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Interaction of positive allosteric modulators with human and *Drosophila* recombinant GABA receptors expressed in *Xenopus laevis* oocytes

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1 A comparative study of the actions of structurally diverse allosteric modulators on mammalian (human $\alpha_3\,\beta_2\,\gamma_{2L}$) or invertebrate (*Drosophila melanogaster Rdl* or a splice variant of *Rdl*) recombinant GABA receptors has been made using the *Xenopus laevis* oocyte expression system and the two electrode voltage-clamp technique.

2 Oocytes preinjected with the appropriate cRNAs responded to bath applied GABA with a concentration-dependent inward current. EC_{50} values of 102 ± 18 μM; 152 ± 10 μM and 9.8 ± 1.7 μM were determined for human $\alpha_3\,\beta_1\,\gamma_{2L}$, *Rdl* splice variant and the *Rdl* receptors respectively.

3 Pentobarbitone enhanced GABA-evoked currents mediated by either the mammalian or invertebrate receptors. Utilizing the appropriate GABA EC_{10}, the EC_{50} for potentiation was estimated to be 45 ± 1 μM, 312 ± 8 μM and 837 ± 25 μM for human $\alpha_3\,\beta_1\,\gamma_{2L}$, *Rdl* splice variant and *Rdl* receptors respectively. Maximal enhancement (expressed relative to the current induced by the EC_{10} concentration of GABA where this latter response = 1) at the mammalian receptor (10.2 ± 1 fold) was greater than that at either the *Rdl* splice variant (5.5 ± 1.3 fold) or *Rdl* (7.9 ± 0.8 fold) receptors.

4 Pentobarbitone directly activated the human $\alpha_3\,\beta_1\,\gamma_{2L}$ receptor with an EC_{50} of 1.2 ± 0.03 mM and had a maximal effect amounting to 3.3 ± 0.4 fold of the response evoked by the EC_{10} concentration of GABA. Currents evoked by pentobarbitone were blocked by 10–30 μM picrotoxin and potentiated by 0.3 μM flunitrazepam. Pentobarbitone did not directly activate the invertebrate GABA receptors.

5 5α-Pregnan-3α-ol-20-one potentiated GABA-evoked currents mediated by the human $\alpha_3\,\beta_1\,\gamma_{2L}$ receptor with an EC_{50} of 87 ± 3 nM and a maximal enhancement of 6.7 ± 0.8 fold of that produced by the GABA EC_{10} concentration. By contrast, relatively high concentrations (3–10 μM) of this steroid had only a modest effect on the *Rdl* receptor and its splice variant.

6 A small direct effect of 5α-pregnan-3α-ol-20-one (0.3–10 μM) was detected for the human $\alpha_3\,\beta_1\,\gamma_{2L}$ receptor (maximal effect only 0.08 ± 0.01 times that of the GABA EC_{10}). This response was antagonized by 30 μM picrotoxin and enhanced by flunitrazepam (0.3 μM). 5α-Pregnan-3α-ol-20-one did not directly activate the invertebrate GABA receptors.

7 Propofol enhanced GABA-evoked currents mediated by human $\alpha_3\,\beta_1\,\gamma_{2L}$ and *Rdl* splice variant receptors with EC_{50} values of 3.5 ± 0.1 μM and 8 ± 0.3 μM respectively. The maximal enhancement was similar at the two receptor types (human 11 ± 1.8 fold; invertebrate 8.8 ± 1.4 fold that of the GABA EC_{10}).

8 Propofol directly activated the human $\alpha_3\,\beta_1\,\gamma_{2L}$ receptor with an EC_{50} of 129 ± 10 μM, and at a maximally effective concentration, evoked a current amounting to 3.5 ± 0.5 times that elicited by a concentration of GABA producing 10% of the maximal response. The response to propofol was blocked by 10–30 μM picrotoxin and enhanced by flunitrazepam (0.3 μM). Propofol did not directly activate the invertebrate *Rdl* splice variant receptor.

9 GABA-evoked currents mediated by the human $\alpha_3\,\beta_1\,\gamma_{2L}$ receptor were potentiated by etomidate ($EC_{50} = 7.7\pm0.2$ μM) and maximally enhanced to 8 ± 0.8 fold of the response to an EC_{10} concentration of GABA. By contrast, the *Rdl*, or *Rdl* splice variant forms of the invertebrate GABA receptor were insensitive to the positive allosteric modulating actions of etomidate. Neither the mammalian nor the invertebrate receptors, were directly activated by etomidate.

10 δ-Hexachlorocyclohexane enhanced GABA-evoked currents with EC_{50} values of 3.4 ± 0.1 μM and 3.0 ± 0.1 μM for the human $\alpha_3\,\beta_1\,\gamma_{2L}$ receptor and the *Rdl* splice variant receptor respectively. The maximal enhancement was 4.5 ± 0.3 and 10.3 ± 0.3 fold that produced by the appropriate EC_{10} concentration of GABA for the mammalian and invertebrate receptors respectively. δ-Hexachlorocyclohexane did not directly activate either receptor type.

11 Loreclezole potentiated GABA-evoked currents with an EC_{50} of 7.4 ± 0.2 μM and 20 ± 1 μM for the human $\alpha_3\,\beta_1\,\gamma_{2L}$ and *Rdl* splice variant receptors respectively. A maximal enhancement of 1.9 ± 0.2 and 6.9 ± 0.2 fold (relative to the response produced by an EC_{10} concentration of GABA) was found for the mammalian and invertebrate receptors respectively. Loreclezole did not directly activate either receptor type.

12 Both the invertebrate *Rdl* receptor and its splice variant function efficiently as homo-oligomeric complexes upon expression in *Xenopus laevis* oocytes. This feature, combined with the differential pharmacology of the invertebrate and human receptors towards a variety of positive allosteric modulators, may be useful in future studies designed to determine drug binding domains on the receptor protein.

Keywords: Human recombinant $GABA_A$ receptor; *Drosophila* recombinant GABA receptor; 5α-pregnan-3α-ol-20-one; pentobarbitone; propofol; etomidate; loreclezole; δ-hexachlorocyclohexane

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Introduction

The function of the $GABA_A$ receptor is enhanced by a variety of structurally diverse agents including benzodiazepines, barbiturates, intravenous and volatile anaesthetics such as propofol, etomidate, halothane and enflurane and depressant steroids such as alphaxalone and 5α-pregnan-3α-ol-20-one (see Sieghart, 1995). Many of these positive allosteric modulators appear to bind to distinct sites on the $GABA_A$ receptor protein to produce their functional effects. With the exception of the benzodiazepines, progress in determining the nature and location of these binding sites has been hampered by the lack of suitable radioligands. Either their relatively low affinities for the receptor, or their relatively high lipid solubilities, or a combination of these two properties, make direct binding studies impossible.

The $GABA_A$ receptor is a multisubunit (probably pentameric) structure and the expression of different combinations of subunits results in the formation of $GABA_A$ receptors with distinctive physiological and pharmacological properties (see Smith & Olsen, 1995; Sieghart, 1995). Benzodiazepines exhibit strict subunit requirements for binding and function. This feature, together with the technique of site-directed mutagenesis, has been utilized to define better their binding domains on the receptor protein complex (see Smith & Olsen, 1995). Although not investigated systematically, many of the allosteric agents described above do not exhibit an absolute subunit specificity. Therefore, the utilization of such non-discriminating mammalian vertebrate subunits to define drug binding sites may not be fruitful for such compounds.

Recently a cDNA (*Rdl*) encoding for a GABA receptor from *Drosophila melanogaster* has been isolated. When expressed in *Xenopus* oocytes this subunit forms functional, presumably homomeric GABA-gated chloride channels (ffrench-Constant *et al.*, 1993). We have demonstrated that a splice variant of *Rdl* also functions efficiently as a homo-oligomeric complex and exhibits a distinctive pharmacological profile when compared to its vertebrate counterparts (Chen *et al.*, 1994). Hence, this subunit may be of value in future chimaera, or mutagenesis studies aimed at defining binding sites for structurally diverse allosteric modulators.

As a prelude to such studies, the present investigation has examined the GABA enhancing and direct agonist effects of four chemically distinct intravenous anaesthetic agents, propofol, etomidate, 5α-pregnan-3α-ol-20-one and pentobarbitone, together with the positive allosteric modulators δ-hexachlorocyclohexane and loreclezole. The actions of these agents have been compared for *Xenopus* oocytes expressing the human $\alpha_3\,\beta_1\,\gamma_{2L}$ subunit combination and the invertebrate *Rdl* and *Rdl* splice variant subunits. A prelimary account of part of this work has appeared in abstract form (Belelli *et al.*, 1994, 1995).

Methods

cDNAs encoding the human α_3, β_1 and γ_{2L} $GABA_A$ receptor subunits were linearized at the *Hpa I*, *BamH I* and *Not I* sites in the pCDM8 vector respectively. The cDNAs coding for the *Drosophila Rdl* subunit in the NB40 vector, and its splice variant in SK(-) Bluescript vector, were linearized at unique *Not I* and *Apa I* sites respectively. cRNA transcripts were prepared according to standard protocols (Hope *et al.*, 1993). The integrity of the transcripts was examined by denaturing gel electrophoresis prior to injection. The cRNA transcripts were injected (30–50 nl of 1 mg ml^{-1} cRNA) into *Xenopus laevis* oocytes (Stage V–VI) which had previously been defolliculated by treatment with 2 mg ml^{-1} collagenase 'A' (Boehringer-Mannheim) for 3 h at room temperature (20–23°C) in Barth's saline with Ca^{2+} salts omitted. Injected oocytes were individually maintained at 19–20°C for up to 12 days in 96-well plates containing 200 μl of standard Barth's solution (composition in mM: NaCl 88, KCl 1, $NaHCO_3$ 2.4, HEPES 15, $Ca(NO_3)_2$ 0.5, $CaCl_2$ 0.5 and $MgSO_4$ 1.0; adjusted to pH 7.6 with NaOH). The solution was supplemented with 0.1 mg ml^{-1} gentamicin.

Oocytes were used for experimentation 2–12 days after cRNA injection. The methodology was essentially as previously described (Hope *et al.*, 1993; Chen *et al.*, 1994). Briefly, electrical recordings were made from oocytes voltage-clamped at −60 mV using an Axoclamp 2A, or a GeneClamp 500 amplifier (Axon Instruments, U.S.A.) in the two-electrode voltage-clamp mode. The oocytes were held in a chamber (0.5 ml) and continuously superfused (7–10 ml min^{-1}) with frog Ringer solution (composition in mM: NaCl 120, KCl 2.0, $CaCl_2$ 1.8, HEPES 5.0; adjusted to pH 7.4 with NaOH). The voltage-sensing and current-passing electrodes were filled with 3 M KCl and had resistances of 1–2 MΩ when measured in frog Ringer solution. Agonist-induced responses were low pass filtered at 250 Hz and recorded onto magnetic or digital audio tapes (DAT) *via* a Racal Store 4DS F.M. tape recorder, or a Biologic DTR 1204 DAT recorder respectively and simultaneously displayed on a chart recorder. The peak amplitude of agonist-evoked currents was measured manually. All drugs were applied by the superfusion system. For each oocyte, a maximal concentration of GABA (3 mM) was applied and the resultant peak current amplitude determined. This concentration of GABA was reapplied at 30 min intervals until the current amplitude was consistent to within ±2% over three GABA challenges. The current amplitude recorded from oocytes expressing the recombinant invertebrate receptors usually stabilized within three to four applications of GABA and remained constant for up to 8 h. Some oocytes expressing the human recombinant $\alpha_3\,\beta_1\,\gamma_{2L}$ subunits also stabilized within this test period. However, for the majority (≈70%) of oocytes expressing these subunits, the current amplitude induced by the maximal concentration of GABA increased substantially with repeated applications over a period of 3 to 4 h and in some extreme cases for up to 6 h. Once stabilized, the current amplitude remained constant throughout the experimental period. In all cases, care was taken to ensure that the amplitude of the GABA-activated current had stabilized before proceeding with the experiment.

