British Journal of Pharmacology (1996) 117, 521-527

Barbiturate interactions at the human $GABA_A$ receptor: dependence on receptor subunit combination

S.A. Thompson, P.J. Whiting & [1]K.A. Wafford

Merck Sharp & Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Eastwick Road, Harlow, Essex, CM20 2QR

1 Human $GABA_A$ receptors containing different α and β subunits with a γ2s subunit were expressed in *Xenopus* oocytes and the effects of pentobarbitone on these subunit combinations were examined by electrophysiological recording of GABA currents with the two-electrode voltage-clamp method.

2 Pentobarbitone has previously been shown to have three actions on $GABA_A$ receptors: a potentiation of GABA responses, a direct activation of $GABA_A$ receptors and, at high concentrations, a block of the GABA chloride channel. In this study pentobarbitone activity consisted of the above mentioned three components on all the subunit combinations tested. However, the affinities and efficacies varied with receptor subtype.

3 Potentiation of GABA by pentobarbitone occurred over the same concentration-range for all the subunits with affinities in the range of 20-35 μM. The degree of potentiation obtained, however, varied from 236% of GABA EC_{20} on $\alpha1\beta2\gamma2$s to 536% on $\alpha6\beta2\gamma2$s.

4 Examination of the direct effect of pentobarbitone revealed that the type of α subunit present determines both the degree of affinity and efficacy obtained. Receptors containing an $\alpha6$ subunit produced maximum direct responses to pentobarbitone larger than that obtainable with maximum GABA (150% to 170% of maximum GABA). The maximum direct pentobarbitone response obtainable with other α subunits ranged between 45% of maximum GABA for $\alpha5\beta2\gamma2$s to 82% for $\alpha2\beta2\gamma2$s. The affinity of the direct action of pentobarbitone on $\alpha6\beta2\gamma2$s was 58 μM compared to affinities for the other α subunits ranging from 139 μM on $\alpha2\beta2\gamma2$s to 528 μM on $\alpha5\beta2\gamma2$s.

5 The type of β subunit present did not influence the direct action of pentobarbitone to the same extent as the α subunit. There were no significant differences between affinity or efficacy on oocytes expressing $\alpha6$ and $\gamma2$s with $\beta1$, $\beta2$ or $\beta3$. Affinities and efficacies on oocytes expressing $\alpha1$ and $\gamma2$s with $\beta1$, $\beta2$ or $\beta3$ were significantly different with pentobarbitone having a higher affinity and efficacy on $\alpha1\beta3\gamma2$s followed by $\alpha1\beta2\gamma2$s and then $\alpha1\beta1\gamma2$s.

6 The direct effect of pentobarbitone was blocked by picrotoxin but not by competitive antagonists, such as bicuculline or SR95531, indicating that the direct agonist activity of pentobarbitone was not mediated via the GABA binding site.

7 For the first time the influence of the various α and β subunits on the effects of pentobarbitone were demonstrated. The results indicate that $GABA_A$ receptors containing $\alpha6$ subunits have both a higher affinity and efficacy for direct activation by pentobarbitone, and reveal that pentobarbitone binds to more than one site on the $GABA_A$ receptor, and these are dependent on receptor subunit composition.

Keywords: Pentobarbitone; γ-aminobutyric acid; *Xenopus* oocytes; two-electrode voltage-clamp; $GABA_A$ subunit

Introduction

The cellular mechanism of anaesthesia has been debated for a number of decades. It is now generally accepted that a common feature of general anaesthetic agents is positive modulation of the inhibitory function of the neurotransmitter γ-aminobutyric acid (GABA) through $GABA_A$ receptors (Olsen, 1988; Tanelian *et al.*, 1993; Franks & Lieb, 1994; Zimmerman *et al.*, 1994). Electrophysiological and neurochemical studies have shown that general anaesthetic agents can have three mechanisms of action, namely (i) a potentiation of the GABA response (Evans, 1979; Study & Barker, 1981; Lin *et al.*, 1992), (ii) a direct activation of $GABA_A$ receptors (Robertson, 1989; Franks & Lieb, 1994) and (iii) at high concentrations, a block of the GABA chloride channel (Schwartz *et al.*, 1986; Peters *et al.*, 1988; Robertson, 1989). The potentiation of the GABA response has been shown to be due to an increase in the ion channel open time (Mathers & Barker, 1980; Study & Barker, 1981; Jackson *et al.*, 1982; Macdonald *et al.*, 1989). This mechanism of action differs from benzodiazepines which increase the frequency of channel openings (Rogers *et al.*, 1994).

