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Subunit-dependent interaction of the general anaesthetic etomidate with the γ-aminobutyric acid type A receptor

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1 The GABA modulating and GABA-mimetic actions of the general anaesthetic etomidate were examined in voltage-clamp recordings performed on *Xenopus laevis* oocytes induced, by cRNA injection, to express human recombinant γ-aminobutyric acid$_A$ ($GABA_A$) receptor subunits.

2 Currents mediated by recombinant receptors with the ternary subunit composition $\alpha_x\beta_y\gamma_{2L}$ (where x = 1,2,3 or 6 and y = 1 or 2), in response to GABA applied at the appropriate EC_{10}, were enhanced by etomidate in a manner that was dependent upon the identity of both the α and β subunit isoforms.

3 For the β_2-subunit containing receptors tested, the EC_{50} for the potentiation of GABA-evoked currents by etomidate (range 0.6 to 1.2 μM) was little affected by the nature of the α subunit present within the hetero-oligomeric complex. However, replacement of the β_2 by the β_1 subunit produced a 9–12 fold increase in the etomidate EC_{50} (6 to 11 μM) for all α-isoforms tested.

4 For α_1, α_2 and α_6, but not α_3-subunit containing receptors, the maximal potentiation of GABA-evoked currents by etomidate was greater for β_2- than for β_1-subunit containing receptors. This was most clearly exemplified by receptors composed of $\alpha_6\beta_1\gamma_{2L}$ compared to $\alpha_6\beta_2\gamma_{2L}$ subunits, where a maximally effective concentration of etomidate potentiated currents evoked by GABA at EC_{10} to 28 ± 2% and 169 ± 4% of the maximal GABA response, respectively.

5 For α_1 subunit-containing receptors, the potency and maximal potentiating effect of either pentobarbitone or propofol was essentially unaffected by the β subunit isoform contained within the receptor complex. The potency of the anaesthetic neurosteroid 5α-pregnan-3α-ol-20-one was marginally higher for β_1 rather than the β_2 subunit-containing receptor, although its maximal effect was similar at the two receptor isoforms.

6 The GABA-mimetic action of etomidate was supported by β_2- but not β_1-subunit containing receptors, whereas that of pentobarbitone or propofol was evident with either β isoform. For β_2-subunit containing receptors, both the agonist EC_{50} and the maximal current produced by etomidate were additionally influenced by the α isoform.

7 It is concluded that the subtype of β-subunit influences the potency with which etomidate potentiates GABA-evoked currents and that the β isoform is a crucial determinant of the GABA-mimetic activity of this compound. The nature of the α-subunit also impacts upon the maximal potentiation and activation that the compound may elicit. Such pronounced influences may aid the identification of the site that recognises etomidate. More generally, these results provide a clear example of structural specificity in anaesthetic action.

Keywords: $GABA_A$ receptor; $GABA_A$ receptor α, β and γ subunits; intravenous general anaesthetics; etomidate; propofol; pentobarbitone; 5α-pregnan-3α-ol-20-one

Introduction

The γ-aminobutyric acid type A ($GABA_A$) receptor is an anion selective ligand-gated ion channel that mediates the majority of the inhibitory actions of GABA within the central nervous system. The receptor is composed of a pentamer of structurally homologous subunits which, in mammals, may be drawn from α_{1-6}, β_{1-3}, δ and γ_{1-3} subunit families (Sieghart, 1995; Smith & Olsen, 1995). The precise subunit composition of $GABA_A$ receptor isoforms is an important determinant of their pharmacological and biophysical properties (Whiting *et al*., 1995; McKernan & Whiting, 1996). Drugs that selectively potentiate GABA-ergic neurotransmission, or include such an action within a wider spectrum of effects, can produce behavioural actions that include anxiolytic, anti-convulsant, sedative, and, most strikingly, general anaesthetic actions (Sieghart, 1995). Although the latter has traditionally been linked to the ability of anaesthetic molecules to partition into the lipid phase of the plasma membrane (Franks & Lieb, 1994; Little, 1996) to effect changes in membrane fluidity or volume, there is now an impressive corpus of evidence demonstrating that the majority of clinically useful and experimental anaesthetics potentiate the actions of GABA at the $GABA_A$ receptor (Tanelian *et al*., 1993; Franks & Lieb, 1994; Lambert *et al*., 1995).

The sheer diversity of anaesthetic structures that modulate $GABA_A$ receptor function, when combined with exquisite structure activity requirements within certain classes of agent, such as the pregnane steroids (Lambert *et al*., 1995), militates against the existence of a common binding site for these agents. Furthermore, the effects of binary combinations of chemically distinct general anaesthetics on $GABA_A$ receptor function, support the presence of discrete binding domains on the receptor protein for these agents (e.g. Hales & Lambert, 1991; Belelli *et al*., 1996). The interaction of benzodiazepines with the $GABA_A$ receptor is dependent upon its subunit composition (Smith & Olsen, 1995). This information, together with that obtained from domain exchange and site-directed mutagenesis experiments, has allowed for the tentative identification of the amino acids which may contribute to the benzodiazepine binding pocket (Smith & Olsen, 1995). By contrast, equivalent studies with general anaesthetics such as the barbiturates and the steroids did not initially reveal an absolute subunit preference and as a consequence there has been little progress in determining the structural determinants of anaesthetic binding

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to the $GABA_A$ receptor. However, more recently a distinctive interaction of anaesthetic steroids with α_6-subunit-containing receptors and pentobarbitone and propofol with α_4- and α_6-subunit-containing receptors has been reported (Lambert *et al*., 1996; Thompson *et al*., 1996; Wafford *et al*., 1996). Furthermore, the differential interaction of anaesthetics with an invertebrate recombinant GABA receptor (Chen *et al*., 1994; Belelli *et al*., 1996) and the insensitivity of the retinal ρ-subunit (Cutting *et al*., 1991) to intravenous anaesthetics (Shimada *et al*., 1992; Mihic & Harris, 1996) may offer alternative approaches for future investigations.