To investigate the enhancement of agonist-evoked responses by putative allosteric modulators, a concentration of GABA producing a peak current approximately 10% of the maximum (EC_{10}) was determined on each oocyte for the human and invertebrate GABA receptors (see below). Positive allosteric modulators were pre-applied for 30 to 60 s prior to co-application with the appropriate concentration of GABA. The direct agonist action of the compounds was also investigated in the absence of GABA, and where evident, was expressed relative to the current induced by an EC_{10} concentration of GABA. Concentration-response data obtained for GABA or modulators acting directly as agonists were fitted, by use of Fig P version 6c (Biosoft, Cambridge, U.K.), with the sigmoidal function:

$$\frac{I}{I_{max}} = \frac{[A]^{n_H}}{[A]^{n_H} + [EC_{50}]^{n_H}}$$

where [A] is the concentration of GABA or the modulator; I_{max} is the maximum inward current evoked by GABA, or by the modulator; I is the inward current produced by concentration [A] of GABA or modulator; EC_{50} is the concentration of GABA, or modulator, required to evoke a response amounting to 50% of their own maximal response and n_H is the Hill coefficient. This equation was also used to evaluate the GABA enhancing effects of the allosteric modulators, in which case I_{max} is the current evoked by GABA in the presence of a maximal enhancing concentration of the modulator; I is the inward current evoked by GABA in the presence of con-

centration [A] of the modulator; EC_{50} is the concentration of modulator producing a half-maximal enhancement of the GABA-evoked current and n_H = the Hill coefficient. In order to compare the degree of potentiation elicited by different allosteric modulators, the peak amplitude of GABA-evoked responses recorded in the presence of the modulator are expressed relative to the response evoked by a concentration of GABA producing 10% of the GABA maximum response in the absence of the modulator. The current induced by an EC_{10} concentration of GABA is normalized to equal 1. The specific involvement of both human and invertebrate GABA receptors in the direct effects of modulatory agents was assessed by examining whether the evoked currents were susceptible to block by picrotoxin at concentrations of 30 μM and 1 μM respectively. For human GABA receptors only, direct effects of modulators were additionally examined for sensitivity to potentiation by flunitrazepam (300 nM). Experiments were conducted at ambient temperature (18–22°C). Quantitative data are reported as the mean ± s.e.mean. The s.e.mean associated with EC_{50} values is that derived from the fitted curve.

Drugs used

The reagents used in the study were: γ-aminobutyric acid (GABA), sodium pentobarbitone, 5α-pregnan-3α-ol-20-one (5α3α), δ-hexachlorocyclohexane (δ-HCH) all obtained from Sigma, 2,6-diisopropylphenol (propofol) from Aldrich, loreclezole and etomidate from (Janssen). Stock solutions of all drugs were prepared daily. Propofol and 5α3α were prepared as 1 or 10 mM stocks in 100% ethanol and 100% dimethylsulphoxide (DMSO) respectively and subsequently diluted into frog Ringer solution with a maximal final vehicle concentration of 0.1% vol/vol, which alone had no effect upon GABA-activated currents. All other drugs were prepared as concentrates in frog Ringer solution.

Results

Oocytes preinjected (2–12 days earlier) with cRNA encoding human α_3, β_1 and γ_{2L} subunits responded to bath applied GABA (100 μM) with an inward current at a holding potential of −60 mV. The GABA-induced current was concentration-dependent with a threshold effect at 1 μM and a maximal response occurring at 3 mM (Figure 1). Analysis of the GABA concentration-response curve yielded an EC_{50} of 102 ± 18 μM (n = 4) and a Hill coefficient of 1.2 ± 0.1 (n = 4). We have previously demonstrated (Chen *et al.*, 1994), under identical recording conditions, that oocytes injected with cRNA coding for the *Rdl* splice variant respond to GABA with a concentration-dependent (0.03–3 mM) inward current, with an EC_{50} of 152 μM and a Hill coefficient of 1.7 (see Figure 1). Here, the *Rdl* receptor was found to be substantially more sensitive to GABA (1–300 μM), with a calculated EC_{50} of 9.8 ± 1.7 μM and a Hill coefficient of 1.6 ± 0.1 (n = 5; see Figure 1). The positive allosteric actions of pentobarbitone at the $GABA_A$ receptor are well established (see Sieghart, 1995). Consistent with these observations, pentobarbitone (1 μM–300 μM) produced a concentration-dependent enhancement of GABA-evoked currents recorded from oocytes expressing human α_3 β_1 γ_{2L} subunits (Figures 2 and 3). The concentration-effect curve for pentobarbitone was bell-shaped. Potentiation was apparent with 3 μM pentobarbitone and was maximal (10.2 ± 1 times the response induced by the GABA EC_{10}, n = 4) at a hundred fold higher concentration of the barbiturate. The EC_{50} for pentobarbitone, calculated over the range 1–300 μM, was 45 ± 1 μM (see Table 1 and Figure 3). At the higher concentration of 1 mM, the enhancement by pentobarbitone was reduced (Figure 3). In the absence of GABA, pentobarbitone (300 μM–3 mM) produced concentration-dependent inward currents with an EC_{50} of 1.2 ± 0.03 mM and a steep Hill coefficient of 2.7 ± 0.2. Such currents were blocked by 10–30 μM picrotoxin and enhanced by 0.3 μM flunitrazepam (Figure 2).

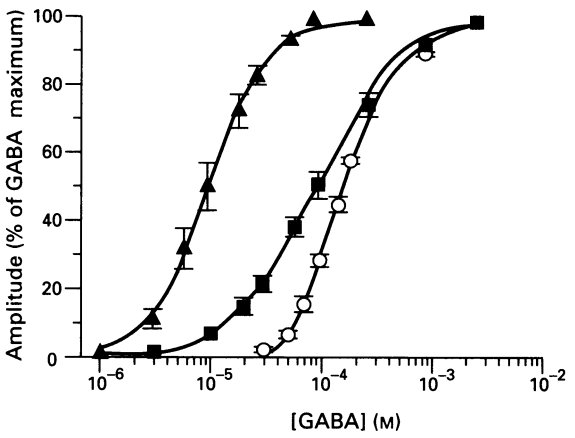

Figure 1 The properties of the GABA-induced currents recorded under voltage-clamp from *Xenopus laevis* oocytes expressing human and invertebrate recombinant GABA receptors. Concentration-dependence of the inward current response evoked by GABA (expressed as a percentage of the current produced by a saturating concentration of GABA) for the human $\alpha_3\beta_1\gamma_{2L}$ (■) the *Rdl* (▲) and the *Rdl* splice variant (○) recombinant receptors. Curves were fitted using a logistic equation (see Methods) and yielded respective EC_{50} and pseudo-Hill coefficients for GABA of 102 ± 11 μM, 1.2 ± 0.1 (human $\alpha_3\beta_1\gamma_{2L}$); 9.8 ± 1.7 μM, 1.64 ± 0.11 (*Rdl.*), and 152 ± 10 μM; 1.7 ± 0.07 (*Rdl* splice variant). Data points represent the mean with s.e.mean of observations made from at least 4 oocytes. All experiments were conducted at a holding potential of −60 mV. The data for the GABA-concentration response curve for the *Rdl* splice variant are taken from Chen *et al.* (1994) and are presented for comparative purposes.

The maximal current (3.3 ± 0.4 times that produced by the GABA EC_{10} concentration, n = 9) occurred in response to 3 mM pentobarbitone (Figures 2 and 3). Concentrations of pentobarbitone > 1 mM elicited a complex response consisting of an initial peak followed by a decline which was succeeded upon wash-out by the redevelopment of an inward current. The magnitude of the latter was concentration-dependent, and amounted to 7.2 ± 0.2 times that produced by the GABA EC_{10}, n = 6) upon washout of the highest concentration (6 mM) of pentobarbitone tested (Figures 2 and 3).

Pentobarbitone (30 μM–1 mM) also produced a concentration-dependent enhancement of GABA-evoked currents recorded from oocytes expressing the splice variant of *Rdl*. Potentiation of the GABA-evoked current was evident at 30 μM and was maximal at 1 mM (5.5 ± 1.3 fold, n = 4) and demonstrated an EC_{50} of 312 ± 8 μM; see Table 1; Figures 2 and 3). Higher concentrations of pentobarbitone (i.e. 3 mM) were associated with a potentiation of smaller magnitude. Picrotoxin is a potent antagonist of the receptor encoded by the splice variant of *Rdl* (Chen *et al.*, 1994). However, although pentobarbitone (≥ 100 μM) occasionally induced a small inward current in the absence of GABA, this response was not blocked by a relatively high concentration of picrotoxin (1 μM; not shown). Furthermore, such currents were observed on some non-injected oocytes and are, therefore, not mediated through the GABA receptor. Pentobarbitone (30 μM–6 mM) also enhanced GABA-evoked currents recorded from oocytes expressing the *Rdl* subunit. Potentiation was evident at 30–100 μM and was maximal at 3 mM (7.9 ± 0.8 fold, n = 3) with an EC_{50} of 837 ± 25 μM (Figure 3, Table 1). Low concentrations (30 μM–1 mM) of pentobarbitone produced quantitatively similar degrees of potentiation at the *Rdl* receptor and its splice variant. However, whereas 3 mM pentobarbitone produced maximal enhancement of GABA responses mediated by the *Rdl* receptor, this concentration of barbiturate lay upon the descending limb of the bell-shaped concentration-response relationship observed for pentobarbitone at the splice variant form of the receptor.