The $GABA_A$ receptor is a multigene family ($\alpha1-6$, $\beta1-3$, $\gamma1-3$ and δ) that is formed by co-assembly of different glycoprotein subunits in the arrangement $\alpha\beta\gamma$ or $\alpha\beta\delta$ (for review see Whiting *et al.*, 1995). In addition to the GABA binding site, which when occupied by GABA directly opens the chloride selective anion channel, a number of modulatory sites have been identified which modulate the activity of the receptor/channel complex.

In this paper we describe the contribution of various receptor subunits to the potentiation of GABA responses and the direct activation of $GABA_A$ receptors by pentobarbitone. This is the first paper showing the influence that the $\alpha6$ subunit has on the direct action of pentobarbitone. Preliminary results have been presented in abstract form (Thompson *et al.*, 1995).

Methods

Human $GABA_A$ receptor cDNAs

cDNAs encoding human $\alpha1$, $\alpha2$, $\alpha3$, $\alpha5$, $\beta1$, $\beta2$, $\beta3$ and $\gamma2$ subunits have been described elsewhere (Hadingham *et al.*, 1993a,b). Cloning and sequencing of cDNAs encoding the

[1] Author for correspondence.

human α6 subunit will be described elsewhere (Hadingham *et al.*, 1995 unpublished).

Xenopus *oocyte expression*

Adult female *Xenopus laevis* were anaesthetized by immersion in a 0.4% solution of 3-aminobenzoic acid ethylester for 30–45 min (or until unresponsive). Ovary tissue was removed via a small abdominal incision and Stage V and VI oocytes were isolated with fine forceps. After mild collagenase treatment to remove follicle cells (Type 1A, 0.5 mg ml^{-1}, for 8 min), the oocyte nuclei were directly injected with 10–20 nl of injection buffer (NaCl 88 mM, KCl 1 mM, HEPES 15 mM, at pH 7, filtered through nitrocellulose) or sterile water containing different combinations of human $GABA_A$ subunit cDNAs engineered into the expression vector pCDM8 or pcDNAI/Amp. Following incubation for 24 h, oocytes were placed in a 50 μl bath and perfused at 4–6 ml min^{-1} with modified Barth's medium (MBS) consisting of (mM): NaCl 88, KCl 1 HEPES 10, $MgSO_4$ 0.82, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.91, $NaHCO_2$ 2.4, at pH 7.5. Cells were impaled with two 1–3 MΩ electrodes containing 2 M KCl and voltaged clamped between −30 and −80 mV.

Pentobarbitone concentration-response curves

In a single oocyte we were able to examine both the direct action of pentobarbitone and the potentiation of GABA. Following a maximal response to GABA (3 mM), constant responses to an EC_{20} concentration were obtained, an EC_{20} concentration being defined as the concentration of agonist that produces 20% of the maximal response for that agonist. The direct action of pentobarbitone was observed followed immediately by the potentiation of the GABA EC_{20}. Between each concentration of pentobarbitone constant responses to the GABA EC_{20} were obtained. In some subunit combinations only the direct action of pentobarbitone was studied and in these cells no GABA EC_{20} responses were obtained. In all cases the drugs were applied until the peak of the response was observed. At least 3 min of wash time was allowed between each drug application, to prevent receptor desensitization. Concentration-response curves were calculated by using a non-linear square fitting programme to the equation $f(x) = B_{max}/[1 + (EC_{50}/x)^{nH}]$ where x is the drug concentration, EC_{50} is the concentration of drug eliciting a half-maximal response and n_H is the Hill coefficient.

GABA antagonists

GABA antagonists (picrotoxin, bicuculline and SR 95531) were preapplied for 30 s before GABA or pentobarbitone addition.

