British Journal of Pharmacology (1998) 124, 13–20
http://www.stockton-press.co.uk/bjp

The effect of a transmembrane amino acid on etomidate sensitivity of an invertebrate GABA receptor

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1 The γ-aminobutyric acid (GABA)-modulatory and GABA-mimetic actions of etomidate at mammalian $GABA_A$ receptors are favoured by β_2- or β_3- versus β_1-subunit containing receptors, a selectivity which resides with a single transmembrane amino acid ($\beta_{2\ N290}$, $\beta_{3\ N289}$, $\beta_{1\ S290}$). Here, we have utilized the *Xenopus laevis* oocyte expression system in conjunction with the two-point voltage clamp technique to determine the influence of the equivalent amino acid (M314) on the actions of this anaesthetic at an etomidate-insensitive invertebrate GABA receptor (*Rdl*) of *Drosophila melanogaster*.

2 Complementary RNA-injected oocytes expressing the wild type *Rdl* GABA receptor and voltage-clamped at -60 mV responded to bath applied GABA with a concentration-dependent inward current response and a calculated EC_{50} for GABA of 20 ± 0.4 μM. Receptors in which the transmembrane methionine residue (M314) had been exchanged for an asparagine (Rdl_{M314N}) or a serine (Rdl_{M314S}) also exhibited a concentration-dependent inward current response to GABA, but in both cases with a reduced EC_{50} of 4.8 ± 0.2 μM.

3 Utilizing the appropriate GABA EC_{10}, etomidate (300 μM) had little effect on the agonist-evoked current of the wild type *Rdl* receptor. By contrast, at Rdl_{M314N} receptors, etomidate produced a clear concentration-dependent enhancement of GABA-evoked currents with a calculated EC_{50} of 64 ± 3 μM and an E_{max} of $68 \pm 2\%$ (of the maximum response to GABA).

4 The actions of etomidate at Rdl_{M314N} receptors exhibited an enantioselectivity common to that found for mammalian receptors, with 100 μM **R**-(+)-etomidate and **S**-(−)-etomidate enhancing the current induced by GABA (EC_{10}) to $52 \pm 6\%$ and $12 \pm 1\%$ of the GABA maximum respectively.

5 The effects of this mutation were selective for etomidate as the GABA-modulatory actions of 1 mM pentobarbitone at wild type *Rdl* ($49 \pm 4\%$ of the GABA maximum) and Rdl_{M314N} receptors ($53 \pm 2\%$ of the GABA maximum) were similar. Additionally, the modest potentiation of GABA produced by the anaesthetic neurosteroid 5α-pregnan-3α-ol-20-one ($Rdl = 25 \pm 4\%$ of the GABA maximum) was not altered by this mutation ($Rdl_{M314N} = 18 \pm 3\%$ of the GABA maximum).

6 Etomidate acting at β_1 (S290)-containing mammalian $GABA_A$ receptors is known to produce only a modest GABA-modulatory effect. Similarly, etomidate acting at Rdl_{M314S} receptors produced an enhancement of GABA but the magnitude of the effect was reduced compared to Rdl_{M314N} receptors.

7 Etomidate acting at human $\alpha_6\beta_3\gamma_{2L}$ receptors is known to produce a large enhancement of GABA-evoked currents and at higher concentrations this anaesthetic directly activates the $GABA_A$ receptor complex. Mutation of the human β_3 subunit asparagine to methionine ($\beta_{3\ N289M}$ found in the equivalent position in *Rdl* completely inhibited both the GABA-modulatory and GABA-mimetic action of etomidate (10–300 μM) acting at $\alpha_6\beta_{3\ N289M}\gamma_{2L}$ receptors.

8 It was concluded that, although invertebrate and mammalian proteins exhibit limited sequence homology, allosteric modification of their function by etomidate can be influenced in a complementary manner by a single amino acid substitution. The results are discussed in relation to whether this amino acid contributes to the anaesthetic binding site, or is essential for transduction. Furthermore, this study provides a clear example of the specificity of anaesthetic action.

Keywords: GABA; GABA receptor; general anaesthetic; etomidate; pentobarbitone; 5α-pregnan-3α-ol-20-one; neurosteroid

Introduction

Although general anaesthetics have been clinically used for over 150 years (Rushman *et al*., 1996), the molecular basis of their actions remains obscure. The striking correlation between the anaesthetic potency and lipid solubility of structurally dissimilar agents, described by the Meyer-Overton rule, suggests a hydrophobic site of action, most frequently equated with the lipid component of the nerve cell membrane (Little, 1996). However, the 'cut-off' phenomenon, in which higher members of a homologous series of *n*-alcohols or *n*-alkanes lose anaesthetic activity despite increased lipophilicity, is a long standing exception to the Meyer-Overton rule (Franks & Lieb, 1987). Further challenges are provided by polyhalogenated and perfluorinated compounds which do not demonstrate the anaesthetic activity predicted by the rule (Koblin *et al*., 1994). Moreover, perturbations of membrane structure do not provide a mechanism of anaesthesia *per se* and are of questionable relevance, since the effects induced by clinically relevant concentrations of anaesthetics are mimicked by modest changes in temperature which do not induce anaesthesia (Franks & Lieb, 1987).

Against this background, membrane proteins have received increasing attention as potential sites of anaesthetic action, leading to the identification of certain members of the transmitter-gated ion channel family as plausible targets (Franks & Lieb, 1987; 1994; Harris *et al*., 1995). Although

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the diversity of chemical structures capable of inducing anaesthesia militates against a common site of anaesthetic action, it has nonetheless emerged that the majority of clinically useful general anaesthetics and many experimental anaesthetics share the ability to enhance the actions of γ-amimobutyric acid (GABA) at the $GABA_A$ receptor. Such observations neither prove the involvement of GABA in anaesthesia, nor exclude the participation of alternative ion channels or receptors, but enhancement of inhibitory tone within the CNS has logical appeal as a mechanism of anaesthetic action.

