced sensitivity to the effects of ethanol on Cl^- flux, loss of righting reflex, and hypothermia. The mutant mice show normal responses to benziodiazepine and barbiturate. These results appear to implicate this specific γ of PKC in the action of ethanol to enhance GABAR current. While the basic studies of ethanol action on GABARs have suggested a relationship between PKC phosphorylation and GABAR function, this study directly links the loss of PKCγ function with loss of ethanol behavioral effects to loss of an interaction between ethanol and GABAR function. Thus it establishes a firm relationship between the pharmacological actions of ethanol, GABA function, and PKCγ function.

However, a number of important questions remain. If PKC is important in regulating GABAR function (up or down?), then why is basal GABAR function not altered in the PKCγ null mutant? Why don't the other six PKC isoforms [α, β_I, β_{II}, δ, ε, or ξ (62)] provide the phosphorylation function lost by PKCγ? Why are membrane vesicles (microsacs) produced from cortex and cerebellum insensitive to ethanol in the PKCγ null mutant mice despite the widespread presence of the

phosphorylation of other PKC isoforms? Why is the γ2L serine-343 important for ethanol action while phosphorylation of the closely adjoining γ2L-subypte serine-327 site and the β-subunit serine site is not relevant? Is the loss of sensitivity to ethanol due to loss of direct phosphorylation of the GABAR by PKCγ, or does it result from phosphorylation of an additional intracellular protein? While the PKCγ isoform is not detectable until 7 days of age and does not reach adult levels until 14–28 days of age, it remains possible that these are subtle developmental alterations produced by its absence which indirectly influence ethanol sensitivity. None of these questions were directly answered by this study. However, this study does strongly suggest that phosphorylation of a central nervous system protein is involved in the action of GABAR current by ethanol and the behavioral effects of ethanol, and the availability of PKCγ null mutant mice provides an important new tool to be employed in the study of PKC function in the central nervous system and for characterizing the link between the behavioral actions of ethanol, PKC phosphorylation, and GABAR function. This study, therefore, provides a considerable stimulus for continued research in this area to identify the responsible mechanisms underlying the behavioral actions of ethanol.

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