The interaction of the general anaesthetic etomidate with different $GABA_A$ receptor isoforms has not been investigated systematically. This anaesthetic is utilized clinically, partly as a consequence of its lack of effect upon sympathetic outflow and autonomic reflexes during induction (Ebert *et al*., 1992). Early electrophysiological studies demonstrated a GABA-mimetic action of this anaesthetic in spinal cord and autonomic ganglia (Evans & Hill, 1978) and GABA-modulated actions in hippocampal slices (Ashton & Wauquier, 1985). Voltage-clamp experiments in which both recombinant and native $GABA_A$ receptors were used confirmed that low concentrations of etomidate potentiate GABA-evoked currents and higher concentrations are GABA-mimetic (Robertson, 1989; Belelli *et al*., 1996). At the single channel level, concentrations of the anaesthetic which may approximate to those achieved clinically, have no effect upon the elementary conductance of $GABA_A$ receptors of rat hippocampus, but produce a prolongation of the receptor-channel open time and opening frequency (Yang & Uchida, 1996). In neurochemical studies, etomidate enhances the binding of [^{3}H]-GABA and of [^{3}H]-diazepam (Thyagarajan *et al*., 1983) to rat brain homogenates and inhibits the binding of [^{35}S]-*tert*-butylbicyclophosphorthionate ([^{35}S]-TBPS) to both native and recombinant receptors (Olsen *et al*., 1986; Ticku & Rastogi, 1986; Slany *et al*., 1995).

We have previously demonstrated that etomidate enhances GABA-evoked currents recorded from *Xenopus laevis* oocytes expressing the human $\alpha_3\beta_1\gamma_{2L}$ recombinant subunit combination (Belelli *et al*., 1996). However, the anaesthetic did not activate the $GABA_A$ receptor complex, a feature readily demonstrable with mammalian native $GABA_A$ receptors (Robertson, 1989; Belelli *et al*., 1996). One interpretation of these disparate findings is that the interaction of etomidate is subunit specific. Here, we demonstrate that both the GABA-modulatory and GABA-mimetic actions of this anaesthetic are dictated by the subunit complement of the $GABA_A$ receptor. Since the receptor composition varies between CNS structures (Whiting *et al*., 1995; McKernan & Whiting, 1996), it can be anticipated that the physiological actions of etomidate will exhibit a regional selectivity that may contribute to its anaesthetic profile. In addition, the subunit-selective action of etomidate might, in the future, allow a better definition of the anaesthetic binding domain on the receptor protein.

Methods

Preparation of transcripts and oocyte injection

cDNAs encoding the human α_1, α_2, α_3, α_6, β_1, β_2 and γ_{2L} $GABA_A$ receptor subunits (obtained from Dr P. Whiting, Merck, Sharp and Dohme, Harlow, U.K.) were linearized by use of the appropriate restriction enzymes in the pCDM8 vector. cRNA transcripts were prepared according to standard protocols (Hope *et al*., 1993). The integrity of the transcripts was determined by electrophoresis through a 1% agarose/formaldehyde gel alongside standard RNA size markers. cRNA transcripts were injected (50 nl of 1 mg ml^{-1} cRNA per subunit) into *Xenopus laevis* oocytes (stage V–VI) which had been defolliculated by a 2–3 h incubation at 18–23°C in a Ca^{2+}-free Barth's saline supplemented with 2 mg ml^{-1} collagenase 'A' (Boehringer-Mannheim). Injected oocytes were subsequently maintained at 19–20°C for up to 12 days in 96 well microtitre plates containing 200 μl of Barth's saline (composition in mM: NaCl 88, KCl 1, $NaHCO_3$ 24, $MgSO_4$ 1, $CaCl_2$ 0.5, $Ca(NO_3)_2$ 0.5, HEPES 15; pH 7.5) supplemented with gentamicin (1 mg ml^{-1}).

Electrophysiological recordings

Electrophysiological experiments with oocytes were conducted 2–12 days after cRNA injection. Agonist evoked currents were recorded under voltage-clamp at a holding potential of −60 mV by an Axoclamp 2A amplifier (Axon Instruments, U.S.A.) in the two electrode voltage-clamp recording mode. The voltage-sensing and current-passing electrodes were filled with 3 M KCl and had resistances of 0.5–1.5 MΩ when measured in extracellular recording solution containing (in mM): NaCl 120, KCl 2, $CaCl_2$ 1.8 and HEPES-NaOH 5 (pH 7.4). Oocytes held in a 0.5 ml chamber were continuously superfused with this solution at a rate of 7–10 ml min^{-1}, and all agonist, antagonist and modulating drugs were applied via their inclusion within the superfusate, Agonist-evoked currents were low-pass filtered at a corner frequency of 200 Hz (Bessel characteristic) and stored on magnetic or digital audio tape with a Racal Store 4DS FM tape recorder or Biologic DAT 1204 recorder respectively, and simultaneously displayed on a chart recorder. All experiments were performed at ambient temperature (18–22°C).

The agonist potency of GABA at recombinant $GABA_A$ receptors composed of $\alpha_X\beta_Y$ and γ_{2L} subunits (where α_X is α_1, α_2, α_3 or α_6, and β_Y is β_1 or β_2) is affected by the composition of the hetero-oligomeric complex (Ebert *et al*., 1994). For several anaesthetic agents which act as positive allosteric modulators of $GABA_A$ receptor mediated currents, potentiation involves an apparent increase in agonist affinity (Harris *et al*., 1995; Belelli *et al*., 1996). To provide a quantitative comparison of the modulating effects of anaesthetic agents at $GABA_A$ receptors of differing subunit composition and apparent affinity towards GABA, it was therefore necessary to utilize concentrations of agonist that produced an equivalent, submaximal, response. For each oocyte and hetero-oligomeric receptor examined, the maximal peak current response to a saturating concentration of GABA (3 mM) was first determined and shown to be stable over time (Belelli *et al*., 1996). The concentration of GABA which evoked a response amounting to 10% of the maximal current (i.e. EC_{10}) was subsequently determined and employed to investigate the effects of the modulating agents. Positive allosteric regulation 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 30–60 s before their co-application with the appropriate concentration of GABA. Potential agonist actions of the anaesthetics were investigated in the absence of GABA and, where evident, responses were expressed as a percentage of the maximal response to GABA. Concentration-effect relationships for either the GABA-modulating or GABA-mimetic actions of the anaesthetics were iteratively fitted, 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 modulator at concentration [A], I_{max} is the amplitude of the response in the presence of a maximally effective concentration of modulator, EC_{50} is the concentration of modulator producing half maximal enhancement and n_H is the Hill coefficient. Concentration-effect relationships for the direct agonist action of the anaesthetics were similarly fitted where I represents the amplitude of the current evoked by anaesthetic concentration [A], I_{max} is the amplitude of the response in the presence of a maximally effective concentration of anaesthetic and EC_{50} is

the concentration of anaesthetic producing half-maximal response.

Quantitative data are presented as the mean ± s.e.mean. Differences between I_{max} values were tested for statistical significance by Student's unpaired t test, (Graphpad Instat; Graphpad, CA, U.S.A.).