The $GABA_A$ receptor is a composed of five subunits drawn from the products of a multigene family (α_{1-6}, β_{1-3}, γ_{1-3}, δ and ε) that exhibit a differential distribution within the CNS (Smith & Olsen, 1995; Whiting *et al*., 1995; Davies *et al*., 1997). Establishing the influence that $GABA_A$ receptor subunit composition plays in determining positive allosteric regulation by anaesthetics may help explain regional variations in anaesthetic sensitivity within the CNS. Additionally, the identification of recombinant receptors that respond differentially to general anaesthetics may help define protein domains that form the anaesthetic binding pocket. As an example of this approach, we have previously demonstrated that the GABA-modulatory and GABA-mimetic actions of the clinically utilized general anaesthetic etomidate are markedly enhanced at $GABA_A$ receptors incorporating β_2 or β_3 versus a β_1 subunit (Belelli *et al*., 1997a,b; Hill-Venning *et al*., 1997). Such an effect is selective, since the actions of barbiturates, neurosteroids and propofol are unaffected by the β-subunit isoform contained within the receptor complex. A single amino acid difference between β_2 or β_3 (N289) and β_1 (S290) subunits (see Table 1, 9′) largely accounts for the differential activities of etomidate (Belelli *et al*., 1997a,b). This residue is postulated to reside at a homologous position within the ion channel forming domain (M2) of the β subunits.

By contrast with vertebrate $GABA_A$ receptors, a recombinant receptor isolated from *Drosophila melanogaster (Rdl)* is insensitive to etomidate, but is modulated by anaesthetics that include propofol and pentobarbitone (Chen *et al*., 1994; Belelli *et al*., 1996). The *Rdl* receptor exhibits limited (30–38%) sequence identity (Hosie *et al*., 1997) to vertebrate (e.g. α,β,γ) subunits and is thought to have branched long before the emergence of such subunits. However, the amino acids that are thought to contribute to the anion selective channels of the invertebrate and mammalian receptors exhibit a much greater degree of identity (60%, see Table 1) presumably as consequence of their common function. Sequence alignment of the putative channel domain of *Rdl* and mammalian β subunits (Table 1) reveals a methionine residue (M314) occupying a position equivalent to either β_2 or β_3 N289 or β_1 S290. Here, we demonstrated that substitution of M314 of the *Rdl* receptor by an asparagine residue imparts etomidate sensitivity to this receptor. Furthermore, as established for mammalian $GABA_A$ receptors (Olsen *et al*., 1986), the mutant *Rdl* receptor exhibited a clear preference for **R**-(+)- versus the **S**-(−)-enantiomer of etomidate. Interestingly, the reciprocal mutation (N289M) introduced into the mammalian β_3 subunit eliminated the actions of etomidate. Hence, although these proteins are widely separated in phylogeny and exhibit limited overall homology, a single amino acid substitution can restore some commonality in pharmacological profile.

Methods

Site-directed mutagenesis

The cDNA encoding the *Drosophila Rdl* GABA receptor subunit (ffrench-Constant *et al*., 1991) was provided by Dr Rousch (Cornell University) in the vector pNB40 (Brown & Kafatos, 1988). A 1840 base pair *Bam* HI-*Not* I fragment of *Rdl* was cloned into the vector pcDNA1Amp (Invitrogen BV, The Netherlands), to allow the preparation of single-stranded template DNA and site-specific mutants were generated by standard methods (Kunkel, 1985). Two mutagenesis reactions were primed individually by the oligonucleotides M1 (5′-ACAACCGTGTTAACAAT GACCACTTT G**AAC**TCGTC-AACA-3′) and M2 (5′-ACAACCGTGTTAACAATGAC-CACTTTG**AGC**TCGTCAACA-3′) to substitute a methionine residue at position 314 to asparagine (Rdl_{M314N}) and serine (Rdl_{M314S}), respectively. In both cases the mutated codon is highlighted in bold. The underlined sequences represent a silent mutation incorporating a novel *Hpa1* restriction site, to facilitate the rapid screening for mutant clones by restriction analysis. The success of the mutagenesis reaction was confirmed by dideoxynucleotide sequencing (*fmol* DNA Sequencing System, Promega, U.K.). The mutated *Bam* HI-*Not*I fragment was subcloned back into the original *Rdl*-pNB40 construct.

Preparation of transcripts and oocyte injection

cDNAs encoding the human α_6, β_3, $\beta_{3\,N289M}$, $\beta_{3\,N289S}$ and γ_{2L} $GABA_A$ receptor subunits in the pcDM8 vector were provided by Dr Paul Whiting (Merck, Sharp and Dohme, Harlow, Essex, U.K.). The cDNAs for α_6 and γ_{2L} $GABA_A$ receptor subunits were linearized at unique *Xho I* sites, those for the β_3, *Rdl* and Rdl_{M314N} at *Not* I and that for $\beta_{3\,N289M}$ and $\beta_{3\,N289S}$ at an *Xba I* site. cRNA transcripts were prepared according to standard protocols (Hope *et al*., 1993). Denaturing gel electrophoresis was utilized to check the

Table 1 Sequence alignment of the M2 domains of *Drosophila melanogaster* (*Rdl*) GABA, and human $GABA_A$ and glycine receptor subunits