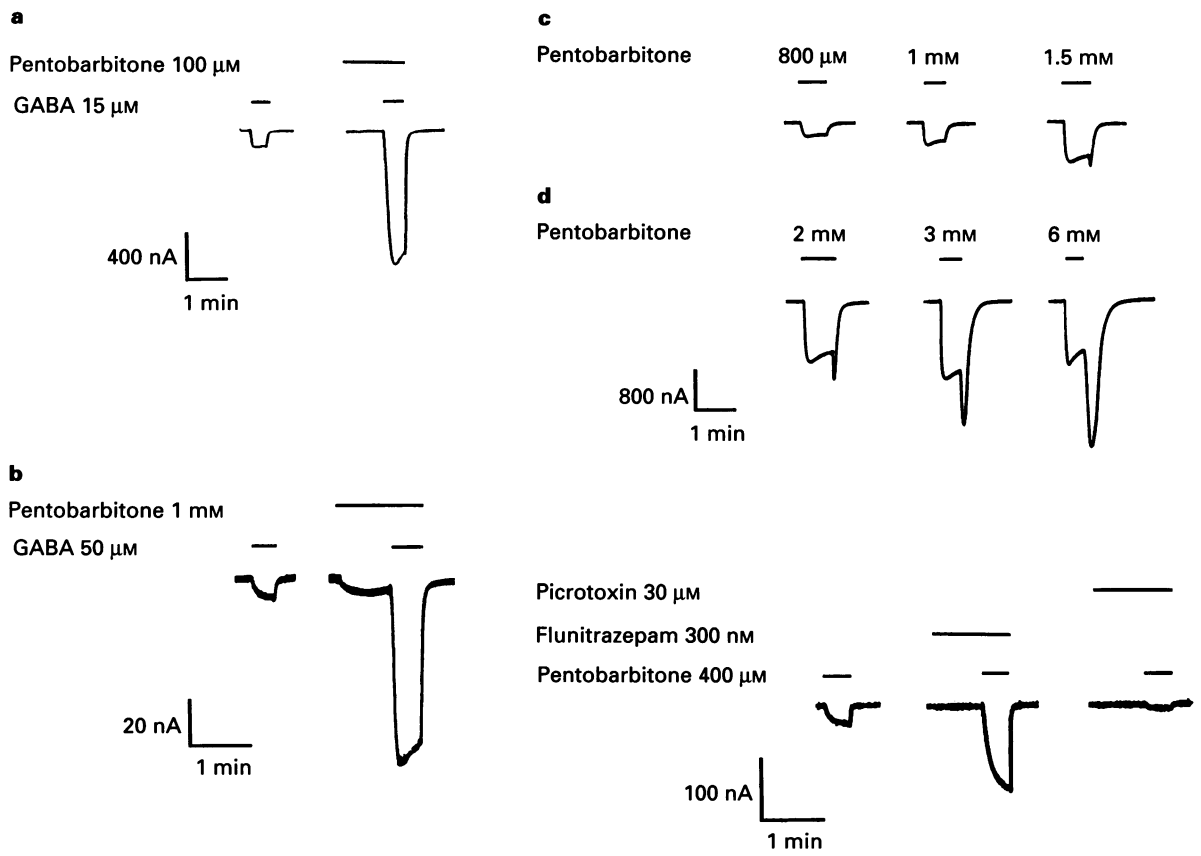

Figure 2 Pentobarbitone acts as a positive allosteric modulator of both mammalian and invertebrate recombinant GABA receptors. (a) Bath applied pentobarbitone (100 μM) greatly potentiates the current-evoked by 15 μM GABA (approximate EC_{10} in this example) recorded from an oocyte expressing the human $\alpha_3\beta_1\gamma_{2L}$ subunit combination. (b) Similarly, 1 mM pentobarbitone greatly enhanced the current evoked by 50 μM GABA (approximate EC_{10} for this cell) recorded from an oocyte expressing the *Drosophila Rdl* splice variant. Note that the small inward current elicited by pentobarbitone alone is not due to the activation of the invertebrate GABA receptors (see text). (c) In the absence of GABA, bath application of relatively high concentrations (0.8–6.0 mM) of pentobarbitone to oocytes expressing the human $\alpha_3\beta_1\gamma_{2L}$ subunit combination induces a concentration-dependent inward current. Note that concentrations of pentobarbitone $\geq$ 1.5 mM elicit a complex response consisting of an initial peak, a fade towards baseline in the continued presence of the barbiturate, and upon washout, a redevelopment of the inward current ('wash-out' current). (d) Currents induced by pentobarbitone (400 μM) are potentiated by the co-application of flunitrazepam (300 nM) and blocked by picrotoxin (30 μM). Drug effects were examined upon the same oocyte expressing the human $\alpha_3\beta_1\gamma_{2L}$ subunit combination. All records were obtained at a holding potential of −60 mV.

5α-pregnan-3α-ol-20-one (5α3α)

Some pregnane steroids are potent positive allosteric modulators of mammalian $GABA_A$ receptors (see Lambert *et al.*, 1995; Sieghart, 1995). In the present study, 5α3α (1 nM–1 μM) produced a concentration-dependent enhancement of GABA-evoked currents recorded from oocytes expressing the human $\alpha_3\ \beta_1\ \gamma_{2L}$ subunit combination (Figure 4). Potentiation was evident at 1 nM and maximal at 1 μM (6.7±0.8 fold, $n=5$) with an EC_{50} of 87±3 nM (Table 1). In the absence of GABA, 5α3α (0.3–10 μM) induced a small inward current with a maximal effect (at 10 μM) of only 0.08±0.01 times that of an EC_{10} GABA concentration ($n=4$). The small magnitude of the steroid-induced currents precluded the determination of an EC_{50} value. Steroid-induced currents were antagonized by picrotoxin (30 μM) and enhanced by flunitrazepam (0.3 μM, see Figure 4c). Presumably, this current results from a direct activation of the $GABA_A$ receptor complex by the steroid (see Lambert *et al.*, 1995).

We have previously reported that GABA currents recorded from the oocytes expressing the splice variant form of *Rdl* are unaffected by concentrations of 5α3α (0.1–1 μM) that produce a robust enhancement of GABA-evoked currents mediated by vertebrate $GABA_A$ receptors. Relatively high concentrations (3–10 μM) of the steroid can, however, elicit a modest enhancement of the GABA current mediated by the *Rdl* splice variant (Chen *et al.*, 1994). For comparison, these data are shown in Figure 4b and d. In this study, the *Rdl* receptor was found to be similarly insensitive and 10 μM 5α3α produced only a small enhancement of the GABA-evoked current (2.3±0.2 fold, $n=4$; data not illustrated).

The interaction of 5α-pregnan-3α-ol-20-one and pentobarbitone

It is well established that chemically disparate compounds may interact with the $GABA_A$ receptor to produce a common effect, namely an enhancement of the GABA-mediated current (see Sieghart, 1995). Whether these structurally distinct compounds interact with a common binding site(s) on the receptor protein is not known. To begin investigating this question, we have determined the effects of binary combinations of 5α3α and pentobarbitone upon the $\alpha_3\ \beta_1\ \gamma_{2L}$ receptor. The enhancement by 5α3α of GABA currents recorded from oocytes expressing rat brain $GABA_A$ receptors is associated with a sinistral shift of the GABA concentration-response relationship (Woodward *et al.*, 1992). In agreement with that study, 5α3α (60 nM–1 μM) produced a concentration-dependent reduction of the estimated GABA EC_{50}. A maximal shift, of approximately 11.4 fold, occurred when 5α3α was applied at a concentration of 0.6 μM (Figure 5a). Increasing the steroid concentration to 1 μM produced no further shift of the GABA EC_{50} (data not shown). Similarly, pentobarbitone produced a concentration-dependent reduction of the EC_{50} for GABA,

with 30 and 60 μM pentobarbitone producing approximately 3.3 and 7.3 fold shifts respectively (Figure 5b). Unfortunately, higher concentrations of the barbiturate could not be investigated due to complications arising from the direct agonist effects of this compound. In the presence of a maximally effective concentration (600 nM) of 5α3α, the shifts in the GABA EC_{50} produced by 30 μM and 60 μM pentobarbitone (i.e. 3.5 and 8.2 fold respectively) were similar to those produced in the absence of the steroid. This result suggests that these compounds act at discrete loci. On-going experiments are investigating the influence of binary combinations of the other agents investigated here.

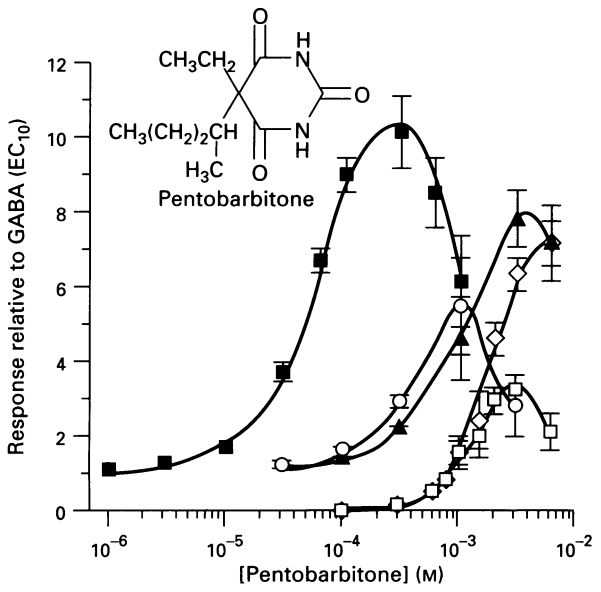

Figure 3 Pentobarbitone produces a concentration-dependent enhancement of GABA-evoked currents recorded from oocytes expressing mammalian or invertebrate GABA receptors. The graph illustrates the relationship between the concentration of bath applied pentobarbitone (logarithmic scale) and the current produced (on a linear scale and expressed relative to the current induced by an EC_{10} concentration of GABA where that response is normalised to equal 1). Data give the potentiation of GABA at the human $\alpha_3\beta_1\gamma_{2L}$ receptor (■), the *Rdl* receptor (▲) and the *Rdl* splice variant receptor (○). Additionally, the peak direct current elicited by pentobarbitone alone at the human $\alpha_3\beta_1\gamma_{2L}$ receptor is plotted (□). As depicted in Figure 2, the washout of relatively high concentrations of pentobarbitone was associated with the development of a 'washout' current. The concentration-dependency of this current is also illustrated (◇). Each data point represents the mean with s.e.mean of data obtained from 4–9 oocytes. All data were obtained from oocytes voltage-clamped at −60 mV. The data for the *Rdl* splice variant are reproduced from Chen *et al.* (1994). The inset shows the chemical structure of pentobarbitone.

Propofol

The general anaesthetic, propofol, is a positive allosteric modulator of the $GABA_A$ receptor (Hales & Lambert, 1991; Lin *et al.*, 1992; Hara *et al.*, 1993; Orser *et al.*, 1994). Consistent with these reports, propofol (0.3–30 μM) produced a concentration-dependent potentiation of GABA-evoked currents recorded from oocytes expressing the human $\alpha_3\ \beta_1\ \gamma_{2L}$ subunit combination (Figure 6). Enhancement of the GABA current was discernible with concentrations of propofol as low as 0.3 μM and was maximal at 30 μM (11 ± 1.8 fold, $n=4$). The EC_{50} value was estimated to be 3.5 ± 0.1 μM (Table 1).

In addition to promoting the interaction of GABA with the $GABA_A$ receptor, propofol can directly activate the receptor channel complex (Hales & Lambert, 1991; Hara *et al.*, 1993; Orser *et al.*, 1994). In the absence of GABA, propofol induced a concentration-dependent (10–300 μM) inward current with a maximal effect (at 300 μM) amounting to 3.5 ± 0.5 times ($n=5$) that produced by the EC_{10} concentration of GABA. At a higher concentration (1 mM), the magnitude of the current was reduced. Analysis of the concentration-response curve yielded an EC_{50} of 129 ± 10 μM and a steep Hill slope of 3.8 ± 0.8. Propofol-induced currents were antagonized by picrotoxin (10 μM–30 μM) and enhanced by flunitrazepam (0.3 μM) and therefore, probably result from the direct activation of the $GABA_A$ receptor complex by the anaesthetic.

