Volume 324, number 2, 140–142 FEBS 12564 June 1993

Recombinant $GABA_A$ receptor function and ethanol

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Received 6 April 1993

Different combinations of cloned subunits of the rat brain $GABA_A$ receptor were expressed in *Xenopus* oocytes. Possible effects of ethanol on the expressed GABA-induced chloride current were determined. The consequence of replacing the γ_{2S} subunit by the alternatively spliced variant γ_{2L} was specifically tested on the responsiveness to ethanol. A significant stimulation of the GABA response was only observed at very high concentrations (> 60 mM) of ethanol. No differential response was observed between subunit combinations containing different γ_2 subunit splice variants.

γ-Aminobutyrate receptor; *Xenopus* oocyte; Ion channel, Ethanol; Expression

1. INTRODUCTION

$GABA_A$ transmission has long been hypothesized to be a target of ethanol action (for review see [1,2]). Reynolds et al. [3] have recently reported that low concentrations (<10 mM) of ethanol stimulate GABA currents of some but not all neuronal cells of different brain areas. Many electrophysiologists have failed to observe such ethanol effects (see references in [2,3]). The reason for these diverse findings is not known. A partial inverse benzodiazepine agonist, Ro 15-4513, which has been shown to antagonize ethanol effects at the behavioural level (for review see [2]) prevented the above-mentioned ethanol stimulation [3].

Work with recombinant $GABA_A$ receptor subunits has shown that triple combinations $\alpha_x\beta_2\gamma_{2S}$ (x = 1–6) have a high affinity for this compound, which can be displaced by the benzodiazepine diazepam, except for x = 4 and x = 6 [4–8]. The investigated recombinant receptors containing α_1 or α_6 are not functionally modulated by ethanol [5,8]. Wafford et al. have first found an effect of ethanol on recombinant $GABA_A$ receptors [9,10], showing that ethanol sensitivity is associated specifically with a consensus phophorylation site for protein kinase C that occurs in the γ_{2L} subunit, and not in the shorter splice variant γ_{2S} [11,12], in the mouse recombinant subunit combination $\alpha_1\beta_1\gamma_2$.

We have a longstanding interest in the functional modulation of $GABA_A$ receptor channels by drugs and by second messenger reactions. In the course of this work, we have studied the functional properties of numerous different recombinant subunit combinations expressed in *Xenopus* oocytes (e.g. [13]). In our hands, ethanol at moderate concentrations does not stimulate, directly or indirectly, the response of these different functional channels to the agonist GABA (γ-aminobutyrate).

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2. MATERIALS AND METHODS

The cDNAs coding for the subunits of the rat $GABA_A$ receptor channel were transcribed, capped and tailed with poly(A) as described earlier [14–16]. Combinations of the transcripts were co-precipitated in ethanol and stored at −20°C. Isolation of the follicles from the frogs, culturing of the follicles, injection with mRNA, removal of the cell layers, and electrophysiological experiments were performed at room temperature as described before [13,17]. GABA-induced ion currents were measured using the two-electrode voltage-clamp technique. GABA was applied by bath perfusion several times in order to establish a control response, in intervals that allowed full recovery of the channel from desensitization. Where not specially mentioned, ethanol was subsequently co-applied with GABA. The GABA concentration used to determine the effect of ethanol depended on the subunit combination under investigation. In each case, a GABA concentration was used which elicited 10–50% of the maximal current amplitude. A fast perfusion system was used that allowed a rate of medium range of 70% within < 0 5 s [13].

3. RESULTS AND DISCUSSION

During the course of the past years, we have expressed $GABA_A$ receptors in *Xenopus* oocytes after injection with total chick brain poly(A^+) mRNA or different recombinant subunit combinations obtained by in vitro transcription of the corresponding cDNAs. In electrophysiological experiments we have tested these combinations for their functional response to various drugs (e.g. [13]). Some of these drugs were dissolved in ethanol and required therefore test applications of the

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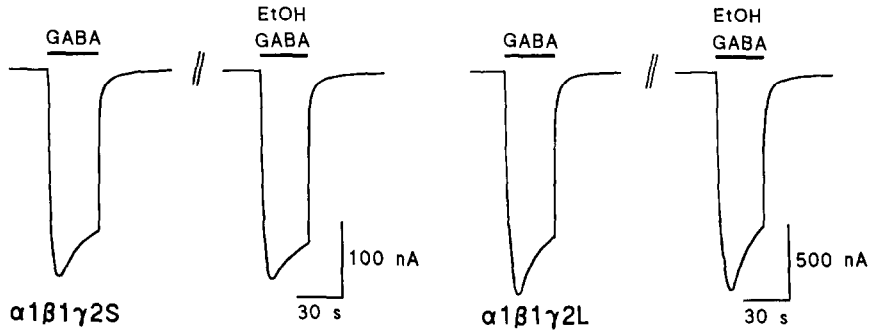

Fig. 1. Lack of stimulation by ethanol (20 mM) of the response to GABA of recombinant $\alpha_1\beta_1\gamma_{2S}$ and $\alpha_1\beta_1\gamma_{2L}$ receptor channels. The experiment was carried out as indicated in section 2 The bar indicates time period of application of GABA or GABA and ethanol, respectively.

drug vehicle alone. Concentrations of 10–100 mM ethanol did not affect GABA responses in oocytes injected with total chick brain mRNA. We also never observed a significant effect of ethanol (13 mM) in many different batches of oocytes expressing various recombinant subunit combinations (α_1(F64L)$\beta_1\gamma_{2S}$, α_1(F64L)$\beta_2\gamma_{2S}$, $\alpha_5\beta_1\gamma_{2S}$, $\alpha_5\beta_2\gamma_{2S}$, and $\beta_2\gamma_{2S}$).