	1′	2′	3′	4′	5′	6′	7′	8′	9′	10′	11′	12′	13′	14′	15′	16′	17′	18′	19′	20′	21′	22′	23′	24′
GABA *Rd1*	T	P	A	R	V	A	L	G	V	T	T	V	L	T	M	T	T	L	**M**	S	S	T	N	A
GABA β_3	S	S	A	R	V	A	L	G	I	T	T	V	L	T	M	T	T	I	**N**	T	H	L	R	E
GABA β_1	S	A	A	R	V	A	L	G	I	T	T	V	L	T	M	T	T	I	**S**	T	H	L	R	E
GABA ρ	V	P	A	R	V	P	L	G	I	T	T	V	L	T	M	S	T	I	**I**	T	G	V	N	A
GABA α_1	V	P	A	R	T	V	F	G	V	T	T	V	L	T	M	T	T	L	**S**	I	S	A	R	N
Gly α_1	A	P	A	R	V	G	L	G	I	T	T	V	L	T	M	T	T	Q	**S**	S	G	S	R	A
Gly β	S	A	A	R	V	P	L	G	I	F	S	V	L	S	L	A	S	E	**C**	T	T	L	A	A

The amino acid which is important for the action of etomidate at GABA β and *Rdl* subunits, and for enflurane and ethanol at GABA α,β,ρ and glycine (Gly) α subunits (Mihic *et al*., 1997), is shown in bold, 19′.

integrity of transcripts before injection. Such cRNA transcripts were injected (30–50 nl of 1 mg ml^{-1}) into *Xenopus laevis* oocytes (stage V–VI) which had previously been defollicated by treatment with 2 mg ml^{-1} collagenase 'A' (Boehringer-Mannheim) for 3 h at room temperature (20–23°C) in nominally calcium-free Barth's saline. 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.

Electrophysiological recordings

Oocytes were used for electrophysiological experiments 2–12 days after cRNA injection essentially as previously described (Chen *et al*., 1994). Electrical recordings were made from oocytes voltage-clamped at a holding potential of −60 mV by utilizing either an Axoclamp 2A or a Gene Clamp 500 amplifier (Axon Instruments, U.S.A.) in the two-electrode voltage-clamp mode.

Oocytes were held in a recording chamber (0.5 ml) and continually superfused (7–10 ml min^{-1}) with frog Ringer solution (composition in mM: NaCl 120, KCl 2, $CaCl_2$ 1.8, HEPES 5, adjusted to pH 7.4 with NaOH). Current-passing and voltage-sensing intracellular electrodes were fabricated such that they exhibited resistances of 0.5–1.25 MΩ (when filled with 3 M KCl and measured in frog Ringer solution). All agonists and anaesthetics were applied by the superfusion system. For each oocyte a maximal concentration of GABA (1 mM and 3 mM for the invertebrate and mammalian receptor, respectively) was applied once every 20 min until the peak inward current response produced was stable (Belelli *et al*., 1996). The magnitude of the GABA enhancing actions of anaesthetics is dependent upon the concentration of GABA utilized (Harris *et al*., 1995; Belelli *et al*., 1996). Hence, for such experiments the concentration of GABA which evoked a response amounting to 10% of the current produced by a saturating concentration of GABA (ie EC_{10}) was determined for each oocyte and used. Anaesthetic regulation of the receptor was quantified as the increase in the peak amplitude of the GABA-evoked current and data were normalized by expressing the observed response as a percentage of the maximal GABA response. Anaesthetics were pre-applied for 60 s before their co-application with the appropriate concentration of GABA. Concentration-effect relationships for either GABA, or the GABA-enhancing actions of the anaesthetics were fitted iteratively by use of Fig P version 6c, with the four parameter logistic equation:

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

where for GABA modulation, I is the amplitude of the GABA-evoked current in the presence of the anaesthetic at concentration [A], I_{max} is the amplitude of the response in the presence of a maximally effective concentration of anaesthetic producing half-maximal enhancement and n_H is the Hill coefficient. Concentration-effect relationships for GABA were similarly fitted where I now represents the amplitude of the current evoked by the agonist at concentration [A], I_{max} is the amplitude of the response in the presence of a maximally effective concentration of GABA and EC_{50} is the concentration of agonist producing a half-maximal response. Quantitative data are presented as the mean ± s.e.mean.