Drugs used

γ-Aminobutyric acid, sodium pentobarbitone and picrotoxin (all Sigma) were freshly prepared as stock solutions in saline. A concentrated stock solution of propofol (2,6-diisopropylphenol, 300 mM, Aldrich) was prepared in 100% ethanol, whereas concentrates (10 mM) of flunitrazepam and 5α-pregnan-3α-ol-20-one (both Sigma) were prepared in 100% dimethylsulphoxide as the solvent. Etomidate was obtained from Janssen as a concentrate in alcohol (Hypnomidate for injection, containing etomidate chloride equivalent to 125 mg etomidate free base ml^{-1}) and the concentrate was diluted into saline. The maximal final vehicle concentration for these drugs was 0.2% vol/vol, which was without effect in the presence, or absence, of GABA.

Results

The influence of the β isoform on the actions of etomidate at the GABA$_A$ receptor

For oocytes expressing the $\alpha_1\beta_2\gamma_{2L}$ subunit combination, etomidate produced a concentration-dependent enhancement of the inward current response induced by the bath application of GABA at EC_{10} (Figure 1). The calculated EC_{50} for etomidate, determined over the range 10 nM – 30 μM, was 1.2 ± 0.1 μM and maximal potentiation (127 ± 12% of the GABA maximum; $n = 4$) was produced by 30 μM of the anaesthetic (Figure 1, Table 1). A higher concentration of etomidate (100 μM) was associated with a potentiation of reduced magnitude, giving rise to a bell-shaped concentration-response relationship. At concentrations greater than those required to produce a substantial enhancement of the GABA-mediated response, etomidate (10 – 300 μM), in the absence of GABA, evoked an inward current response (Figures 1 and 2, Table 1) which was enhanced by flunitrazepam (300 nM) and antagonized by picrotoxin (30 μM), confirming the involvement of $GABA_A$ receptors in this effect. The calculated EC_{50} of etomidate for this direct effect was 83 ± 34 μM and the maximal current evoked by 300 μM of the anaesthetic was 19 ± 2% ($n = 4$) of that induced by a saturating concentration of GABA (Figure 1; Table 1). Concentrations of etomidate > 300 μM elicited a complex response consisting of an initial peak, followed by a decline which was succeeded upon wash-out by the transient redevelopment of an inward current (Figure 2). The latter, which has previously been observed for native $GABA_A$ receptors, might be attributable to a low affinity blockade of the chloride channel by etomidate which reverses upon washout before the dissociation of the drug from a higher affinity agonist site (Robertson, 1989).

Etomidate also potentiated GABA-evoked currents recorded from oocytes expressing the $\alpha_1\beta_1\gamma_{2L}$ subunit combination (Figure 1 and Table 1). However, the calculated EC_{50} value for etomidate (determined over the range 300 nM – 100 μM) was approximately 9 fold greater than that derived for the $\alpha_1\beta_2\gamma_{2L}$ subunit combination ($P < 0.005$, Figure 1, Table 1). Furthermore, the maximal potentiation produced by 100 μM etomidate (79 ± 2% of the GABA maximum; $n = 4$) was less than the maximal effect of the anaesthetic on the β_2 subunit-containing receptor ($P < 0.005$, Figure 1, Table 1). A higher concentration of etomidate (300 μM) was associated with a potentiation of reduced magnitude (cf. 100 μM for the $\alpha_1\beta_1\gamma_{2L}$ subunit combination; Figure 1). In contrast to the $\alpha_1\beta_2\gamma_{2L}$ subunit combination, the β_1 subunit-containing receptor appeared relatively insensitive to the direct effects of this anaesthetic. Hence, even a high concentration (1 mM) of etomidate induced a current amounting to only 4 ± 1% of the GABA maximum ($n = 4$). The small magnitude of these currents precluded the calculation of an EC_{50} for this direct effect of etomidate. Furthermore, at these high concentrations (1 – 3 mM), only a proportion (~50%) of this small direct current was blocked by picrotoxin (30 μM) suggesting a component of the response to be mediated by a non-specific action of etomidate. Indeed, un-injected oocytes exhibited small inward currents to high concentrations (3 mM) of etomidate (data not shown). Hence, in summary, replacement of the β_2 by the β_1 subunit in the ternary subunit complex $\alpha_1\beta_X\gamma_{2L}$ reduced the modulating