As it had been published that specifically the γ_{2L} subunit is responsible for conferring ethanol stimulation, we also tested the subunit combinations $\alpha_1\beta_1\gamma_{2S}$ and $\alpha_1\beta_1\gamma_{2L}$. Expression of these two subunit combinations resulted in maximal current amplitudes amounting to several μA. Similar concentrations of GABA were needed to elicit half-maximal current amplitudes in both subunit combinations (not shown). No matter whether the GABA concentration used elicited 10 or 50% of the maximal current response, or whether ethanol was pre-applied for 30 s or not, we never observed with both subunit combinations any significant effect of 20 mM (0.09% w/v) ethanol. Fig. 1 compares effects of ethanol on the expressed subunit combinations $\alpha_1\beta_1\gamma_{2S}$ and $\alpha_1\beta_1\gamma_{2L}$, at a GABA concentration eliciting 10% of the maximal current amplitudes in the corresponding oocytes (3 μM and 1.5 μM, respectively). A range of ethanol concentrations up to 100 mM was tested for stimulation of the GABA response of the subunit combinations $\alpha_1\beta_2\gamma_{2S}$, $\alpha_1\beta_1\gamma_{2S}$ and $\alpha_1\beta_2\gamma_{2L}$, respectively. A GABA concentration eliciting about 10% of the maximal current response was used in each case. Ethanol concentrations up to 40 mM (0.18% w/v) in the β_2 containing subunit combination, and up to 60 mM (0.27% w/v) in the β_1 containing subunit combinations did not significantly stimulate the control current amplitudes (Fig. 2). At 100 mM (0.45% w/v) ethanol, a very small (< 20%) stimulation could be observed with all subunit combinations tested.

It is not clear, why the stimulatory ethanol effect at low concentrations, seen by others [9,10] cannot be reproduced. One possible reason for the discrepancy is the fact that the subunit combination containing γ_{2L} expressed in this earlier work [9,10] consistently much less current than the one containing γ_{2S}, although it is not clear how this should result in ethanol sensitivity of the currents.

Wafford et al. found an absolute requirement of the extra protein kinase C consensus phosphorylation site on γ_{2L} for the observation of ethanol effects. This was shown by loss of ethanol effect after disruption of the site by in vitro mutagenesis [9,10]. Treatment of oocytes expected to result in protein kinase C mediated phosphorylation, and which results in down modulation of GABA currents [18–20], did not abolish ethanol effects [10]. For this down-modulation, the extra phosphorylation site on γ_{2L} is not required [19,20]. From these observations, it was concluded, that the phosphorylation state of the extra phosphorylation site on γ_{2L}, which would render the $GABA_A$ receptor at the target of ethanol action, was different from that of other phosphorylation sites [10].

In summary, our experiments are difficult to reconcile with previous observations by other laboratories. In previous work the bovine subunit combination were used, whereas in the present work rat subunits were combined. The amino acid sequence of bovine and rat subunits are very similar. α_1 and $\gamma_{2S/L}$ sequences differ

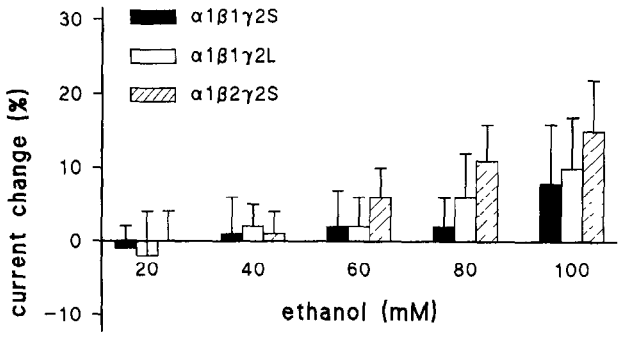

Fig. 2. Effect of higher concentrations of ethanol on the GABA response of $\alpha_1\beta_2\gamma_{2S}$, $\alpha_1\beta_1\gamma_{2S}$, and $\alpha_1\beta_1\gamma_{2L}$. Experiments were carried out at GABA concentrations eliciting about 10% of the maximal current amplitude. Three experiments with pre-application of ethanol were pooled with 3 experiments without pre-application, as no statistical difference was observed between the two sets of data. Effects of ethanol are shown as change of the control current amplitude (mean ± S.D.).

in these two species only in 1 and 3 amino acids, respectively, all located in the N-terminal, extracellular domain [11,15,21]. The β_1 subunit differs in 1 N-terminal residue, and in 9 residues located on the putative cytoplasmic domain between the two transmembrane sequences M3 and M4 [21]. Possibly, these sequence differences, subtle differences in the experimental conditions, or in the posttranslational modification of the $GABA_A$ channel in different batches of oocytes are responsible. Further experimentation is be needed to clarify the situation.

Acknowledgements We thank Prof. H. Reuter for helpful comments on the manuscript and together with Prof. W. Haefely for continuous support. The study was financially supported by the Swiss National Science Foundation.

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