Results

Etomidate (300 μM) had little effect on GABA-evoked currents recorded from oocytes expressing the *Rdl* receptor (14 ± 1% of the GABA maximum; $n = 4$; ie an increase of only 4%). This result is in accord with a previous study on a spliced variant of the *Rdl* receptor (Belelli *et al*., 1996; see Figures 1, 2 and Table 2). For the *Rdl* receptor, mutation of the transmembrane located methionine residue to an asparagine (*Rdl* $_{M314N}$) found in etomidate sensitive β_2 or β_3 subunits (Table 1) produced a dramatic change in the actions of etomidate at this invertebrate GABA receptor. Hence, GABA-evoked currents recorded from oocytes expressing the *Rdl* $_{M314N}$ receptor were now clearly enhanced in a concentration-dependent (10 μM–300 μM) manner by etomidate with a calculated EC_{50} of 64 ± 3 μM ($n = 4$) and an E_{max} of 68 ± 2% occurring with 300 μM of the anaesthetic (Figures 1 and 2). Higher concentrations of etomidate (600 μM) were associated with a reduced enhancement resulting in a bell-shaped concentration-response relationship (Figure 2). This feature is also evident for etomidate on mammalian receptors (Belelli *et al*., 1997a,b; Hill-Venning *et al*., 1997). Additionally, the mutation produced an approximately 4 fold reduction of the EC_{50} for GABA (*Rdl* = 20 ± 0.4 μM; Hill coefficient $(n_H) = 1.7 \pm 0.1$; $n = 9$; *Rdl* $_{M314N} = 4.8 \pm 0.2$ μM; $n_H = 1.7 \pm 0.1$; $n = 5$) with no effect on the Hill coefficient. Etomidate acting at β_1-containing $GABA_A$ receptors produces only a modest GABA modulatory effect (Hill-Venning *et al*., 1997). Similarly here, the mutation of the *Rdl* methionine to a serine residue (equivalent amino acid to β_1 – see Table 1) results in a receptor at which etomidate produces a GABA modulatory effect (Figures 1 and 2), intermediate between *Rdl* wild type and Rdl_{M314N} (600 μM etomidate enhances the GABA evoked current to 39 ± 5% ($n = 3$) of the GABA maximum – greater concentrations of anaesthetic were not investigated due to limited solubility). Similar to Rdl_{M314N}, this mutation produced an approximately 4 fold reduction of the EC_{50} for GABA ($Rdl_{M314S} = 4.8 \pm 0.2$ μM; $n_H = 2.1 \pm 0.1$; $n = 4$) with little effect on the Hill coefficient.

As the asparagine mutation at the invertebrate receptor produced such a dramatic change in the allosteric actions of the previously inert etomidate, it was of interest to determine whether the effects of a structurally distinct anaesthetic, pentobarbitone, which is active at the wild type *Rdl* receptor (Belelli *et al*., 1996; Hosie & Sattelle, 1996) were also modified. By contrast with etomidate, the actions of pentobarbitone were little influenced by the mutation. Hence, 1 mM pentobarbitone enhanced GABA-evoked currents to 49 ± 4% ($n = 4$) and 53 ± 2% ($n = 4$) for the *Rdl* and Rdl_{M314N} receptors, respectively. Examination of the pentobarbitone concentration-response relationship revealed the mutation to produce only a modest reduction of the EC_{50} (837 ± 64 μM; 394 ± 47 μM for *Rdl* and *Rdl* $_{M314N}$, respectively) and a small decrease rather than an increase of the E_{max} (72 ± 7%; 53 ± 2% for *Rdl* and *Rdl* $_{M314N}$ respectively; see Figure 3).

The anaesthetic neurosteroid 5α-pregnan-3α-ol-20-one at the relatively high concentration of 10 μM produced only a modest enhancement (25 ± 4% of the GABA maximum; $n = 4$) of GABA-evoked currents recorded from oocytes expressing the wild type *Rdl* receptor. Similarly, this concentration of neurosteroid produced a limited enhancement (18 ± 3% of the GABA maximum; $n = 3$) of GABA-evoked responses mediated by Rdl_{M314N} receptors. Hence, the exchange of the *Rdl* transmembrane methionine to an asparagine residue selectively influences etomidate with little or no effect on the activity of pentobarbitone or 5α-pregnan-3α-ol-20-one.