Drugs

Drugs used were: γ-amino-n-butyric acid (GABA; Sigma), pentobarbitone sodium (Sagatal; Rhone Merieux), picrotoxin (Sigma), (−)-bicuculline methiodide (Sigma), SR95531 (Research Biochemicals Inc). Solutions of GABA were prepared in MBS, of bicuculline, picrotoxin and SR95531 in dimethylsulphoxide (DMSO) while pentobarbitone sodium was supplied as a 60 mg ml^{-1} solution in ethanol. The highest concentration of DMSO or ethanol vehicle perfusing the oocyte was 0.1% and 1.2% respectively neither of which had effects when applied alone at these concentrations.

Data analysis

Arithmetic mean values or geometric mean values were calculated from data obtained from a number (n) of different cells. The statistical significance of differences between mean values were assessed by Student's one-tailed t tests.

Results

Potentiation of the GABA response

Effects of pentobarbitone were studied on $GABA_A$ receptor subtypes expressing β2γ2s in the presence of different α subtypes. Potentiation of the GABA EC_{20} response by pentobarbitone occurred at approximately 10 fold lower concentrations than the direct channel activation of pentobarbitone on α1β2γ2s, α2β2γ2s, α3β2γ2s and α5β2γ2s whereas on α6β2γ2s both effects occurred over the same concentration-range. Measurement of potentiation of the GABA EC_{20} response by pentobarbitone included the direct activation component. The affinity for the potentiation of GABA EC_{20} responses by pentobarbitone was similar on all subunits tested (between 20 and 35 μM); however, the maximum percentage increase in GABA EC_{20} varied from 236% on α1β2γ2s to 536% on α6β2γ2s (Figure 1 and Table 1). Concentrations of pentobarbitone greater than 100 μM produced no further increase in the degree of potentiation and bell shaped concentration-response curves were observed (Figure 1). The slopes for the potentiation of the GABA response were all less than 2.0. A typical representative trace showing the effects of pentobarbitone on GABA responses in oocytes expressing human α1β2γ2s and α6β2γ2s $GABA_A$ receptors is shown in Figure 2.

Direct effect of pentobarbitone on receptors containing different α subunits

The direct effects of pentobarbitone varied according to the α subunit present (Figure 3a and Table 2). Oocytes expressing receptors containing α6 subunits produced currents to pentobarbitone alone which were larger than those obtainable with a maximal concentration of GABA. These currents ranged from 154% of the maximal GABA response on α6β1γ2s to 168% on α6β2γ2s. The other α subunits (α1, α2, α3, α5) only produced currents to pentobarbitone which were smaller than those obtained with a maximal concentration of GABA. These efficacies ranged from 45% of the maximal GABA response on α5β2γ2s to 83% on α2β2γ2s. Affinities for the direct effect of pentobarbitone on α6 containing receptors were higher (between 53 and 77 μM) than those obtained on receptors containing α1, α2, α3 or α5 (between 140 and 540 μM) (Table 2). It is interesting to note that the slopes obtained for the direct action of pentobarbitone were higher than those obtained for the potentiation of GABA, suggesting a greater degree of co-operativity for direct channel activation than GABA.

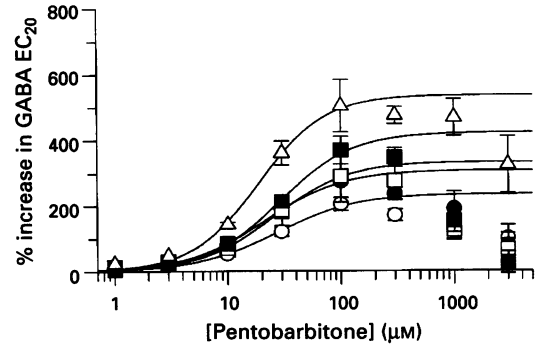

Figure 1 The effect of pentobarbitone (potentiation and direct effect) on GABA EC_{20} responses on oocytes expressing human α1β2γ2s, α2β2γ2s, α3β2γ2s, α5β2γ2s and α6β2γ2s $GABA_A$ receptors: (○) α1β2γ2s (n=6); (●) α2β2γ2s (n=3); (□) α3β2γ2s (n=4); (■) α5β2γ2s (n=5) and (△) α6β2γ2s (n=5). Each point represents the arithmetric mean (± s.e.mean) calculated as a percentage increase of the GABA EC_{20}. As inhibition was observed at high concentrations curves were fitted to the maximum response.