Propofol (0.3–100 μM) also produced a concentration-dependent enhancement of GABA-evoked currents recorded from oocytes expressing the splice variant of *Rdl* (Figure 6). The effective concentration-range was similar to that for the human $\alpha_3\ \beta_1\ \gamma_{2L}$ combination. Hence, potentiation was evident at 1.0 μM and was maximal at 100 μM (8.8 ± 1.4 fold, $n=4$) with an EC_{50} of 8 ± 0.3 μM (Table 1). At concentrations ⩾10 μM, small inward currents to propofol alone were observed on some oocytes (see Figure 6). However, such currents are unlikely to result from direct stimulation of the GABA receptor because they were insensitive to picrotoxin (1 μM) and occasionally were observed on uninjected oocytes.

Table 1 Quantification of the potency and maximal effect of various allosteric modulators acting on human recombinant $\alpha_3\ \beta_1\ \gamma_{2L}$ $GABA_A$ receptors and *Drosophila Rdl* splice variant GABA receptors.

	Human ($\alpha_3\ \beta_1\ \gamma_{2L}$)			*Drosophila*			
Allosteric Modulator	*Effective concentration range*	*EC_{50}*	*Maximum effect*	*Effective concentration range*	*EC_{50}*	*Maximum effect*	*EC_{50} Drosophila/ EC_{50} Human*
Propofol	0.3–30 μM	3.5 ± 0.1 μM	11 ± 1.8 (30 μM)	1–100 μM	8.0 ± 0.3 μM	8.8 ± 1.4 (100 μM)	2.3
Etomidate	1–100 μM	7.7 ± 0.2 μM	8.0 ± 0.8 (100 μM)	Minimal effect at 100 μM	–	–	–
5α-Pregnan-3α-ol-20-one	1 nM–1 μM	87 ± 3 nM	6.7 ± 0.8 (1 μM)	3–10 μM	ND	2.0 ± 0.2 (10 μM)	–
δ-Hexachlorocyclohexane	0.3–10 μM	3.4 ± 0.1 μM	4.5 ± 0.3 (10 μM)	0.3–30 μM	3.0 ± 0.1 μM	10.3 ± 0.3 (30 μM)	0.9
Loreclezole	3–30 μM	7.4 ± 0.2 μM	1.9 ± 0.2 (30 μM)	3–100 μM	20.3 ± 0.5 μM	6.9 ± 0.2 (100 μM)	2.7
Pentobarbitone	1–300 μM	45 ± 1 μM	10.2 ± 1 (300 μM)	30 μM–1 mM	312 ± 8 μM	5.5 ± 1.3 (1 mM)	6.9
Pentobarbitone*	1–300 μM	45 ± 1 μM		30 μM–3 mM	837 ± 25 μM	7.9 ± 0.8 (3 mM)	18.6

The maximum effect is expressed relative to the response elicited by an EC_{10} concentration of GABA (see Methods) and where that response is normalised to equal 1. The concentration of modulator producing the maximum effect is given in parentheses. ND: not determined. *Indicates the effect of pentobarbitone on the *Rdl* subunit.

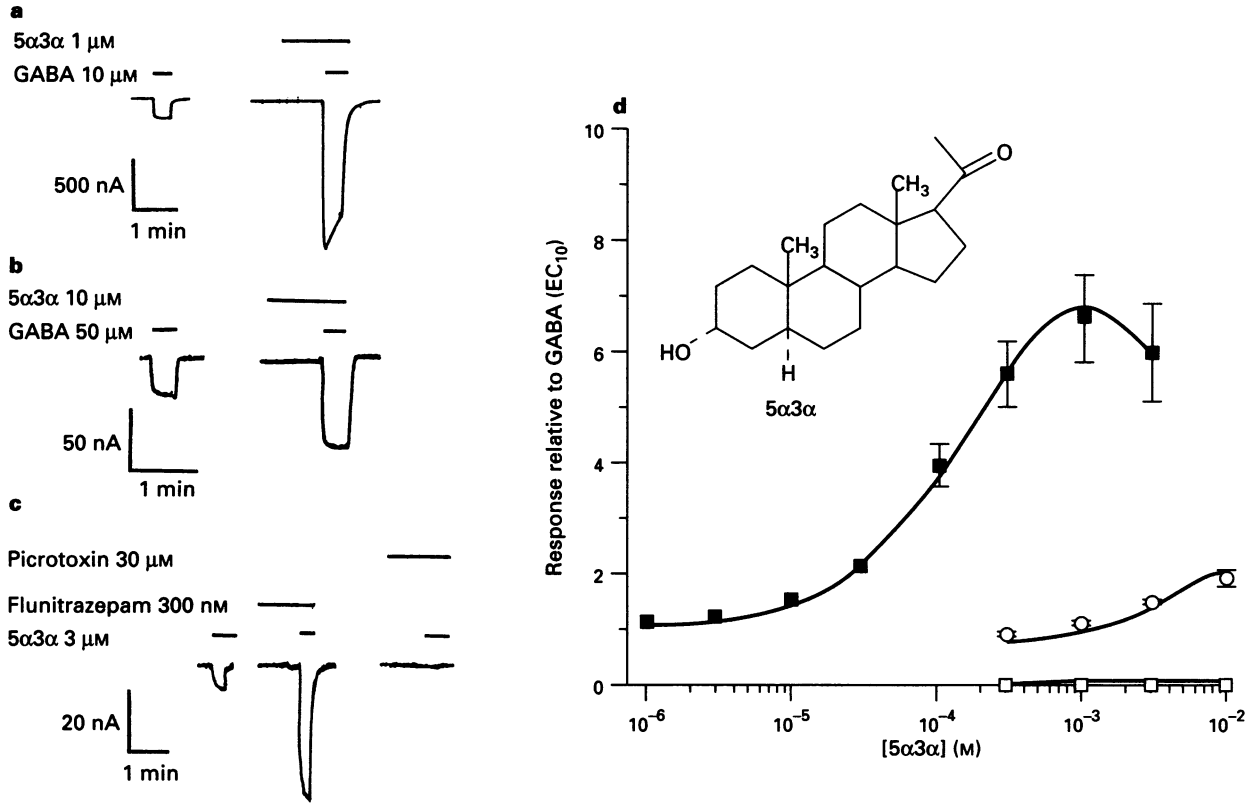

Figure 4 5α-Pregnan-3α-ol-one (5α3α) acts as a positive allosteric modulator of mammalian $GABA_A$ receptors, but has little effect on the invertebrate *Rdl* splice variant receptor. (a) Bath application of 1 μM 5α3α greatly potentiates the current evoked by 10 μM GABA (approximate EC_{10} in the exemplar cell) recorded from an oocyte expressing the human $\alpha_3\beta_1\gamma_{2L}$ subunit combination. (b) A much higher concentration of 5α3α (10 μM) produces only a modest enhancement of the current evoked by 50 μM GABA (approximately the EC_{10} for this oocyte) from a cell expressing the invertebrate *Rdl* splice variant receptor. (c) In the absence of GABA, the bath application of 3 μM 5α3α induced a relatively small inward current for oocytes expressing the human $\alpha_3\beta_1\gamma_{2L}$ subunit combination. Such currents were enhanced by flunitrazepam (300 nM) and blocked by picrotoxin (30 μM). (d) Graphical depiction of the relationship between the concentration of bath-applied 5α3α (logarithmic scale) and the current elicited by an EC_{10} concentration of GABA (on a linear scale and expressed relative to the current induced by an EC_{10} concentration of GABA where that response is normalized to equal 1). Data illustrated are for human $\alpha_3\beta_1\gamma_{2L}$ receptors (■) and the *Rdl* splice variant receptor (○). Also plotted is the peak direct current elicited by 5α3α alone (□) for human $\alpha_3\beta_1\gamma_{2L}$ receptors. Data points represent the mean with s.e.mean of data obtained from 4-8 oocytes. Curves were fitted as described in Methods. The inset shows the chemical structure of 5α3α. All data were obtained from oocytes voltage-clamped at a holding potential of −60 mV.

Etomidate

The intravenous general anaesthetic etomidate produced a concentration-dependent (1–100 μM) enhancement of the GABA-evoked current recorded from oocytes expressing the human $\alpha_3\ \beta_1\ \gamma_{2L}$ receptor subunits (Figure 7). Enhancement was apparent at 1 μM and maximal at 100 μM etomidate (8±0.8 fold, $n=4$) with an EC_{50} of 7.7±0.2 μM. Etomidate did not directly activate the human receptor. By contrast to the clear allosteric regulation by etomidate of human recombinant receptors, the compound produced little effect (1.2±0.1 times that produced by the EC50 concentration of GABA, $n=4$) upon the GABA-evoked current recorded from oocytes pre-injected with the *Rdl* splice variant. The *Rdl.* receptor was similarly insensitive to this anaesthetic with an even higher concentration of 100 μM producing a response of only 12.2±1.3% (1.18±0.1 times $n=3$) that produced by the EC_{10} concentration of GABA (data not illustrated).

δ-Hexachlorocyclohexane (δ-HCH)

In confirmation of a previous report (Woodward *et al.*, 1992), δ-HCH produced a concentration-dependent enhancement of GABA-evoked currents recorded from oocytes expressing the human $\alpha_3\ \beta_1\ \gamma_{2L}$ subunit combination (Figure 8, Table 1). This effect was apparent at 300 nM and maximal at 10 μM (4.5±0.3 fold, $n=4$). The EC_{50} was calculated to be 3.4±0.1 μM. In contrast to pentobarbitone, propofol and 5α3α, high concentrations (10–30 μM) of δ-HCH, in the absence of GABA, did not induce an inward current. Higher concentrations were not investigated due to limitations imposed by solubility. δ-HCH also produced a concentration-dependent (0.3–30 μM) enhancement of GABA-evoked currents recorded from oocytes expressing the splice variant of the *Rdl.* subunit (Figure 8, Table 1). The EC_{50} (3.0±0.1 μM) for this effect was similar to that determined for the human recombinant receptor. However, the maximal enhancement produced by the compound at a concentration of 30 μM (10.3±0.3 fold, $n=4$) was substantially greater than that occurring with the $\alpha_3\ \beta1_1\ \gamma_{2L}$ receptor (4.5±0.3 fold, $n=4$; see Figure 8 and Table 1). Again, in the absence of GABA, high concentrations (10–30 μM) of δ-HCH did not induce an inward current.