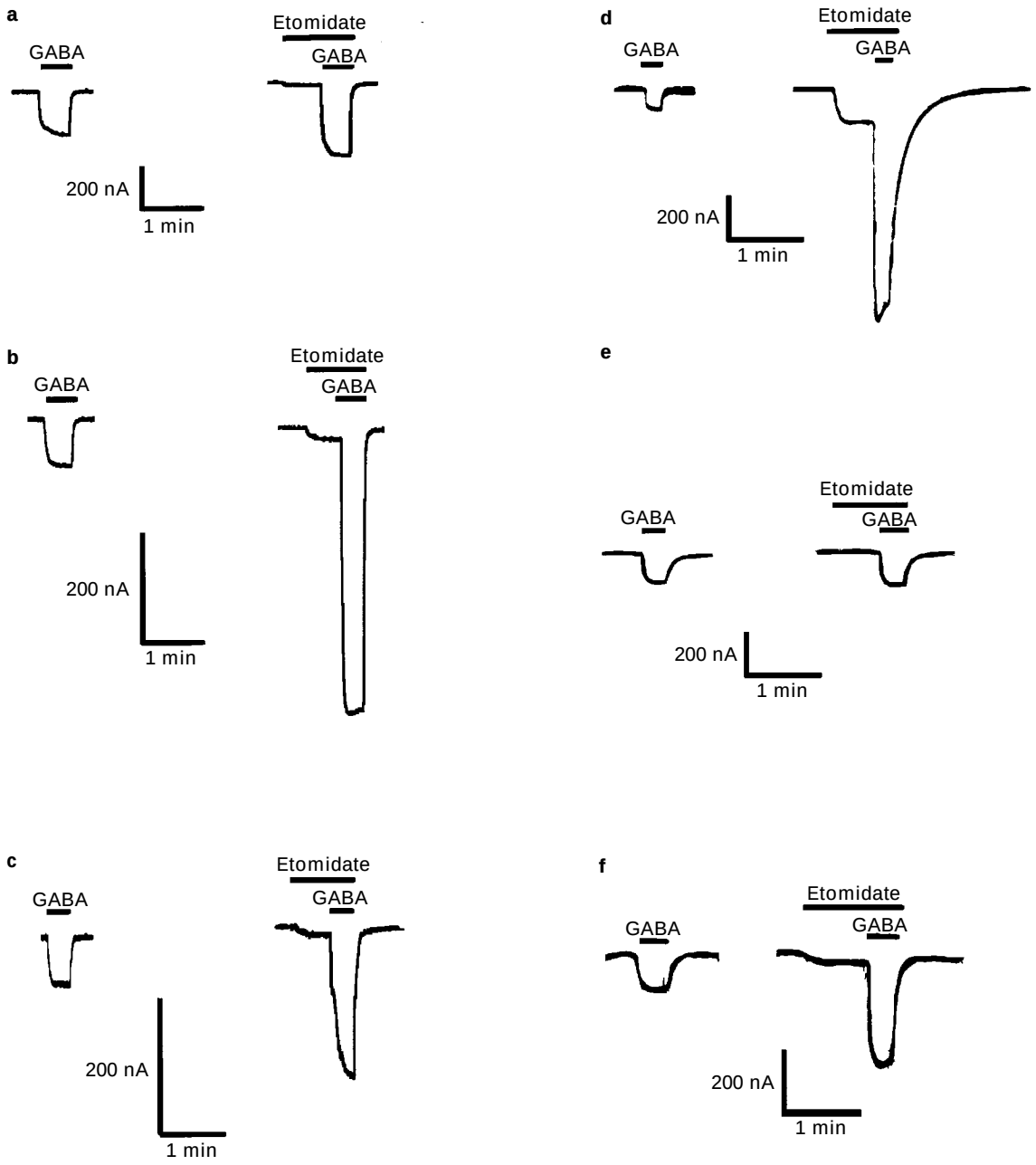

Figure 1 A single amino acid governs the etomidate sensitivity of invertebrate (*Rdl*) and human ($\alpha_6\beta_3\gamma_{2L}$) GABA receptors. Illustrated are traces which demonstrate that: (a) etomidate (300 μM) had little or no modulatory effect on GABA (14 μM)-evoked responses mediated by *Rdl* wild type receptors expressed in an oocyte. (b) GABA (1.4 μM)-evoked currents recorded from an oocyte expressing *Rdl* receptors in which a methionine residue located within the second transmembrane region of the protein was exchanged for an asparagine i.e. Rdl_{M314N} (the equivalent amino acid in the β_3 subunit) was greatly enhanced by 300 μM etomidate. (c) Etomidate (300 μM) enhancement of GABA (2 μM)-evoked currents recorded from an oocyte expressing *Rdl* receptors, in which the transmembrane methionine had been exchanged for a serine residue ie Rdl_{M314S} (the equivalent amino acid in the β_1 subunit), was intermediate between that of wild type and Rdl_{M314N} receptors. (d) The potentiation by etomidate (10 μM) of the inward current response evoked by GABA (1 μM) at receptors composed of wild type $\alpha_6\beta_3\gamma_2$ subunits expressed in oocytes. Note that the pre-application of this concentration of etomidate produced an inward current due to the GABA-mimetic effect of the anaesthetic. (e) Etomidate (10 μM) had no effect on GABA (1.2 μM)-evoked currents recorded from oocytes expressing α_6, γ_2 and a β_3 subunit in which a transmembrane located asparagine residue was mutated to a methionine i.e. $\beta_{3\ N289M}$ (the equivalent residue in the *Rdl* subunit). (f) The enhancement of the GABA (1 μM)-evoked current by etomidate (10 μM) was greatly reduced at receptors composed of α_6, γ_2 and a β_3 subunit in which a transmembrane located asparagine residue was mutated to a serine i.e. $\beta_{3\,N289S}$ (the equivalent residue in the β_1 subunit). All recordings were made from *Xenopus laevis* oocytes voltage-clamped at -60 mV and each pair of traces was taken from the same cell. For each oocyte, a concentration of GABA which produced a response amounting to 10% of the maximum GABA response (EC_{10}) was determined and used.

Etomidate is optically active. The interaction of this anaesthetic with mammalian $GABA_A$ receptors and the induction of general anaesthesia is favoured by **R**-(+) etomidate *cf* the **S**-(−)isomer (Olsen *et al*., 1986). For the Rdl_{M314N} receptor, the GABA modulatory effect of 100 μM **R**-(+)etomidate (52±6% of the GABA maximum, $n=4$) was greater than that produced by 100 μM **S**-(−)etomidate (12±1% of the GABA maximum, $n=3$). This enantioselectivity is in accord with that observed for mammalian ($\alpha_6\beta_3\gamma_{2L}$) $GABA_A$ receptors (Belelli *et al*., 1997a,b). These data suggest