Direct effect of pentobarbitone on receptors containing different β subunits

The contribution of the β subunit to the direct effect of pentobarbitone was examined by co-expressing different β subunits (β1, β2 or β3) with α6 and γ2s or α1 and γ2s. The type of β subunit present did not influence the direct action of pentobarbitone to the same extent as the α subunit. There were no significant differences (Student's *t* test) between affinity or efficacy on oocytes expressing human α6β1γ2s, α6β2γ2s and α6β3γ2s $GABA_A$ receptors (Figure 3b and Table 2). The affinity of pentobarbitone on α1β1γ2s, α1β2γ2s and α1β3γ2s (540 μM, 314 μM and 189 μM respectively) were all significantly different ($P < 0.05$ Student's *t* test) whereas only the efficacy on α1β1γ2s (33%) was significantly different from that of α1β2γ2s (66%) and α1β3γ2s (75%) (Figure 3b and Table 2). It appears that the type of β subunit present does affect affinity and efficacy obtained with pentobarbitone but when an α6 subunit is present it is this subunit which determines the response to pentobarbitone. These results suggest that the

Table 1 Summary of the data obtained with pentobarbitone on the potentiation of GABA EC_{20} responses on oocytes expressing various human $GABA_A$ receptors

Subunit combination	n	*EC_{50} (μM)*	*Maximum % increase in GABA EC_{20}*	*Slope*
α1β2γ2s	6	25.6 (20.9, 31.4)	236 ± 32	1.7 ± 0.3
α2β2γ2s	3	20.2 (16.7, 24.5)	299 ± 10	1.4 ± 0.1
α3β2γ2s	4	25.4 (20.7, 31.1)	313 ± 161	2.0 ± 0.7
α5β2γ2s	5	25.7 (24.2, 27.4)	403 ± 42	1.4 ± 0.1
α6β2γ2s	5	20.7 (18.9, 22.6)	536 ± 75	1.7 ± 0.3
α1β1γ2s	4	34.7 (30.7, 39.2)	308 ± 27	1.4 ± 0.2
α1β2γ2s	6	25.6 (20.9, 31.4)	236 ± 32	1.7 ± 0.3
α1β3γ2s	3	19.4 (18.9, 19.9)	315 ± 30	1.4 ± 0.3

Values for the maximum and the slope are the arithmetic mean (± s.e.mean) and for the EC_{50} are the geometric mean (−s.e.mean + s.e.mean) from *n* cells.