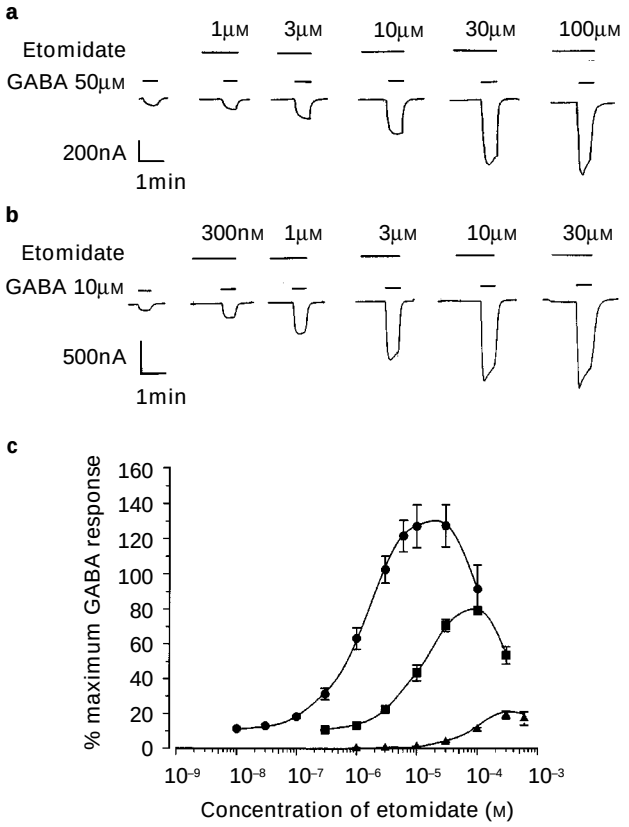

Figure 1 The isoform of the β subunit influenced both the GABA-modulating and GABA-mimetic actions of etomidate. (a) Traces illustrating the concentration-dependent enhancement by etomidate (1 – 100 μM) of the inward current evoked by GABA, at EC_{10}, bath-applied to *Xenopus laevis* oocytes expressing the human $\alpha_1\beta_1\gamma_{2L}$ receptor combination. In this and subsequent illustrations, periods of drug application are indicated by the horizontal bars above the current records. (b) Comparative records depicting enhancement by etomidate (300 nM – 30 μM) of GABA (EC_{10})-evoked currents recorded from oocytes expressing the $\alpha_1\beta_2\gamma_{2L}$ receptor combination. Note that the anaesthetic is a more potent modulator of the $\alpha_1\beta_2\gamma_{2L}$ subunit combination and, that at the highest concentration of etomidate tested (30 μM), there is evidence of a small inward current before the co-application of GABA. (c) Graphical depiction of the relationship between the concentration of etomidate in the medium (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 (3 mM) of GABA). Data show the potentiation of GABA at the $\alpha_1\beta_1\gamma_{2L}$ (■) and the $\alpha_1\beta_2\gamma_{2L}$ (●) receptors. Additionally, the peak direct current elicited by etomidate alone at the $\alpha_1\beta_2\gamma_{2L}$ receptor (▲) is plotted. Little or no direct current was evident for the $\alpha_1\beta_1\gamma_{2L}$ receptor. Each point represents the mean of data obtained from 4 – 5 oocytes, which were voltage-clamped at a holding potential of −60 mV; vertical lines show s.e.mean. Note that the EC_{50} values for etomidate (and other anaesthetics) quoted in the text and Table 1 were calculated from curve fits restricted to the ascending limb of the concentration-effect relationship. Curves illustrated in this and subsequent figures were fitted 'free-hand' and have no theoretical significance.

Table 1 A comparison of the GABA-modulating and GABA-mimetic actions of etomidate across different recombinant $GABA_A$ receptors expressed in *Xenopus laevis* oocytes

Subunit combination	*Etomidate*			
	Modulating EC_{50} (μM)	*Modulating E_{max} (%)*	*Agonist EC_{50} (μM)*	*Agonist E_{max} (%)*
$\alpha_1\beta_2\gamma_2$	1.2±0.1	127±12	83±34	19±2
$\alpha_1\beta_1\gamma_2$	10.8±1.1	79±2	ND	4±1 (1 mM)
$\alpha_2\beta_2\gamma_2$	0.75±0.1	108±1	55±24	26±6
$\alpha_2\beta_1\gamma_2$	6.3±0.3	65±3	ND	5±1 (1 mM)
$\alpha_3\beta_2\gamma_2$	1.0±0.1	88±6	108±4	9±1
$\alpha_3\beta_1\gamma_2$	8.1±0.9	75±8	ND	<2 (1 mM)
$\alpha_6\beta_2\gamma_2$	0.6±0.04	169±14	22±1	51±15
$\alpha_6\beta_1\gamma_2$	7.4±0.6	28±2	ND	5±2 (1 mM)

The numbers in parentheses are the maximum concentrations of etomidate tested as an agonist for the β_1-containing receptors. The E_{max} is expressed as a percentage of the maximum response to GABA. ND = not determined due to the small magnitude of the current induced by etomidate. All data were obtained from 4–6 oocytes voltage-clamped at −60 mV.

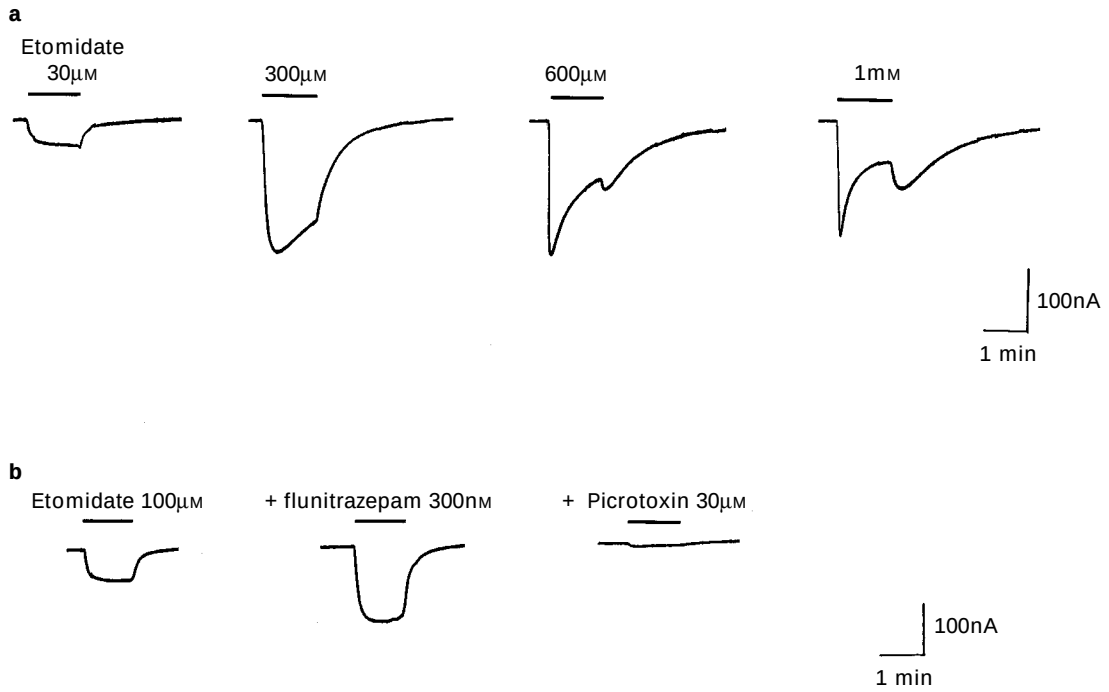

Figure 2 Etomidate directly activated the human $\alpha_1\beta_2\gamma_{2L}$ receptor. Bath applied etomidate (30 μM – 1 mM) induced a concentration-dependent inward current when applied to oocytes expressing the $\alpha_1\beta_2\gamma_{2L}$ receptor. Note that at the higher concentrations of the anaesthetic tested ($\geqslant$300 μM) the current faded in the continued presence of the agonist. With 600 μM and 1 mM etomidate, redevelopment of the inward current was apparent upon wash-out of the drug. (b) The current induced by 100 μM etomidate was enhanced by the co-application of flunitrazepam (300 nM) and antagonized by picrotoxin (30 μM). All records were obtained at a holding potential of −60 mV.

potency and maximal potentiating effect of etomidate. Furthermore, the ability of the anaesthetic to activate directly the $GABA_A$ receptor-channel complex was dramatically reduced.