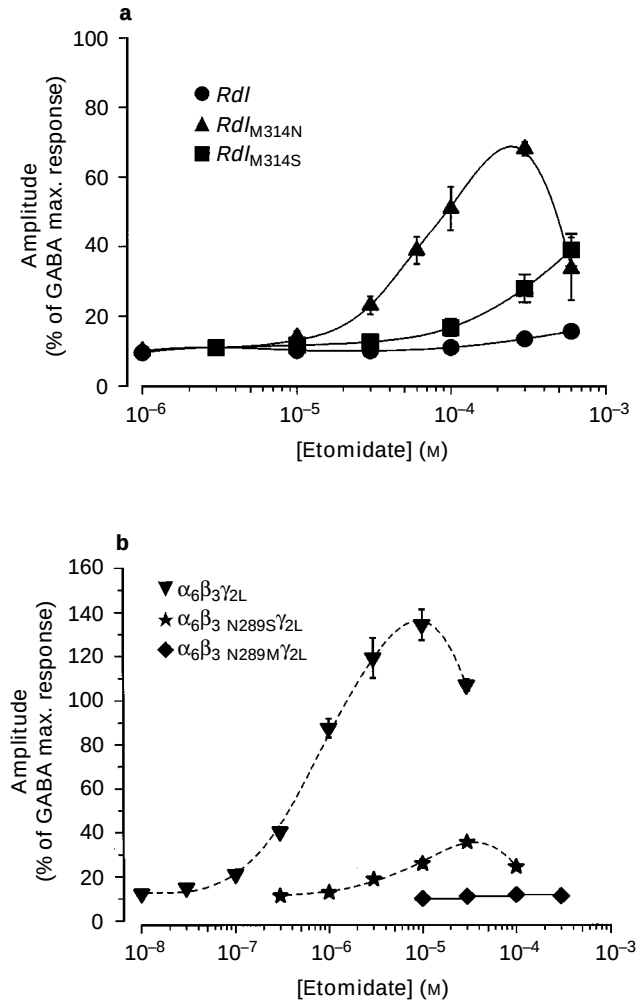

Figure 2 A single transmembrane amino acid governs etomidate sensitivity of an invertebrate and a human GABA receptor. The graph depicts the relationship between the concentration of etomidate (logarithmic scale) and the peak amplitude of the GABA-evoked current (on a linear scale and expressed relative to the maximum current induced by a saturating concentration 1–3 mM of GABA) (a) invertebrate GABA receptors, *Rdl*, Rdl_{M314S} and Rdl_{M314N}, and at (b) human $\alpha_6\beta_3\gamma_{2L}$, $\alpha_6\beta_{3\,N289S}\,\gamma_{2L}$ and $\alpha_6\beta_{3\,N289M}\,\gamma_{2L}$ receptors. Each point represents the mean of data obtained from 3–4 oocytes which were voltage-clamped at a holding potential of −60 mV, vertical lines show the s.e.mean. Note that the EC_{50} values for etomidate (where calculated–see Table 2) were determined from curve fits restricted to the ascending limb of the concentration-effect relationship. Curves illustrated here were fitted 'free-hand' and have no theoretical significance. The data for $\alpha_6\beta_3\gamma_{2L}$ and $a_6\beta_{3\,N289S}\,\gamma_{2L}$ (dotted lines) were taken from (Belelli *et al*., 1997) and are shown here for comparison.

that not only has the point mutation imparted etomidate sensitivity to the invertebrate receptor, but additionally this protein now exhibits the appropriate anaesthetic stereoselectivity common to the mammalian $GABA_A$ receptor.

For $GABA_A$ receptors, the GABA-mimetic actions of etomidate are also influenced by the same β subunit amino acid (Belelli *et al*., 1997a,b). However, although the substitution of the methionine residue to an asparagine imparted GABA modulatory actions to the invertebrate receptor, even relatively high concentrations of the anaesthetic were not GABA-mimetic. A small inward current induced by relatively high concentrations of etomidate ($\geqslant$100 μM) was evident on some oocytes (eg see Figure 1), but was insensitive to picrotoxin (1 μM) and exhibited a current-voltage relationship which was inconsistent with a chloride-mediated current. Furthermore, a similar current was also observed on some uninjected oocytes. Collectively, these observations suggest that this current is not a consequence of a GABA-mimetic action of etomidate. Although pentobarbitone acts as a GABA

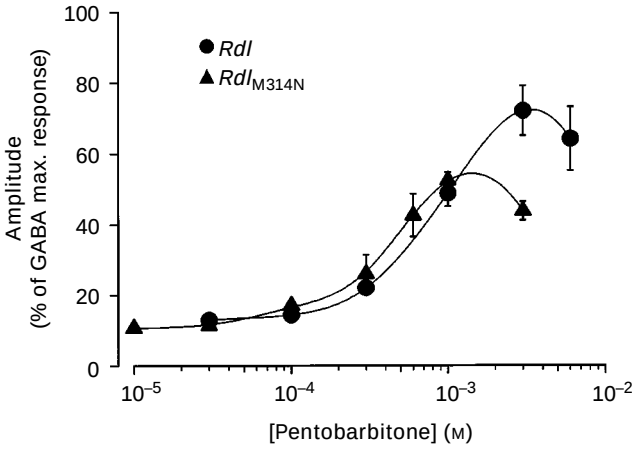

Figure 3 The positive allosteric effects of pentobarbitone at the invertebrate GABA receptor (*Rdl*) were not influenced by transmembrane amino acid 314. The graph depicts the relationship between the concentration of pentobarbitone (logarithmic scale) and the peak amplitude of the GABA-evoked current (on a linear scale and expressed relative to the maximum current induced by a saturating concentration of 1 mM GABA). Data illustrate the potentiation of GABA by pentobarbitone at *Rdl* and Rdl_{M314N} receptors. Each point represents the mean of data obtained from 3–4 oocytes which were voltage-clamped at a holding potential of −60 mV; vertical lines show the s.e.mean. Note that the EC_{50} values for pentobarbitone were calculated from curve fits restricted to the ascending limb of the concentration-effect relationship. Curves illustrated here were fitted 'free-hand' and have no theoretical significance.