Loreclezole

Recent studies have revealed the anticonvulsant loreclezole to produce a potent and relatively large enhancement of GABA-evoked currents recorded from cells expressing recombinant receptors which contain either the β_2 or β_3 subunit (Wafford *et al.*, 1994). By contrast, those receptors which include a β_1 subunit are only modestly affected by relatively large concentrations of this compound (Wafford *et al.*, 1994). Here, loreclezole (3–30 μM) produced a concentration-dependent enhancement of GABA-evoked

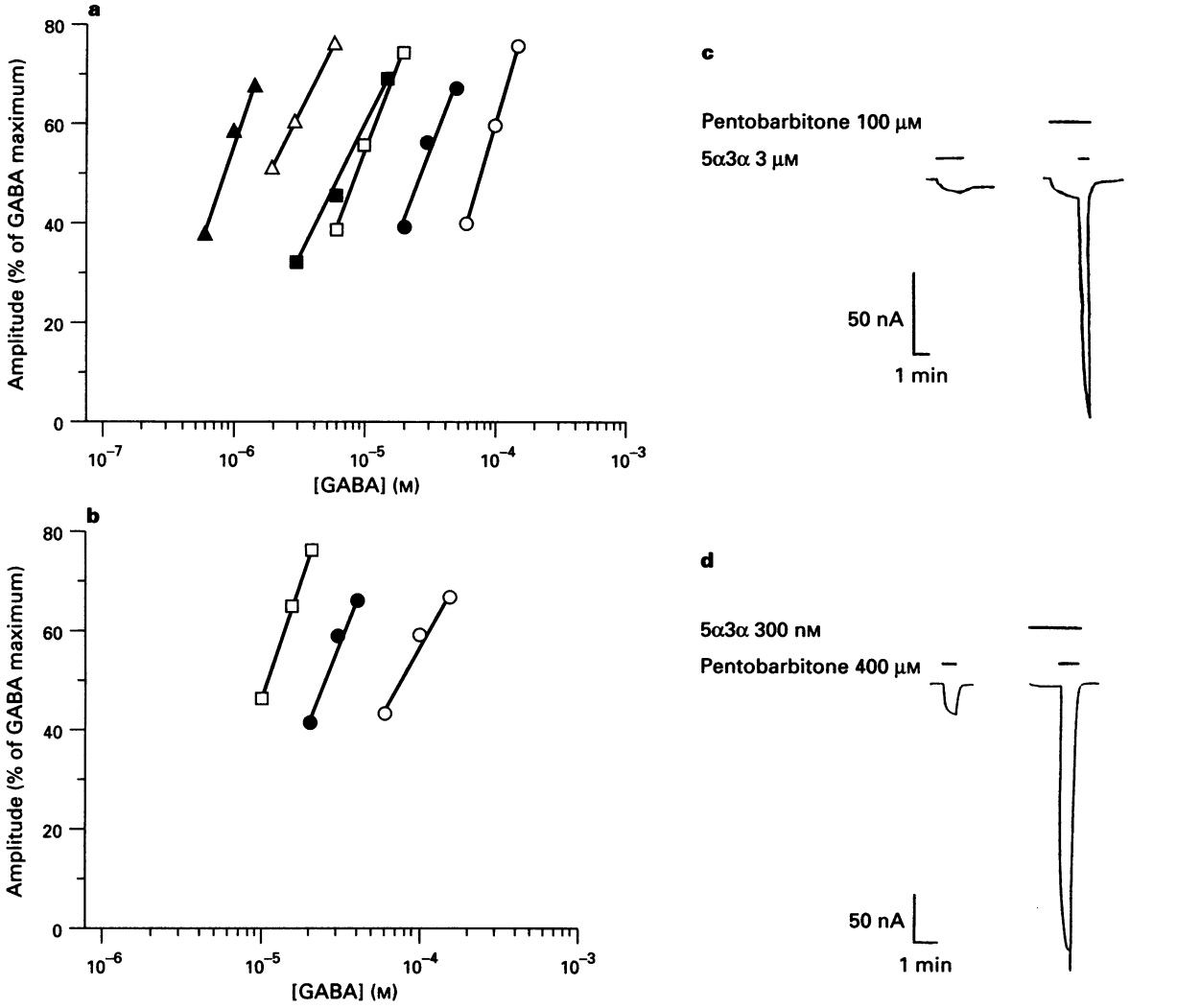

Figure 5 The interactions of 5α-pregnan-3α-ol-20-one (5α3α) and pentobarbitone at the human $\alpha_3\beta_1\gamma_{2L}$ recombinant $GABA_A$ receptor. (a) Graph depicting the relationship between the amplitude of the GABA-evoked current (here expressed as a percentage of the maximal response to GABA, ordinate) and the concentration of bath applied GABA (logarithmic scale, abscissa scale). Currents were recorded in the absence of any modulator (○), in the presence of 5α3α (60 nM, ●; 300 nM, □; 600 nM, ■), and in the combined presence of a maximally effective concentration of 5α3α (i.e. 600 nM, □) and pentobarbitone at concentrations of 30 μM (△) and 60 μM (▲). Under each condition, 3 concentrations of GABA bracketing the EC_{50} were examined. Note that 5α3α produces concentration-dependent and parallel sinistral shifts in the GABA concentration-effect curve over the range 60–300 nM. A further increase in the concentration of the steroid (to 600 nM) produces no further shift. By contrast, pentobarbitone, in the presence of such a saturating concentration of steroid, causes further sinistral shifts in the concentration-effect relationship for GABA. All data were obtained from a single oocyte. (b) Graphical representation of the influence of pentobarbitone alone upon the concentration-effect relationship to GABA in a different oocyte. The points shown are responses recorded in control (○), 30 μM pentobarbitone (●) and 60 μM pentobarbitone (□). The magnitude of the sinistral shifts produced by pentobarbitone are similar to those illustrated in (a) indicating that the effects of the barbiturate and steroid are simply additive and mediated by separate sites. (c) Inward currents to bath-applied 5α3α recorded in control and subsequently in the presence of pentobarbitone. (d) Inward currents to bath applied pentobarbitone recorded in control and subsequently in the presence of 5α3α. All data were obtained at a holding potential of −60 mV.

currents recorded from oocytes expressing the human $\alpha_3\,\beta_1\,\gamma_{2L}$ combination with an EC_{50} of 7.4±0.2 μM ($n=4$; Table 1, Figure 9). However, consistent with previous findings with the β_1 subunit-containing receptors, the maximal enhancement produced by loreclezole (1.9±0.2 fold, $n=4$) was modest. In the absence of GABA, these concentrations of loreclezole (3–30 μM) did not induce an inward current.

Loreclezole (3–100 μM) also produced a concentration-dependent enhancement of the GABA-evoked current recorded from oocytes expressing the splice variant form of the *Rdl* subunit (Table 1, Figure 9). Although requiring relatively high concentrations ($EC_{50}=20\pm1$ μM), the maximal effect (6.9±0.2 fold, $n=3$) which occurred at 100 μM was relatively large. In the absence of GABA, these concentrations of loreclezole did not induce an inward current.

Discussion

The present study has sought to compare the pharmacologies of human and invertebrate recombinant $GABA_A$ receptors with respect to allosteric modulation by a range of structurally dissimilar compounds. Such an assessment is intended to add an additional dimension to an expanding literature detailing

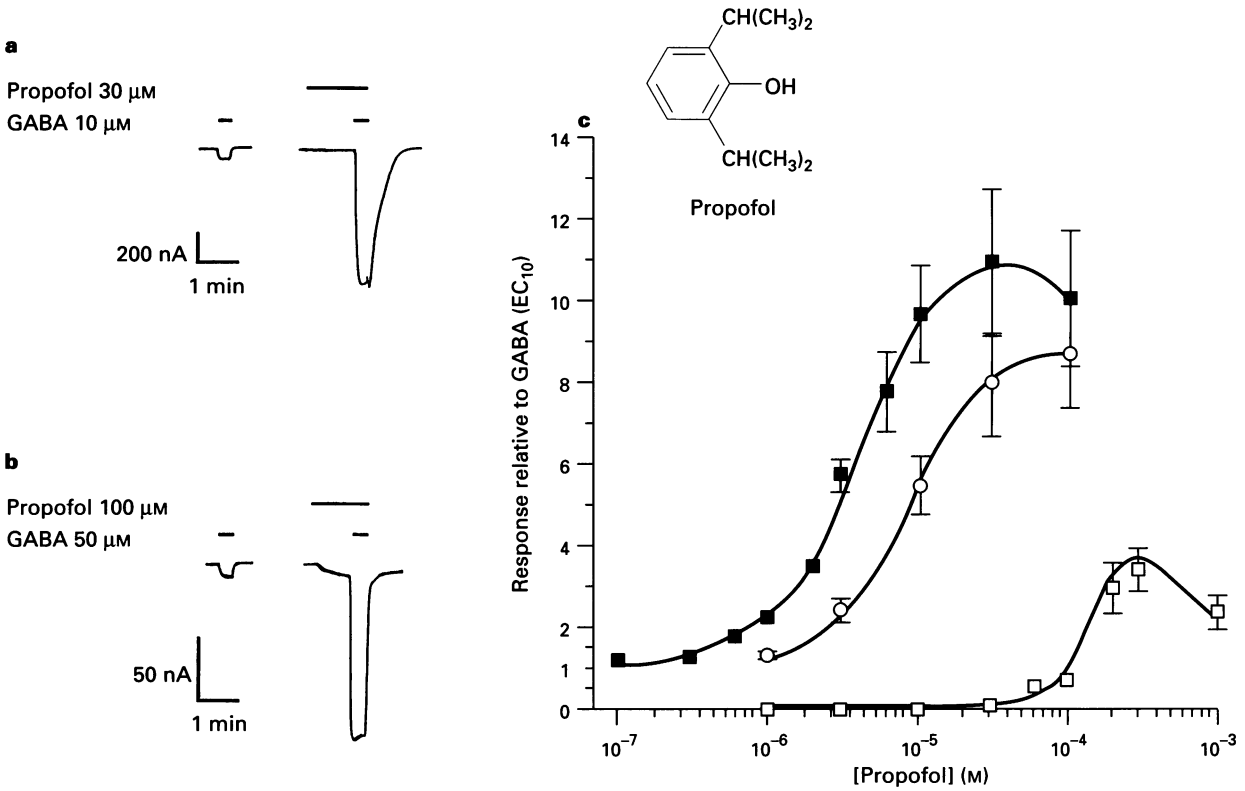

Figure 6 Propofol acts as a positive allosteric modulator of both mammalian and invertebrate recombinant GABA receptors. (a) Bath-applied propofol (30 μM) greatly augments the current evoked by 10 μM GABA (approximate EC_{10} for GABA in this example) recorded from an oocyte expressing the human $\alpha_3\beta_1\gamma_{2L}$ subunit combination. (b) Traces illustrating potentiation by propofol (100 μM) of the current evoked by an EC_{10} concentration of GABA (50 μM in this case) acting at the *Drosophila Rdl* splice variant GABA receptor. Note that the small inward current elicited by propofol alone is not mediated by the invertebrate receptor (see text). (c) Graphical depiction of the relationship between the concentration of bath-applied propofol (logarithmic scale) and the current produced by GABA (on a linear scale and expressed relative to the current induced by an EC_{10} concentration of GABA where that response is normalized to equal 1) when applied at the appropriate EC_{10}. Curves represent data obtained for the human $\alpha_3\beta_1\gamma_{2L}$ receptor (■) and the *Rdl* splice variant receptor (○). Also plotted is the peak direct current elicited by propofol alone (□) for the human $\alpha_3\beta_1\gamma_{2L}$ receptor. Each point is the mean with s.e..mean of data obtained from 4–7 oocytes. Curves were fitted as described in Methods. All data are from oocytes voltage-clamped at −60 mV. The inset gives the chemical structure of propofol.

the relationship between $GABA_A$ receptor subunit composition and drug action (see Siegart, 1995; Smith & Olsen, 1995).