Pentobarbitone, propofol and 5α-pregnan-3α-ol-20-one are not influenced by the isoform of the β subunit

We further investigated whether the subtype of β subunit similarly influenced the interaction of the structurally diverse anaesthetics pentobarbitone, propofol and 5α-pregnan-3α-ol-20-one with the $GABA_A$ receptor. Pentobarbitone (1–300 μM) produced a concentration-dependent potentiation of GABA-evoked currents recorded from ooyctes expressing either $\alpha_1\beta_2\gamma_{2L}$ or $\alpha_1\beta_1\gamma_{2L}$ receptor subunit combinations (Figure 3). However, in contrast to etomidate, neither the calculated EC_{50} value for pentobarbitone (55±4 μM for $\alpha_1\beta_2\gamma_{2L}$; 65±3 μM for $\alpha_1\beta_1\gamma_{2L}$), nor the maximal potentiation produced (128±7%; n=4 for $\alpha_1\beta_2\gamma_{2L}$; 117±9% of the GABA maximum; n=3 for $\alpha_1\beta_1\gamma_{2L}$) was influencend by the nature of the β subunit isoform (Figure 3). In the absence of GABA, pentobarbitone (100 μM–6 mM) evoked a concentration-dependent inward current on oocytes expressing either subunit combination (Figure 3). Such currents were inhibited by picrotoxin (30 μM) and potentiated by flunitrazepam (300 nM; not shown) implicating the $GABA_A$ receptor in this effect. However, in contrast to etomidate, neither the EC_{50} for pentobarbitone (0.6±0.08 mM for $\alpha_1\beta_2\gamma_{2L}$; 1.1±0.02 mM for $\alpha_1\beta_1\gamma_{2L}$), nor the maximal current produced (16±1%; n=3 for $\alpha_1\beta_2\gamma_{2L}$; 16±1% of the GABA maximum, n=3 for the $\alpha_1\beta_1\gamma_{2L}$ receptor combination) were much influenced by the β isoform.

Propofol (30 nM–30 μM) produced a concentration-dependent enhancement of GABA-evoked currents recorded from oocytes expressing either the $\alpha_1\beta_2\gamma_{2L}$ or $\alpha_1\beta_1\gamma_{2L}$ receptor subunit combinations (Figure 3). The calculated EC_{50} for propofol (7±0.3 μM, for $\alpha_1\beta_2\gamma_{2L}$; 4±0.7 μM for $\alpha_1\beta_1\gamma_{2L}$), was similar for either β isoform. The maximal effect of propofol at β_2 subunit-containing receptors (127±5% of the GABA maximum; n=4) was somewhat greater than that observed at β_1 subunit-containing receptors (95±2% of the GABA maximum; n=5; P<0.01; Figure 3). In the absence of GABA, propofol (10–300 μM) evoked a concentration-dependent inward current on oocytes expressing receptors containing either

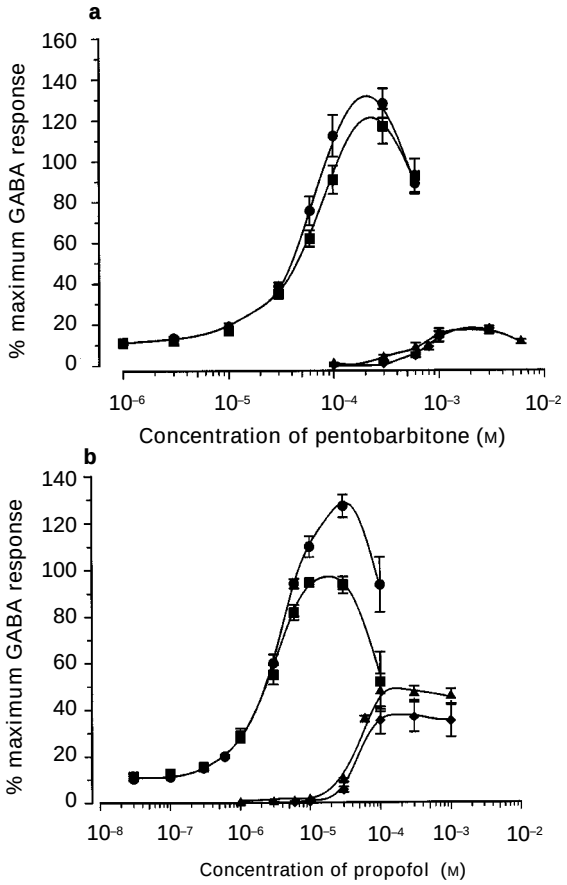

Figure 3 The isoform of the β subunit had little influence upon the GABA-modulating and GABA-mimetic actions of pentobarbitone and propofol. Graph illustrating the relationship between the concentration of (a) pentobarbitone or (b) propofol (logarithmic scale) and the GABA (EC_{10})-evoked current (on a linear scale and expressed relative to the maximum current induced by a saturating concentration (3 mM) of GABA). Data show the potentiation of GABA at the $\alpha_1\beta_1\gamma_{2L}$ (■) and the $\alpha_1\beta_2\gamma_{2L}$ (●) receptor combinations. Additionally, the peak direct currents elicited by these anaesthetics alone were plotted for both $\alpha_1\beta_1\gamma_{2L}$ (◆) and the $\alpha_1\beta_2\gamma_{2L}$ (▲) receptors. Each point represents the mean of data obtained from 3–5 oocytes, which were voltage-clamped at −60 mV; vertical lines show s.e.mean.

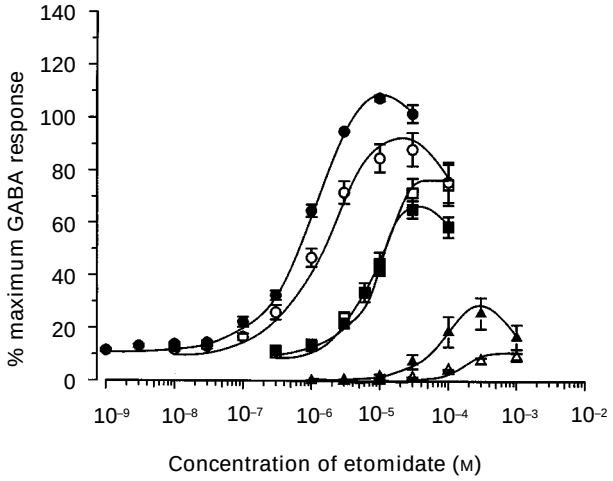

Figure 4 The interaction of etomidate with α_2- and α_3-containing receptors was influenced by the β isoform. The graphs illustrate the relationship between the concentration of etomidate (logarithmic scale) and the GABA (EC_{10})-evoked current (on a linear scale and expressed relative to the maximum current induced by a saturating concentration (3 mM) of GABA) for α_2 subunit-containing and α_3 subunit-containing receptors. Data show the potentiation of GABA at the $\alpha_2\beta_2\gamma_{2L}$ (●) and the $\alpha_3\beta_2\gamma_{2L}$ (○) and $\alpha_2\beta_1\gamma_{2L}$ (■) and $\alpha_3\beta_1\gamma_{2L}$ (□) receptors. Additionally, the peak direct current elicited by etomidate alone is plotted for both the $\alpha_2\beta_2\gamma_{2L}$ (▲) and the $\alpha_3\beta_2\gamma_{2L}$ (△) receptors. Little current was observed for the $\alpha_2\beta_1\gamma_{2L}$ receptor and etomidate induced no current for the $\alpha_3\beta_1\gamma_{2L}$ receptor. Each point represents the mean of data obtained from 4–5 oocytes, which were voltage-clamped at −60 mV; vertical lines show s.e.mean.