Table 2 A comparison of the GABA-modulatory and GABA-mimetic activities of etomidate across wild type and point-mutant GABA receptors expressed in *Xenopus laevis* oocytes

Subunit Composition	*Modulatory EC_{50} (μM)*	*Modulatory E_{max} (%)*	*Agonist EC_{50} (μM)*	*Agonist E_{max} (%)*
Rdl	ND	16±1 (600 μM)	Inactive	Inactive
Rdl_{M314N}	64±3	68±2	Inactive	Inactive
Rdl_{M314S}	ND	39±5 (600 μM)	Inactive	Inactive
*$\alpha_6\beta_3\gamma_{2L}$	0.7±0.06	135±7	23±2.4	96±24
$\alpha_6\beta_{3N289M}\gamma_{2L}$	ND	11.8±0.6 (100 μM)	ND	3.5±0.4 (600 μM)
*$\alpha_6\beta_{3N289S}\gamma_{2L}$	5.7±2.6	36±1	55±6	14±3
*$\alpha_6\beta_1\gamma_{2L}$	7.4±0.6	28±2	ND	5±2
*$\alpha_6\beta_{1S290N}\gamma_{2L}$	1.6±0.3	150±13	79±6	45±13

Numbers in parentheses refer to the maximum concentration of etomidate tested. The E_{max} is expressed relative to the maximal response to GABA. ND = not determined due to limited potentiation, or direct agonist action, or the absence of a clear maximal effect.
* Data from Belelli *et al*. (1997) and Hill-Venning *et al*. (1997) for comparison.

modulator at the wild type *Rdl* receptor it is not GABA-mimetic (Belelli *et al*., 1996). Similarly, pentobarbitone acting at the Rdl_{M314N} receptor was inert in this respect.

We have previously shown that etomidate acting at human $\alpha_6\beta_3\gamma_{2L}$ $GABA_A$ receptors produces a potent and large enhancement of the GABA-evoked current (eg see Figure 1, 2, 3 and Table 2) and at higher concentrations produces a GABA-mimetic effect (Belelli *et al*., 1997a,b). In a preliminary experiment, we demonstrated that mutation of the β_3 subunit asparagine residue 289 to a methionine residue ($\beta_{3\,N289M}$) inhibited the positive allosteric actions of etomidate when coexpressed with α_6 and γ_{2L} receptor subunits (Belelli *et al*., 1997a,b). Here, we have quantified this effect. Acting at $\alpha_6\beta_{3\,N289M}\gamma_{2L}$ receptors, etomidate over a range of concentrations (10–300 μM) produced no enhancement of the GABA-evoked current ($n=4$; see Figures 1 and 2). Furthermore, this mutation completely inhibited the GABA-mimetic actions associated with relatively high concentrations of this anaesthetic at the wild type $\alpha_6\beta_3\gamma_{2L}$ receptor (Figure 1). However, this mutation has not produced a non-specific perturbation of allosteric regulation of the receptor protein as the GABA-enhancing action of the anaesthetic neurosteroid 5α-pregnan-3α-ol-20-one (1 μM) at wild type $\alpha_6\beta_3\gamma_{2L}$ receptors ($90\pm9\%$ of the GABA maximum, $n=6$) was similar to that produced at the $\alpha_6\beta_{3\,N289M}\gamma_{2L}$ receptor ($108\pm9\%$ of the GABA maximum, $n=5$). However, this mutation did cause a modest increase in the calculated EC_{50} for GABA ($\alpha_6\beta_3\gamma_{2L}=14\pm0.8$ μM; $n_H=1\pm0.05$; $n=4$; $\alpha_6\beta_{3\,N289M}\gamma_{2L}=31\pm1$ μM; $n_H=0.9\pm0.03$; $n=4$) with no effect on the Hill coefficient.

Discussion

The cardinal findings of the present study were (i) that a single amino acid substitution (M314N) within the putative channel sequence of the *Rdl* receptor was sufficient to impart an enantioselective sensitivity to etomidate upon the invertebrate receptor and (ii) that the reciprocal mutation (N289M) applied to the β_3 subunit of a mammalian hetero-oligomeric $GABA_A$ receptor essentially abolished both the GABA-modulatory and GABA-mimetic activities of etomidate. This mutational strategy was guided by previous studies highlighting the importance of the β_2/β_3 subunit, and specifically N289, in the actions of etomidate (Belelli *et al*., 1997a,b; Hill-Venning *et al*, 1997; Sanna *et al*., 1997). In addition, we demonstrated that the substitution M314S also rendered the *Rdl* receptor susceptible to modulation by etomidate, but with a much reduced potency for the anaesthetic in comparison to the M314N mutation. This accords with the diminution in the potency and maximal effect of etomidate that is observed when a β_1 (S290) subunit replaces either a β_2 or β_3 (N289) subunit in mammalian $GABA_A$ receptors of otherwise identical composition (Belelli *et al*., 1997a,b; Hill-Venning *et al*., 1997). Such a coherent picture is particularly surprising in view of the modest sequence identity between *Rdl* and mammalian $GABA_A$ receptor subunits (Hosie *et al*., 1997).

The observation that the mutant invertebrate receptor (Rdl_{M314N}) in common with mammalian $GABA_A$ receptors (Olsen *et al*., 1986), exhibits a clear selectivity for the **R**-(+) enantiomer of etomidate has considerable importance. Such enantioselectivity is indicative of a direct interaction with chiral (almost certainly protein) target rather than an indirect action through an achiral lipid membrane. Notwithstanding the fact that some phospholipids, and indeed cholesterol, possess chiral centres (Dickinson *et al*., 1994; Franks & Lieb, 1994), an indirect action of etomidate via the membrane is difficult to reconcile with the effects of the amino acid substitutions described here. Additionally, the demonstration that intracellularly applied etomidate is inert in respect of $GABA_A$ receptor modulation (Belelli *et al*., 1997a,b) militates against the prime perturbation being mediated by a change in membrane structure.