The concentration-effect relationship for GABA determined upon oocytes expressing the human $\alpha_3\ \beta_1\ \gamma_{2L}$ subunit combination yielded an EC_{50} and a Hill coefficient similar to the values reported for receptors formed from the corresponding rat subunits expressed in the same system (Knoflach *et al.*, 1992). Previous studies performed upon naturally occurring $GABA_A$ receptors in their native environment (e.g. Akaike *et al.*, 1987; Peters *et al.*, 1988; Robertson, 1989), or in heterologous expression systems (Parker *et al.*, 1986), have demonstrated pentobarbitone to elicit a triad of effects consisting of potentiation, direct activation and putative channel block, in order of decreasing potency. Similar phenomena occur at recombinant $GABA_A$ receptors of varied tertiary subunit composition (e.g. Sigel *et al.*, 1990; Thompson *et al.*, 1996), including the human $\alpha_3\ \beta_1\ \gamma_{2L}$ subunit combination examined in the present study. The influence of subunit composition upon the various allosteric effects of pentobarbitone have been examined in several studies. For tertiary combinations of subunits, pentobarbitone-induced enhancement of GABA is little affected by the subtype of β subunit present within the oligomeric complex $\alpha_1\ \beta_x\ \gamma_{2s}$ (where x = 1–3; Hadingham *et al.*, 1994). However, a recent report (Thompson *et al.*, 1995b) examining the tertiary combination $\alpha_x\ \beta_2\ \gamma_{2s}$ (where x = 1, 3, 5 or 6), demonstrates that although the nature of the α-subunit has no impact upon the potency with which pentobarbitone potentiates GABA, it is an important determinant of the maximal increase in the GABA response produced by the barbiturate, the possession of an α_6 subunit being particularly favourable in this regard. The presence of a γ subunit is not required for potentiation by pentobarbitone; indeed the introduction of the γ_{2L} subunit into the binary combination $\alpha_1\ \beta_1$ reduces the maximal effect of the barbiturate (Horne *et al.*, 1993).

Pentobarbitone is reported to potentiate GABA-evoked currents recorded from cells expressing solely mammalian α_1 or β_1 subunits as homo-oligomeric complexes, demonstrating that an allosteric site capable of mediating barbiturate-induced potentiation is represented on each of these subunits (Blair *et al.*, 1988; Pritchett *et al.*, 1988). We have previously reported that the splice variant of the *Rdl* subunit is barbiturate-sensitive (Chen *et al.*, 1994). However, the *Rdl* subunit when stably expressed in a *Drosophila* cell line, has been reported to be relatively insensitive to barbiturate modulation (Miller *et al.*, 1994). By contrast, here high micromolar to millimolar concentrations of pentobarbitone produced a clear enhancement of the GABA-evoked current recorded from oocytes expressing the *Rdl* subunit. Whether these differences reside in the different expression systems used, is not known. The *Drosophila* shows relatively little overall homology to the β, ρ, δ, α or γ subfamilies of GABA receptor subunits and a dendrogram analysis suggests, unsurprisingly, that this invertebrate receptor had branched before the separation of the five established GABA receptor channel subunit families (Tyndale *et al.*, 1995). Yet it is clear that these invertebrate homomeric receptors share with vertebrate α or β subunits a binding site that mediates the GABA modulatory actions of pentobarbitone.

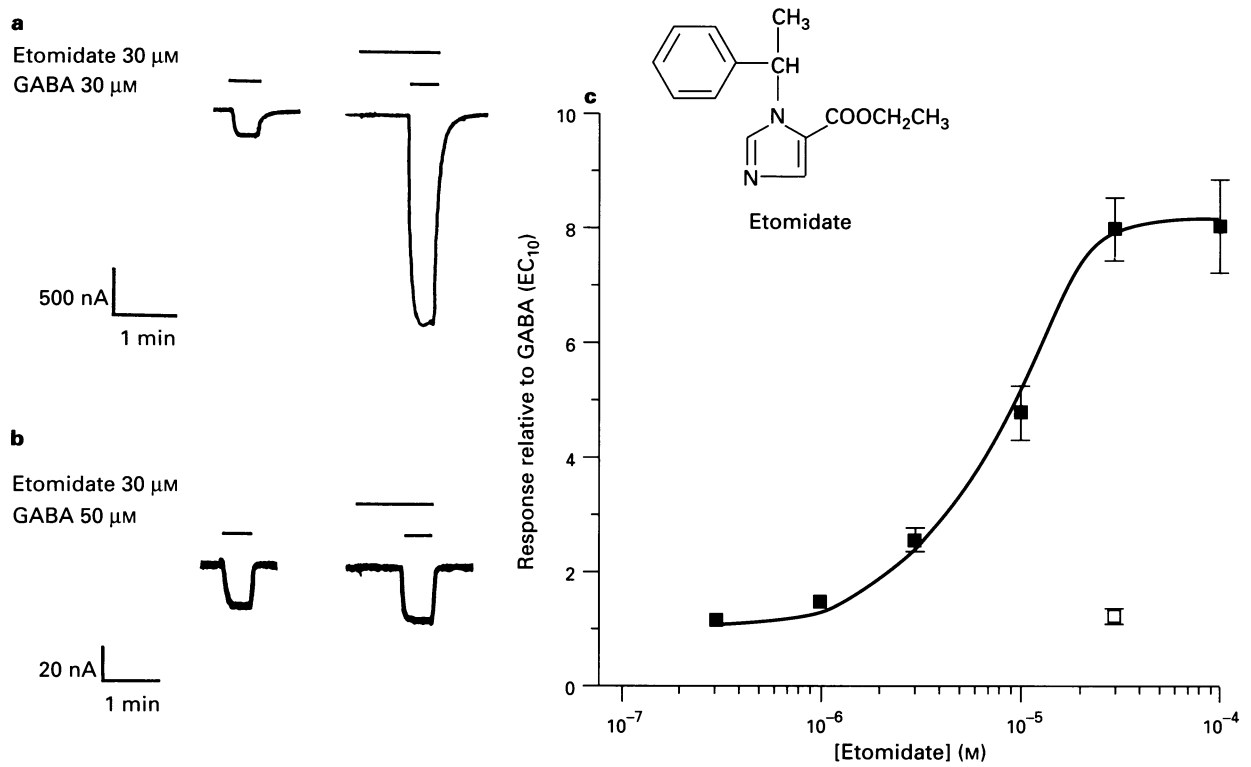

Figure 7 Etomidate acts as a positive allosteric modulator of mammalian $GABA_A$ receptors, but has little effect on the invertebrate *Rdl* splice variant receptor. (a) Bath-applied etomidate (30 μM) greatly potentiates the current evoked by 30 μM GABA (approximate EC_{10} for GABA for this cell) recorded from an oocyte expressing the human $\alpha_3\beta_1\gamma_{2L}$ subunit combination. (b) The same concentration of etomidate has little or no effect on the current evoked by 50 μM GABA (approximately the EC_{10} concentration of GABA in this example) from an oocyte expressing the invertebrate *Rdl* splice variant receptor. (c) Graph illustrating the relationship between the concentration of etomidate (logarithmic scale) and the current produced (on a linear scale and expressed relative to the current induced by an EC_{10} concentration of GABA where that response is normalized to equal 1) by the appropriate GABA EC_{10} acting at human $\alpha_3\beta_1\gamma_{2L}$ receptors (■) or the *Rdl* splice variant receptor (□). Each point represents the mean with s.e.mean of data obtained from 4–5 oocytes. The curve was fitted as described in Methods. All data were obtained from oocytes voltage-clamped at −60 mV. The inset gives the chemical structure of etomidate.

At relatively high concentrations, pentobarbitone directly activates the $GABA_A$ receptor channel complex (e.g. Owen *et al.*, 1986; Akaike *et al.*, 1987; Peters *et al.*, 1988; Robertson, 1989) Indeed, such an action may contribute to the anaesthetic properties of this compound (Schulz & Macdonald, 1981). On oocytes expressing human α_3 β_1 and γ_{2L} subunits, pentobarbitone induced a concentration-dependent inward current which was blocked by the $GABA_A$ receptor antagonist, picrotoxin and enhanced by flunitrazepam, implicating the $GABA_A$ receptor in this effect. Direct activation of the receptor complex by pentobarbitone occurred at higher concentrations (approximately 27 fold difference between the EC_{50} values) than those required for GABA potentiation. This result suggests the presence of distinct high affinity (GABA modulatory) and low affinity (agonist) sites for the barbiturates on the receptor complex. The concept of distinct binding sites coupled to GABA modulation and channel activation is supported by the results obtained with the invertebrate receptors, where pentobarbitone enhanced GABA, but did not directly activate the receptor-channel complex. Hence, these invertebrate subunits appear to possess a barbiturate modulatory site, but not the activation site. Consistent with previous reports (Malherbe *et al.*, 1990; Thompson *et al.*, 1996) analysis of the pentobarbitone-induced current concentration-response curve for the α_3 β_1 γ_{2L} subunit combination reveals a much steeper Hill slope (2.7) than that determined for GABA, suggesting that channel activation by this anaesthetic requires more than one molecule of pentobarbitone. The apparent maximal current induced by pentobarbitone was approximately 3.3 fold that produced by an EC_{10} concentration of GABA. However, at relatively high concentrations the pentobarbitone-induced response was complex, consisting of an initial peak inward current which gradually declined, followed by a transient current increase upon washout of the anaesthetic. This 'washout' or 'rebound' current has been attributed to an unblocking of the pentobarbitone-activated chloride channel by the barbiturate (Akaike *et al.*, 1987; Peters *et al.*, 1988; Robertson, 1989). If the amplitude of the pentobarbitone-induced current upon washout is considered, then the maximal current approaches that induced by GABA. Hence, it is probable that binding of the barbiturate to a third site on the receptor channel complex (the ion channel) reduces the apparent agonist efficacy of this compound.