β isoform. Currents evoked by propofol were blocked by picrotoxin (30 μM) and enhanced by flunitrazepam (300 nM; not shown). Again, in contrast to etomidate, neither the propofol EC_{50} (47 ± 1 μM for $\alpha_1\beta_2\gamma_{2L}$; 46 ± 1 μM for $\alpha_1\beta_1\gamma_{2L}$), nor the maximal effect ($48\pm8\%$, $n=3$ for $\alpha_1\beta_2\gamma_{2L}$; $37\pm2\%$, $n=5$ for $\alpha_1\beta_1\gamma_{2L}$ of the GABA maximum) were much influenced by the β isoform (Figure 3).

The neurosteroid anaesthetic 5α-pregnan-3α-ol-20-one is established as a potent allosteric modulator of the $GABA_A$ receptor (Lambert *et al.*, 1995). Here, 5α-pregnan-3αol-20-one (3 nM–1 μM) produced an enhancement of GABA-evoked currents recorded from oocytes expressing $\alpha_1\beta_2\gamma_{2L}$ or $\alpha_1\beta_1\gamma_{2L}$ receptors. The calculated EC_{50} for this effect was 177 ± 2 nM and 89 ± 6 nM for $\alpha_1\beta_2\gamma_{2L}$ and $\alpha_1\beta_1\gamma_{2L}$ receptors, respectively (not shown). Hence, in contrast to etomidate, the steroid is slightly more potent at the β_1-containing receptor. The maximum potentiation produced ($75\pm4\%$, $n=4$ for $\alpha_1\beta_2\gamma_{2L}$ receptors and $69\pm4\%$ of the GABA maximum, $n=6$ for $\alpha_1\beta_1\gamma_{2L}$ receptors) is not influenced by the β isoform (not shown). We have previously shown that the direct effect of this neurosteroid is small (~1% of the GABA maximum; see Belelli *et al.*, 1996). Therefore, no attempt was made to compare the influence of the β subtype on this aspect of neurosteroid action.

The influence of the α isoform on the actions of etomidate at the $GABA_A$ receptor

The results above clearly demonstrate that for α_1 subunit-containing receptors, the interaction of etomidate is influenced by the isoform of the β subunit. The impact of changing the subtype of the α subunit on the actions of this anaesthetic for β_1- and β_2-containing receptors was additionally investigated. The EC_{50} for the GABA modulating action of etomidate was little influenced by the subtype of α subunit ($\alpha=2$, 3 or 6) assembled within either $\beta_1\gamma_{2L}$ or $\beta_2\gamma_{2L}$ subunit-containing receptors (Table 1, Figures 4 and 5). However, consistent with the results obtained for α_1 subunit-containing receptors, a comparison of $\alpha_x\beta_2\gamma_{2L}$ with $\alpha_x\beta_1\gamma_{2L}$ receptors (where x = 2, 3 or 6) revealed the etomidate EC_{50} to be 7 to 12 fold greater for the β_1- compared to β_2-containing receptors ($P<0.005$). Furthermore, similar to α_1, for α_2 and α_6 the maximal enhancement produced by the anaesthetic was greater for the β_2-compared with the β_1-containing receptors ($P<0.0001$, Table 1, Figures 4 and 5). This difference is most marked when the β subunit is coexpressed with the α_6 subunit ($169\pm14\%$, $n=5$, for the $\alpha_6\beta_2\gamma_{2L}$; $28\pm2\%$ of the GABA maximum for $\alpha_6\beta_1\gamma_{2L}$, $n=4$; Figure 5). For α_3-containing receptors, the replacement of the β_1 by the β_2 subunit produced an 8 fold decrease of the etomidate EC_{50}, but it had little influence on the maximal potentiation produced ($P>0.1$, Figure 4, Table 1). Hence, although for α_1, α_2 and α_6 both the potency and maximal effect of etomidate are greater for the β_2- compared to β_1-containing receptors, the result obtained with the α_3 subunit suggests that these two properties are not intrinsically linked.

For all β_2 subunit-containing receptors ($\alpha_x\beta_2\gamma_{2L}$ where x = 2, 3 or 6) etomidate, in the absence of GABA, evoked a concentration-dependent inward current that was blocked by picrotoxin (30 μM, not shown). By contrast, etomidate produced little or no effect on the corresponding β_1 subunit-containing receptors (Figures 4 and 5, Table 1). Both the agonist potency of etomidate and the maximal effect produced were clearly

influenced by the α isoform and followed the same rank order ($\alpha_6\beta_2\gamma_{2L} > \alpha_2\beta_2\gamma_{2L} > \alpha_1\beta_2\gamma_{2L} > \alpha_3\beta_2\gamma_{2L}$). In summary, the β_1 subunit-containing receptor does not mediate a robust direct effect of etomidate. However, for β_2 subunit-containing receptors, both the potency and the maximal effect are additionally influenced by the α subunit isoform.