Very recently, Mihic *et al*. (1997) identified S290 (alternatively numbered S265) of the β_1 $GABA_A$ receptor subunit to influence modestly the positive allosteric regulation by ethanol and enflurane of recombinant $GABA_A$ receptors assembled from α_1 (or α_2) and β_1 subunits (Mihic *et al*., 1997). Mutation of this amino acid to isoleucine (the homologous residue contained within the GABA ρ_1 subunit which is inhibited by ethanol and enflurane), produced a reduction in GABA potentiation by these agents. More strikingly, the homologous serine residue of both the α_1 and α_2 subunits of the $GABA_A$ receptor, and the α_1 subunit of strychnine-sensitive glycine receptors, was found to exert a profound influence upon positive allosteric regulation by these anaesthetics. Clearly, the nature of the amino acid at this key position within several inhibitory amino acid receptor subunits can effect the allosteric activities of both relatively simple (ethanol, enflurane) and more complex (etomidate) anaesthetic structures (Mihic *et al*., 1997; Peters & Lambert, 1997). However, it is not essential to the activities of all anaesthetics, since potentiation by pentobarbitone, propofol and neuroactive steroids is little affected by the amino acid substitutions at this position which have been studied thus far.

A key question in interpreting the results of this study is whether the point mutations influence the transduction of the allosteric actions of etomidate or whether they impinge upon a discrete binding site for the anaesthetic? At present, this cannot be answered directly. However, it is noteworthy that the acquisition of etomidate sensitivity by the invertebrate receptor is associated with a decrease of the apparent affinity of the receptor for GABA, whereas the loss of etomidate activity at the human $GABA_A$ receptor is accompanied by a reciprocal increase in the apparent affinity for GABA. Given the fact that the mutated residues reside within a transmembrane domain, it is most unlikely that they contribute to the agonist recognition site directly, indicating that the small changes (2–4 fold) in the potency of GABA may result from an alteration of the allosteric properties of the protein as a whole. Consistent with these observations, a previous study has also documented changes in the apparent affinity of GABA at receptors with M2 domain mutations (Chang *et al*., 1996). However, in comparison to their dramatic effect upon the actions of etomidate, the mutations described in the present study exert only a modest effect on the apparent affinity of the receptor for GABA. Furthermore, the mutations do not influence all classes of anaesthetic equally, as the actions of pentobarbitone at the invertebrate receptor and the anaesthetic steroid 5α-pregnan-3α-ol-one at both mammalian and invertebrate receptor were little changed. We suggest that the point mutation of *Rdl* either creates a new binding pocket for etomidate on the invertebrate receptor protein, or that the wild type receptor possesses an etomidate binding site which is functionally inert until the replacement of the methionine residue by an asparagine residue.

Previous mutagenesis studies have identified amino acids located within channel forming regions that influence the block of AMPA receptors by pentobarbitone (Yamakura *et al*., 1995) and the block of muscle nicotinic receptors by isoflurane or hexanol (Forman *et al*., 1995). In those instances, the possibility that the amino acids contribute to the anaesthetic binding site is more persuasive. For both receptors, the

identified amino acids are thought to point into the ion channel lumen (Akabas *et al*., 1994; Burnashev, 1996). Furthermore, the characteristics of the antagonism produced by the anaesthetics are consistent with binding occurring within the ion channel lumen (Forman *et al*., 1995; Yamakura *et al*, 1995).

Isoflurane ($\sim$100–200 μM) acting at *Rdl* receptors produces a modest enhancement of GABA-evoked responses: whereas higher concentrations of the anaesthetic (>400 μM) are associated with a concentration-dependent block. The GABA-enhancing actions of low concentrations of isoflurane are unchanged in an insecticide (picrotoxin) resistant form of the receptor in which an alanine residue located deep within the ion channel domain (6′, see Table 1) is exchanged for a serine (Edwards & Lees, 1997). However, the inhibitory effects associated with higher concentrations of the anaesthetic are now prevented, revealing a large enhancement of the GABA-evoked current. A parsimonious explanation of these data invokes both a high affinity modulatory site and a low affinity channel blocking site for the anaesthetic, the latter being perturbed by the mutation (Edwards & Lees, 1997).

It is far more difficult to conceive a mechanism whereby the binding of a bulky anaesthetic molecule within the ion channel lumen could result directly in an enhanced function of the GABA receptor. However, the orientation of the amino acids which contribute to the ion channel formed by the β subunit, or the *Rdl* receptor, is not known. If the model proposed for the α_1 $GABA_A$ subunit (Xu & Akabas, 1995) can be extended to the β and *Rdl* subunits, then the crucial amino acid would probably project into the interior of the protein, and hence the anaesthetic binding site need not be within the aqueous pore. It is noteworthy that the study of Mihic *et al*. (1997) also identified an additional amino acid residue within the third transmembrane domain of the α_1 and α_2 subunits of the $GABA_A$ receptor, and the α_1 subunit of strychnine-sensitive glycine receptor, which determined allosteric regulation by ethanol and enflurane. Given their potentially close apposition, it is conceivable that the residues within the M2 and M3 domains contribute to an anaesthetic binding pocket (Mihic *et al*., 1997).

Ultimately the binding site *versus* transduction issue will be resolved by physical approaches such as X-ray crystallography. However, the identification of single amino acids which selectively modify the interaction of general anaesthetics with specific synaptic proteins, together with the use of transgenic animal technology should allow for the assessment of the role of that protein in the complex processes which result in general anaesthesia.

We are grateful to Drs P. Whiting and R. Roush for providing the human and *Drosophila* GABA receptor cDNAs respectively and Dr R. McGuire for the enantiomers of etomidate. We thank Dr J. Peters for constructive comments upon the manuscript. This work was supported by grants from the Medical Research Council (M.P. and D.B.) and Organon Teknika (K.Mc).

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(Received December 18, 1997
Revised January 9, 1998
Accepted January 19, 1998)