The location and nature of the pentobarbitone agonist site is not known, although recent mutagenesis studies appear to have confirmed the long held view that it is distinct from that occupied by GABA (Amin & Weiss, 1993). An approach, used successfully for the benzodiazepines (Galzi & Changeux, 1995; Lüddens *et al.*, 1995), and for the anticonvulsant loreclezole (Wingrove *et al.*, 1994), is to identify subunits, or subunit combinations which express a differential pharmacology and to use the techniques of domain exchange and site-directed mutagenesis to define better the amino acid residues which may contribute to the drug binding site. Data presented here suggests that the *Drosophila* subunits may be useful in the future in this respect. To date, the data emerging from studies employing vertebrate subunits appear complex and no clear consensus is evident. The majority of studies agree that pentobarbitone induces a chloride current on oocytes expressing the tertiary combination α, β and γ_2. A recent report (Thompson *et al.*, 1996) examining receptors constructed from $\alpha_x\beta_y\gamma_{2s}$ subunits (where x = 1, 3, 5 or 6 and y = 1, 2 or 3) con-

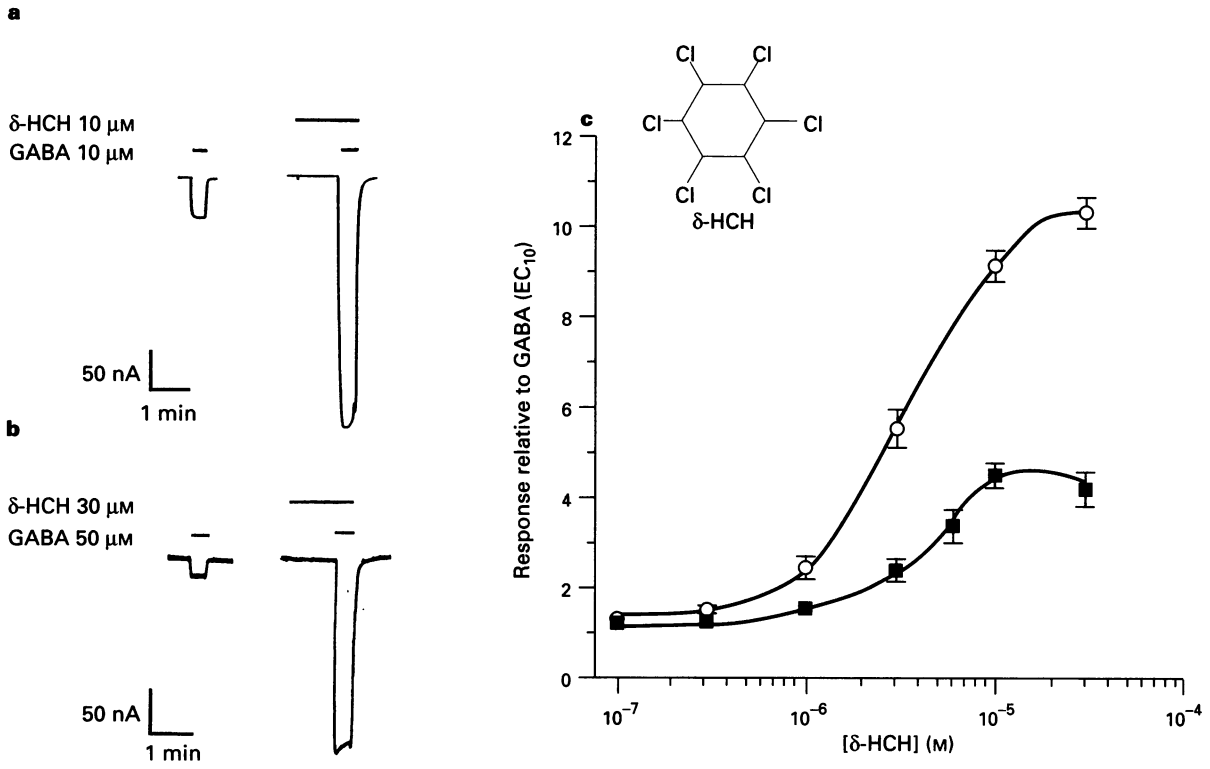

Figure 8 δ-Hexachlorocyclohexane (δ-HCH) acts as a positive allosteric modulator of mammalian $GABA_A$ receptors and the invertebrate *Rdl* splice variant receptor. (a) Bath-applied δ-HCH (10 μM) greatly increases the current evoked by 10 μM GABA (approximate EC_{10} in this oocyte) recorded from a cell expressing the human $\alpha_3\beta_1\gamma_{2L}$ subunit combination. (b) Similarly, the bath-application of 30 μM δ-HCH greatly potentiates the current evoked by 50 μM GABA (approximate EC_{10} in this oocyte) from a cell expressing the invertebrate *Rdl* splice variant receptor. (c) The graph illustrates the relationship between the concentration of δ-HCH (logarithmic scale) and the current produced (on a linear scale and expressed relative to the current induced by an EC_{10} concentration of GABA where that response is normalized to equal 1) by the appropriate GABA EC_{10} acting at human $\alpha_3\beta_1\gamma_{2L}$ receptors (■) or the *Rdl* splice variant receptor (○). Each point represents the mean with s.e.mean of data obtained from 4–5 oocytes. Curves were fitted as described in Methods. All data were collected from oocytes voltage-clamped at −60 mV. The inset gives the chemical structure of δ-HCH.

cluded that, for the direct effect of pentobarbitone, both α and β subunits are determinants of this anaesthetic's affinity for the receptor and the maximal current that it induces. The most striking effects were noted for receptors incorporating an α_6 subunit. Given the discrete location of the α_6 subunit in the cerebellum, this effect may contribute to the behavioural actions of the barbiturates. Pentobarbitone retains agonist activity on the binary α, β combination, implying that the γ subunit is not essential for this effect (Levitan *et al.*, 1988; Malherbe *et al.*, 1990; Sigel *et al.*, 1990; Sanna *et al.*, 1995a). The possession of a β subunit, however, appears crucial (Mihic *et al.*, 1995). In this respect, it is interesting that homomeric human β_1 receptors are sensitive to the agonist actions of pentobarbitone (Sanna *et al.*, 1995a).

In confirmation of previous reports (see Lambert *et al.*, 1995) the naturally occurring steroid 5α3α was found to be a potent positive allosteric modulator of the mammalian recombinant $GABA_A$ receptor, with a clear effect being evident at concentrations as low as 1 nM. The steroid is approximately 500 fold more potent than pentobarbitone in potentiating GABA, although the maximal effect of the barbiturate is greater than that of the steroid (Table 1). At concentrations ⩾300 nM, 5α3α directly activated the receptor but the maximal effect was much less than that produced either by GABA or the anaesthetics, pentobarbitone and propofol. This result is not restricted to recombinant receptors, as we have recently obtained similar data for the $GABA_A$ receptors native to bovine chromaffin cells (unpublished observations). This property distinguishes the steroid from the non-steroidal anaesthetics, pentobarbitone and propofol. The results obtained in this study assessing the influence of binary combinations of the steroid and the barbiturate on either the GABA concentration-response curve, or on the direct agonist actions of these anaesthetics, are consistent with previous electrophysiological (Callachan *et al.*, 1987; Peters *et al.*, 1988) and radioligand binding experiments (Gee *et al.*, 1988; Peters *et al.*, 1988; Turner *et al.*, 1989) and suggest they occupy distinct binding sites on the receptor protein. This would certainly seem to be the case for homomeric receptors assembled from the β_1 subunit which appear insensitive to activation by alphaxalone, yet responsive to pentobarbitone (Sanna *et al.*, 1995a). The differential sensitivity of the invertebrate recombinant receptors to the barbiturate and the steroid further supports the proposal of unique sites. For mammalian $GABA_A$ receptors the steroid, like the barbiturate, does not exhibit a strict subunit requirement (see Lambert *et al.*, 1995) and hence a comparison of the amino acid sequence of the steroid-insensitive invertebrate subunits with their steroid-sensitive mammalian counterparts may be instructive.

A number of investigations have demonstrated the positive allosteric actions of the intravenous anaesthetic, propofol, upon native (Hales & Lambert, 1991; Lin *et al.*, 1992; Hara *et al.*, 1993; Orser *et al.*, 1994; Adodra & Hales, 1995) and recombinant (Jones *et al.*, 1995; Sanna *et al.*, 1995a, b) $GABA_A$ receptors. In the present study, propofol potently enhanced the GABA-evoked current mediated by the $\alpha_3\ \beta_1\ \gamma_{2L}$ recombinant receptor. The EC_{50} for this effect (3.5 μM) is close to that reported for propofol-induced enhancement of GABA-evoked currents recorded from GT1-7 hypothalamic neurones (i.e. 5 μM; Adodra & Hales, 1995). Interestingly, propofol acted to

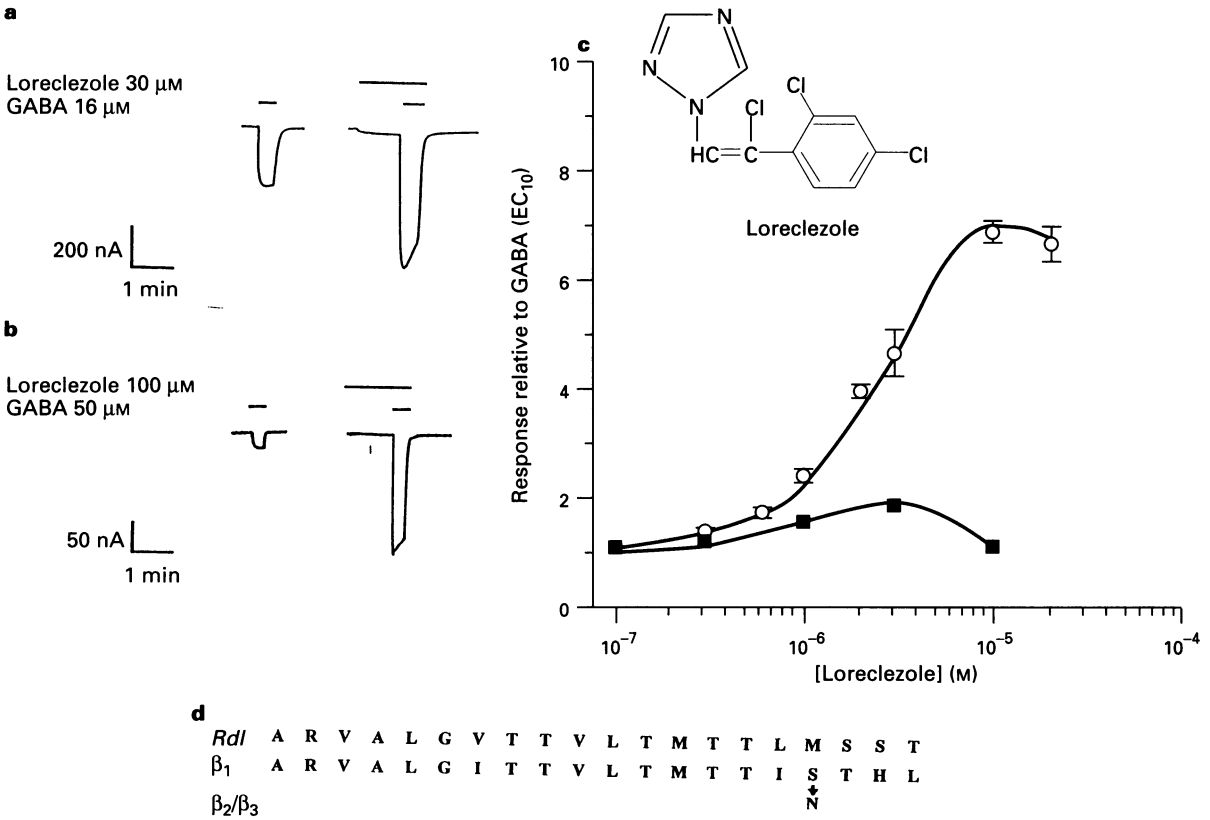

Figure 9 Loreclezole acts as a positive allosteric modulator of mammalian $GABA_A$ receptors and the invertebrate *Rdl* splice variant receptor. (a) The bath application of loreclezole (30 μM) produces a modest enhancement of the current evoked by 16 μM GABA (approximate EC_{10} for this oocyte) recorded from a cell expressing the human $\alpha_3\beta_1\gamma_{2L}$ subunit combination. (b) The bath application of 100 μM loreclezole produces a relatively large enhancement of the current evoked by 50 μM GABA (approximately the EC_{10} in this example) from an oocyte expressing the invertebrate *Rdl* splice variant receptor. (c) The graph illustrates the relationship between the concentration of loreclezole (logarithmic scale) and the current produced (on a linear scale and expressed relative to the current induced by an EC_{10} concentration of GABA where that response is normalized to equal 1) in response to the appropriate GABA EC_{10} applied to the human $\alpha_3\beta_1\gamma_{2L}$ receptor (■) or the *Rdl* splice variant receptor (○). Each point represents the mean with s.e.mean of data obtained from 4 oocytes. Curves were fitted as described in Methods. All data were obtained at a holding potential of −60 mV. The inset gives the chemical structure of loreclezole. (d) Alignment of primary amino acid sequence (single letter code) of the putative M_2 region of the *Rdl* and human β_1, β_2 and β_3 subunits. The asparagine residue (N) identified as conferring loreclezole sensitivity ot the human β subunit is indicated by the downwards arrow. In the interests of clarity, amino acid residues which are conserved across human β subunits are not illustrated in the β_2 and β_3 sequences.