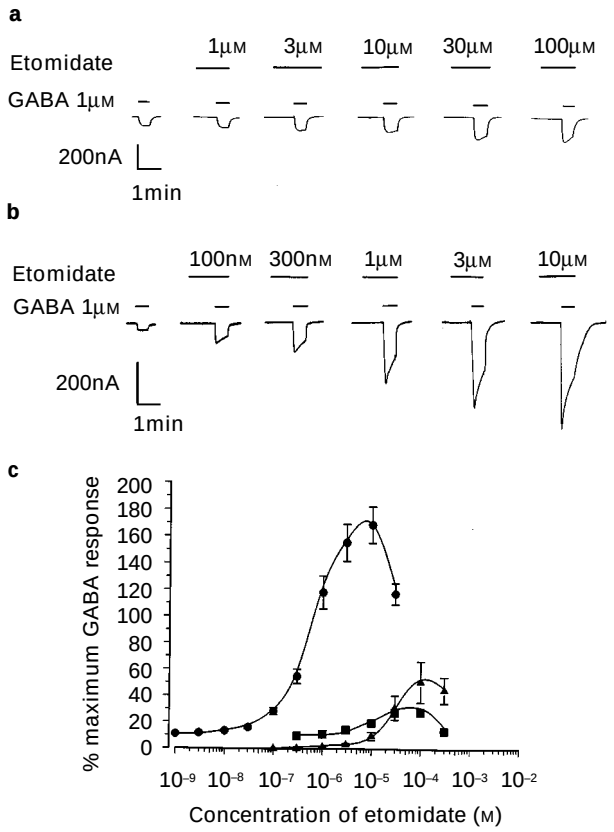

Figure 5 The isoform of the β subunit greatly influenced both the GABA-modulating and GABA-mimetic actions of etomidate for α_6-containing receptors. (a) Etomidate (1–100 μM) produced a concentration-dependent, but modest, enhancement of the inward current produced by GABA, bath applied at EC_{10}, to *Xenopus laevis* oocytes expressing the human $\alpha_6\beta_1\gamma_{2L}$ receptor combination. (b) In comparison, at $\alpha_6\beta_2\gamma_{2L}$ receptors, much lower concentrations of etomidate (100 nM–30 μM) enhanced GABA (EC_{10}) evoked currents and produced a much greater maximal potentiation. (c) Graph illustrating the relationship between the concentration of etomidate (logarithmic scale) and the current elicited by GABA at EC_{10} (on a linear scale and expressed relative to the maximum current induced by a saturating concentration (3 mM) of GABA). Data show the potentiation of GABA at the $\alpha_6\beta_1\gamma_{2L}$ (■) and the $\alpha_6\beta_2\gamma_{2L}$ (●) receptors. Additionally, the peak direct current elicited by etomidate alone at the $\alpha_6\beta_2\gamma_{2L}$ receptor (▲) is plotted. Little or no direct current was evident for the $\alpha_6\beta_1\gamma_{2L}$ receptor. Each point represents the mean of data obtained from 4–6 oocytes, which were voltage-clamped at −60 mV; vertical lines show s.e.mean.

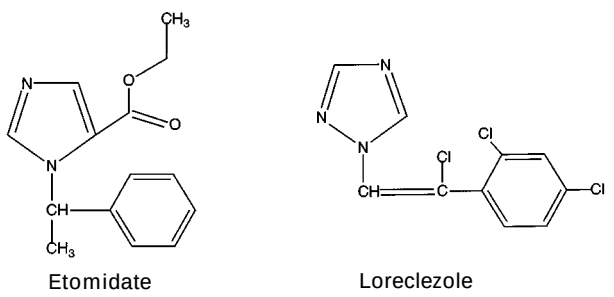

Figure 6 The chemical structures of etomidate and loreclezole.

Discussion

In the present study, etomidate potentiated, in a concentration-dependent manner, GABA-evoked currents mediated by any of the recombinant $GABA_A$ receptors examined. However, both the potency and the magnitude of this effect were clearly dependent on the subtype of the β subunit (β_1 or β_2) expressed within the hetero-oligomeric complex. Hence, for all α subunits examined ($\alpha_{1,2,3}$ and $_6$), etomidate was consistently more potent (7–12 fold) at β_2 compared to β_1 subunit-containing receptors. In preliminary experiments, the GABA-modulating action of etomidate at the $\alpha_6\beta_3\gamma_{2L}$ receptor was found to be similar to that of the corresponding β_2 subunit-containing receptor (D. Belelli, unpublished observations). In contrast to this clear selectivity for β_2 or β_3 subunit-containing receptors, a comparison of the subtype of α subunit within either β_1- or β_2-containing receptors revealed little or no influence of the α subtype on the modulating potency of the anaesthetic. With the exception of α_3 subunit-containing receptors, the maximal enhancement of the GABA-evoked current produced by etomidate was greater for the β_2- than the β_1-containing receptors. This difference is most marked for $\alpha_6\beta_1\gamma_{2L}$ in comparison to $\alpha_6\beta_2\gamma_{2L}$ receptors. However, the example of α_3-containing receptors would suggest the increased maximal effect and the apparent affinity of etomidate for the modulating site are not intrinsically linked. The role of the γ subunit on the GABA modulating actions of etomidate has not been systematically investigated, although an electrophysiological study of HEK293 cells expressing $\alpha_1\beta_1$ and $\alpha_1\beta_1\gamma_{2s}$ subunits revealed that the γ_2 subunit is not essential for activity, but may influence the nature of the pertubation of $GABA_A$ receptor channel kinetics by the anaesthetic (Uchida *et al*., 1995). In summary, the subtype of β subunit is a major determinant of the modulating actions of etomidate, although for some receptors the α subtype may also influence this interaction. The influence of the isoform of the γ subunit remains to be determined.

Although the present investigation was limited to receptors incorporating the α_1 subunit subtype, the nature of the β subunit appeared to exhibit little influence upon the potency, or the maximal effect, of propofol, pentobarbitone or 5α-pregnan-3α-ol-20-one to potentiate GABA-evoked responses (see also Hadingham *et al*., 1993). Hence, the clear β_2 selective actions of etomidate are not shared by propofol, pentobarbitone or 5α-pregnan-3α-ol-20-one. These observations suggest that the modulator binding sites for these anaesthetics are distinct from the etomidate site. In support of this proposal, we have recently demonstrated that pentobarbitone and propofol, but not etomidate, act as positive allosteric modulators of a recombinant GABA receptor isolated from *Drosophila melanogaster*, whereas 5α-pregnan-3α-ol-20-one exerts a modest allosteric action at this invertebrate receptor (Chen *et al*., 1994; Belelli *et al*., 1996).

Previous investigations on native mammalian $GABA_A$ receptors have shown that etomidate, at concentrations generally greater than those required for GABA modulation, can directly activate $GABA_A$ receptors in a picrotoxin- and bicuculline-sensitive manner (Evans & Hill, 1987; Robertson, 1989). In common with the GABA modulating effects of the anaesthetic, the GABA-mimetic action of etomidate is highly dependent upon the β subunit. Indeed, like pentobarbitone and propofol, etomidate can directly activate murine homomeric β_2 or β_3 $GABA_A$ receptors expressed in *Xenopus* oocytes (Cestari *et al*., 1996). Therefore, the agonist binding site for etomidate is clearly represented on these isoforms of β subunit. The $GABA_A$, like the nicotinic, receptor is thought to be composed of five subunits. For the majority of native $GABA_A$ receptors, at least three classes of subunit are proposed to contribute to the hetero-oligomeric complex (McKernan & Whiting, 1996). In the case of recombinant ternary receptors, the GABA-mimetic actions of etomidate are critically dependent on the presence of the β_2 subunit isoform and are minimal, or absent, for β_1-containing receptors. In preliminary

experiments, we found the agonist actions of etomidate to be supported by β_3-containing ternary receptors (D. Belelli, unpublished observations). Therefore, the β isoform affects both the GABA-modulating and GABA-mimetic actions of this anaesthetic, but does not influence the GABA-modulating or GABA-mimetic actions of pentobarbitone or propofol. For β_2 subunit-containing receptors, the EC_{50} for the direct activation of the $GABA_A$ receptor channel complex by etomidate was influenced by the α isoform (range $\sim 22-130$ μM for α_1- and α_6 subunit-containing receptors), whereas the GABA modulating effect was not. Additionally, for β_2-containing receptors, the magnitude of the etomidate-induced current was dependent upon the α subtype (e.g. maximal effect = 9% and 51% of the maximum response to GABA for the α_3- and α_6-containing receptors, respectively; see Table 1). These data strongly suggest that the interaction of this anaesthetic with native $GABA_A$ receptors will be dependent upon the α and β subunit isoform expressed within the pentameric complex. The pharmacological selectivity of etomidate (Shepherd *et al*., 1996) makes it likely that the $GABA_A$ receptor class is an important mediator of the anaesthetic effect of the agent. Indeed, the EC_{50} values determined for the potentiating, but not GABA-mimetic, actions of etomidate at β_2 subunit-containing receptors (range 0.6 to 1.2 μM) are close to the plasma concentration (approximately 2 μM) of the drug required to maintain anaesthesia in patients in which opioid analgesics were co-administered (Fragen *et al*., 1983). However, the free concentration of etomidate in plasma is likely to be considerably lower than this value due to extensive protein binding of the drug (Meuldermans & Heykants, 1976). Conversely, following a bolus injection, the concentration of etomidate in the brain exceeds that measured in plasma (Heykants *et al*., 1975). In view of these facts, it would be unwise to attach too great a significance to the concordance between plasma concentrations of the drug associated with anaesthesia and those affecting β_2 subunit-containing $GABA_A$ receptors.

The differential susceptibility of $GABA_A$ receptor isoforms to allosteric regulation by etomidate may impart a degree of regional selectivity in its actions. A precedent is provided by α_6 subunit-containing receptors which are confined to the granule cells of the cerebellum. A strain of rats has been identified which exhibit pronounced postural impairment and ataxia to benzodiazepines (Korpi *et al*., 1993). Normally, α_6-containing receptors are insensitive to the positive allosteric modulating actions of certain benzodiazepines. However, genetic analysis of these rats reveals a single amino acid mutation of the α_6 subunit which confers sensitivity to such benzodiazepines (Korpi *et al*., 1993). Collectively, these observations suggest that α_6-containing $GABA_A$ receptors may play an important role in cerebellar motor control and its pertubation by drugs. The $\alpha_6\beta_X\gamma_2$ subunit combination is well represented in the rat cerebellum, where the β_1 subunit is a minor component compared to the β_2 and β_3 subunits (Whiting *et al*., 1995; Behringer *et al*., 1996; McKernan & Whiting, 1996). Hence, it is probable that cerebellar granule cells express $GABA_A$ receptors ($\alpha_6\beta_2$or$_3\gamma_2$) which are exceptionally sensitive to both the GABA-modulating and GABA-mimetic actions of etomidate (Table 1, Figure 5).

Clearly, the relative slow application of submaximal concentrations of GABA to an oocyte does not reproduce the situation thought to occur at many central GABA-ergic synapses, where the release of neurotransmitter is rapid and may briefly saturate a relatively small number of postsynaptically located $GABA_A$ receptors (Mody *et al*., 1994). These features restrict the mechanisms by which positive allosteric modulators of the $GABA_A$ receptor can act to enhance fast inhibitory neurotransmission within the central nervous system. Under those conditions, such compounds produce a prolongation of the duration of the inhibitory postsynaptic current (i.p.s.c.) rather than an augmentation of i.p.s.c. peak amplitude (Mody *et al*., 1994). The application of whole-cell clamp techniques may provide an understanding of the role of the α_6 subunit in the inhibitory circuitry of the cerebellum (e.g. Tia *et al*., 1996). It would now be of interest to investigate the influence of etomidate on granule cell miniature i.p.s.cs to determine whether the clear $GABA_A$ receptor subtype selectivity evident in experiments with recombinant receptors has a functional consequence for synaptic transmission.

The clear selectivity of etomidate for β_2 and β_3 over β_1 subunit-containing receptors is reminiscent of the situation for the positive allosteric actions of the anticonvulsant loreclezole (Wingrove *et al*., 1994). Indeed, the structures of etomidate and loreclezole are similar (Figure 6). The preference of loreclezole for the β_3 and β_2 rather than the β_1 subunit appears to be endowed by a single amino acid (an asparagine residue within β_2 and β_3 and a homologous serine residue within β_1) located towards the extracellular side of the M2 domain of the subunit, a region thought to form the lining of the associated chloride ion channel (Wingrove *et al*., 1994). Mutation of this amino acid to a serine residue (as in β_1) results in a reduction of the sensitivity to loreclezole, whereas mutation of the equivalent serine residue of a β_1 subunit to an asparagine enhances loreclezole sensitivity (Wingrove *et al*., 1994). The recent observation that the positive allosteric actions associated with relatively high concentrations of the β-carboline methyl-6,7-dimethoxy-4-ethyl-β-carboline (DMCM; Stevenson *et al*., 1995) are similarly influenced by this residue highlights this domain as an important modulator locus for drug action. It would clearly be of interest to examine the impact of the above mutation upon the sensitivity of the $GABA_A$ receptor towards etomidate. If etomidate and loreclezole do indeed share a common site of action, it remains to be explained why the former possesses anaesthetic activity but the latter does not. Of potential relevance are the limited potentiation of $GABA_A$ receptor-mediated currents that can be achieved with loreclezole in comparison to etomidate (Wafford *et al*., 1994; D. Belelli, unpublished observations) and the absence of a GABA-mimetic action for the former compound (Wafford *et al*., 1994).

In conclusion, the molecular interactions of the general anaesthetic etomidate with the $GABA_A$ receptor are subunit selective. It remains to be determined whether the primary influence of the β isoform subtype is on the anaesthetic binding site, the transduction process, or both. Nevertheless, these observations appear counter-intuitive to a mechanism which invokes a non-specific membrane perturbation by the anaesthetic to produce a secondary change in receptor function.

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