potentiate GABA-evoked currents mediated by the invertebrate receptor with a potency similar to that found for the mammalian receptor (Table 1). In agreement with data obtained for native $GABA_A$ receptors (Hales & Lambert, 1991; Hara *et al.*, 1993; Orser *et al.*, 1994; Adodra & Hales, 1995), at concentrations higher than those required for GABA potentiation, propofol directly activated the $GABA_A$ receptor (approximately 37 fold difference between the propofol EC_{50} values for the GABA potentiating and direct agonist actions). The EC_{50} value for propofol as an agonist in the present work (i.e. 120 μM) is in reasonable agreement with that reported for mouse hippocampal neurones (61 μM; Orser *et al.*, 1994) but is greater than that determined for rat hippocampal neurones (12 μM; Hara *et al.*, 1993) GT1-7 cells (5 μM; Adodra & Hales, 1995) or recombinant receptors composes of α_2 β_1 or α_2 β_1 γ_{2s} subunits expressed in HEK 293 cells (8 and 21 μM respectively; Jones *et al.*, 1995). Differences in experimental protocol, or a subunit specificity of propofol action (see below) may contribute to this variation. Previous studies have reported a Hill slope for receptor activation by propofol greater than unity (Hara *et al.*, 1993; Orser *et al.*, 1994). In the present study, an extremely steep Hill slope of 3.8 was determined. Although this value may be influenced by the 'bell-shaped' concentration-response for propofol activation (Figure 6), it nonetheless approximates to the results of Jones *et al.* (1995) and suggests co-operativity in channel activation by propofol. The maximal current produced by propofol (35% of the maximum current induced by GABA) is in good agreement with that determined for mouse hippocampal neurones and GT1-7 neurones (31% of the maximum current induced by GABA in both cases; Orser *et al.*, 1994; Adodra & Hales, 1995). By contrast to pentobarbitone, high concentrations of propofol were not associated with a 'rebound' current upon washout of the anaesthetic from human recombinant receptors, although such an effect is evident for $GABA_A$ receptors of the mouse hippocampus and GT1-7 cells (Orser *et al.*, 1994; Adodra & Hales, 1995).

The propofol binding site(s) on the $GABA_A$ receptor protein, like those for pentobarbitone, are not defined. Propofol is highly lipophilic (James & Glen, 1980) and might affect the receptor indirectly via an interaction with the surrounding membrane lipid. However, both the GABA modulatory effect and the direct agonist action of propofol exhibit a clear membrane asymmetry, activity being apparent only when the drug is applied extracellularly, observations which make the membrane an unlikely locus of action (Hales & Lambert, 1991). Radioligand binding and chloride flux studies suggest the propofol and pentobarbitone binding sites to be distinct (Concas *et al.*, 1991; Prince & Simmonds, 1992), although the results of some electrophysiological studies investigating the interaction of binary combinations of these anaesthetics are consistent with a common locus of action (Hales & Lambert, 1991; Hara *et al.*, 1993). To date, the subunit specificity of

propofol action has not been investigated systematically. However, the GABA potentiating actions of the anaesthetic showed little or no selectivity across β_1, α_1 γ_{2L}, α_1 β_1, or α_1 β_1 γ_{2s} receptors when expressed in oocytes (Mihic *et al.*, 1995; Sanna *et al.*, 1995b). By contrast, the direct effects of propofol and pentobarbitone exhibited a clear selectivity for the β_1 homomeric receptor (Mihic *et al.*, 1995; Sanna *et al.*, 1995a,b) supporting the hypothesis that the two effects are mediated through distinct binding sites. The present observation that propofol is approximately equieffective in potentiating GABA-evoked currents from the mammalian (α_3 β_1 γ_{2L}) and the invertebrate receptor, but that the direct actions of the anaesthetic are restricted to the mammalian receptor, are consistent with such a proposal.

The insecticide, lindane (γ-hexachlorocyclohexane, γ-HCH), is a non-competitive antagonist of both vertebrate $GABA_A$ receptors and invertebrate GABA receptors (Woodward *et al.*, 1992; Tokutomi *et al.*, 1994; Callachan *et al.*, 1994). By contrast, the δ-isomer enhances GABA-evoked currents recorded from oocytes preinjected with mRNA extracted from rat cortex (Woodward *et al.*, 1992). In the present study, δ-HCH exhibited similar potency at human and the invertebrate GABA receptors, although the maximal effect produced was greater for the latter. The possibility exists that the negative and positive effects of these isomers on the GABA receptor result from the occupation of a common site which induces opposite allosteric changes to the protein. Amino acid sequence analysis of the *Rdl* subunit from fruit flies which are resistant to the non-competitive GABA antagonist, dieldrin, revealed a single nucleotide mutation which produced an alanine (wild-type) to serine (resistant) substitution in the M2 region of the subunit, an area which is proposed to form the lining of the chloride ion channel (ffrench-Constant *et al.*, 1993). Utilizing site-directed mutagenesis, we have reproduced this amino acid change for the splice variant of the *Rdl.* subunit. Receptors formed from this engineered protein exhibit a greatly decreased antagonist potency for the γ-isomer of HCH, but this change has no influence on the positive allosteric effects of the δ-isomer (Belelli *et al.*, 1995). This observation does not support a common binding locus for these isomers.

The positive allosteric actions of the intravenous anaesthetic, etomidate, have been investigated quantitatively mainly at native $GABA_A$ receptors (Ashton *et al.*, 1981; Thyagarajan *et al.*, 1983). In the present study, the potency ($EC_{50} = 7.7$ μM) of etomidate in enhancing the GABA-induced current mediated by the α_3 β_1 γ_{2L} recombinant receptor was remarkably close to the IC_{50} value for inhibition of [^{35}S]-TBPT binding and the EC_{50} values for enhancement of both [^{3}H]-GABA and [^{3}H]-diazepam binding to rat cortical membranes (Thyagarajan *et al.*, 1983; Ticku & Rastogi, 1986). Furthermore, a number of electrophysiological investigations have shown etomidate potentiation of GABAergic inhibition in mammals to occur over a range of concentrations (8–10 μM) similar to those used in the present investigation (Ashton & Wauquier, 1985; Proctor *et al.*, 1986; Yang, 1991).

Interestingly, GABA-evoked currents mediated by the invertebrate receptors, while greatly potentiated by both pentobarbitone and propofol, were insensitive to modulation by etomidate (Table 1). These observations suggest that the GABA modulatory actions of etomidate occur through a site on the receptor protein distinct from that of propofol and pentobarbitone.

In common with many other intravenous general anaesthetics, at concentrations higher than those required for potentiation of GABA, etomidate activates a bicuculline- and picrotoxin-sensitive chloride current in GABA-sensitive neurones (Evans & Hill, 1978; Robertson, 1989). In the present study, we did not observe any direct activation of either the human or invertebrate GABA receptors by etomidate. These findings are consistent with the proposal that the modulatory and agonist-like actions of etomidate may be mediated through distinct, though as yet undefined, sites on the receptor (Uchida *et al.*, 1995). The subunit specificity of either action remain to be investigated systemically. However, the modulatory properties appear to be regionally dependent because in the cerebellum, etomidate produces only a modest enhancement of [^{3}H]-GABA binding (Thyagarajan *et al.*, 1983). Interestingly, the β_3 subunit has been shown to be uniquely sensitive to the agonist-like actions of this anaesthetic (Uchida *et al.*, 1995). Furthermore, etomidate has recently been reported to be a potent allosteric inhibitor of [^{35}S]-TBPS binding in HEK 293 cells transiently transfected with the β_3 subunit (Slany *et al.*, 1995). Collectively, these observations, coupled with the presently observed insensitivity of $\alpha_3\beta_1\gamma_2$ recombinant receptors to activation by etomidate, point to the β subunit as the possible locus of action of the agonist effect of the drug. This proposal is consistent with our recent findings that β_2-containing, but not β_1-containing, recombinant $GABA_A$ receptors are directly activated by the anaesthetic (Hill-Venning *et al.*, 1995).

The anticonvulsant, loreclezole, has recently been shown to potentiate selectively GABA-evoked currents mediated by β_2- or β_3-containing receptors but to have limited action on β_1-containing-receptors (Wafford *et al.*, 1994; Wingrove *et al.*, 1994). In agreement with these findings, in the present work, loreclezole produced a very modest potentiation of the GABA-evoked currents mediated by recombinant receptors composed of α_3 β_1 and γ_2 subunits. Point mutation studies have highlighted the importance of an asparagine residue in the β_2 and β_3 subunits for the actions of loreclezole. This amino acid is located towards the extracellular side of the M2 transmembrane region of the β subunit, a region which is thought to line the pore of the associated chloride channel. Mutation of this amino acid to a serine residue (which occurs naturally in the β_1 subunit) causes a reduction in both the potency and the magnitude of the enhancement of the GABA-evoked currents produced by loreclezole (Wingrove *et al.*, 1994). The reciprocal mutation (serine to asparagine), increases the potency and the magnitude of the effect of loreclezole on β_1-containing receptors. Hence, either the asparagine residue is essential for the loreclezole effect, or the serine residue prevents this anticonvulsant's action. Like the β_1 subunit, all mammalian α and γ subunits have a serine in this equivalent position, whereas the δ subunit and the invertebrate *Rdl* subunit have a methionine residue (Tyndale *et al.*, 1995) see Figure 9. Here, GABA-evoked currents mediated by the invertebrate receptor were greatly potentiated by loreclezole, although the potency of this effect was approximately 20 fold lower than that observed for the β_2 and β_3-containing human $GABA_A$ receptors. Hence, one interpretation of these data is that at least the magnitude of the loreclezole effect can be supported by the unconserved substitution of an asparagine to a methionine residue.

However, a recent study has demonstrated that the mutation of the asparagine residue in the human β subunit to a methionine (equivalent to *Rdl*), results in a receptor which is insensitive to loreclezole (Stevenson *et al.*, 1995). Therefore, either the invertebrate loreclezole binding site is distinct from its mammalian counterpart, or additional binding domains contribute to the attachment of loreclezole to mammalian and invertebrate receptors.

The β-carboline, DMCM, in addition to inhibiting GABA-evoked currents by interacting with the benzodiazepine binding site, has been shown at higher concentrations to potentiate GABA-evoked currents via an interaction with the loreclezole binding site (Stevenson *et al.*, 1995). As the *Rdl* receptor is benzodiazepine-insensitive (Chen *et al.*, 1994), but loreclezole-sensitive, clearly it would be of interest to investigate the actions of this β carboline on the invertebrate receptor.

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