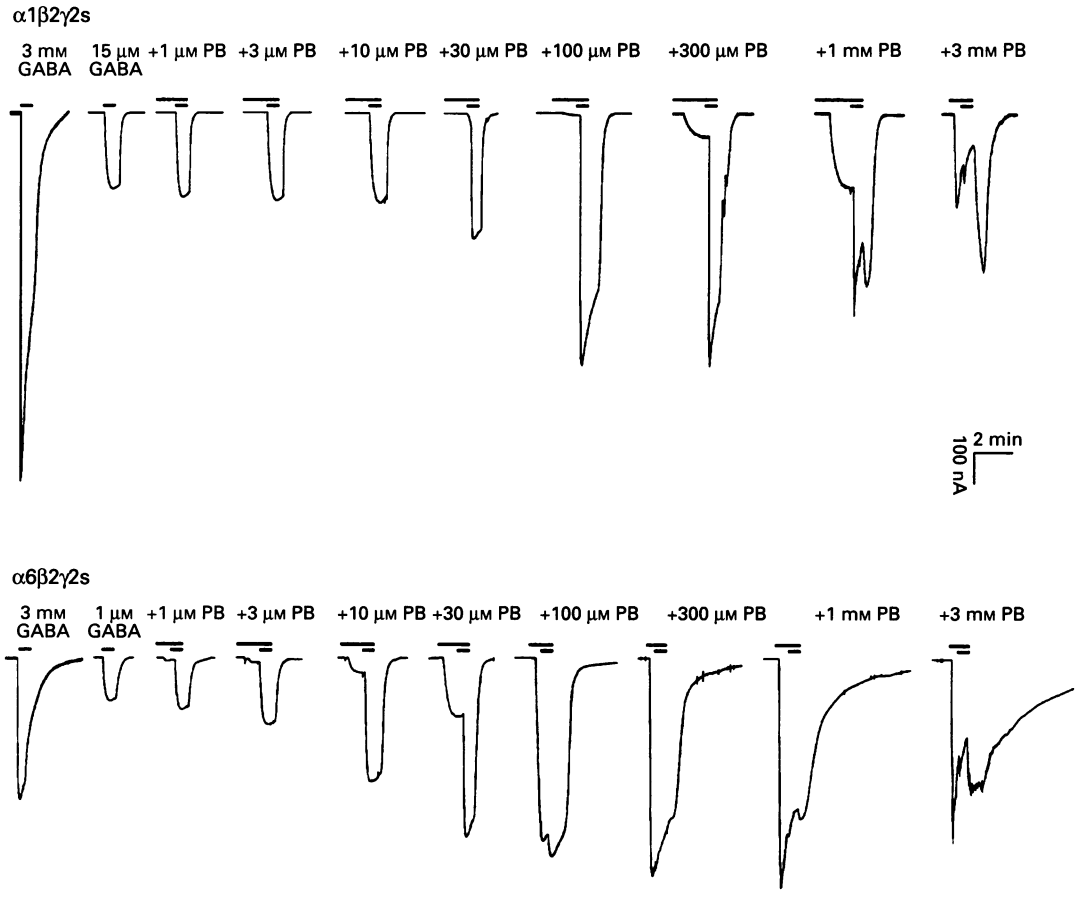

Figure 2 Typical current responses on oocytes expressing human α1β2γ2s and α6β2γ2s $GABA_A$ receptors. A maximum GABA response is followed by approximate EC_{20} concentrations on (a) α1β2γ2s and (b) α6β2γ2s, subsequent responses showing the effects of increasing concentrations of pentobarbitone (PB) on the control GABA response of each receptor subtype.

binding site for the direct action of pentobarbitone is influenced by, or even made up of, determinants from both the α and β subunit.

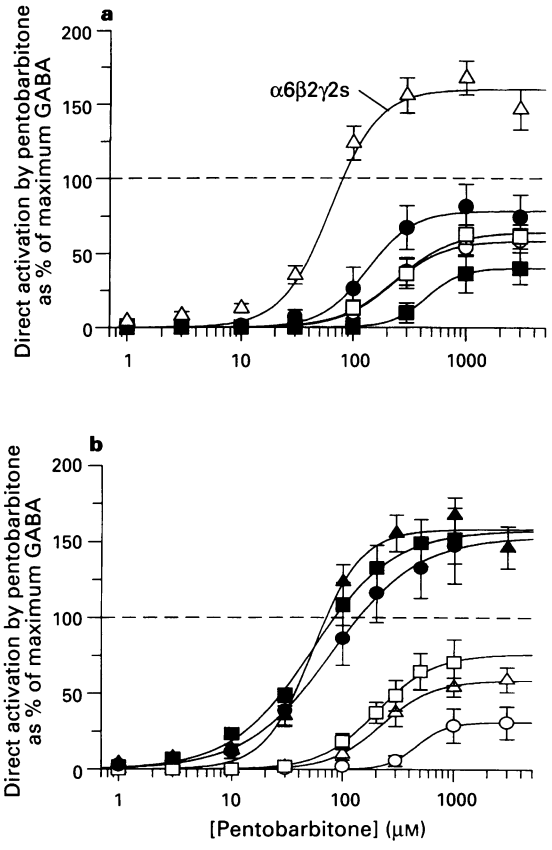

Figure 3 Concentration-response curves for the direct effect of pentobarbitone on oocytes expressing various human $GABA_A$ receptor subtypes. (a) varying α subunits: (○) α1β2γ2s ($n=9$); (●) α2β2γ2s ($n=5$); (□) α3β2γ2s ($n=5$); (■) α5β2γ2s ($n=5$) and (△) α6β2γ2s ($n=10$); (b) varying β subunits: (○) α1β1γ2s ($n=4$); (△) α1β2γ2s ($n=9$); (□) α1β3γ2s ($n=4$); (●) α6β1γ2s ($n=6$); (▲) α6β2γ2s ($n=10$) and (■) α6β3γ2s ($n=5$). Each point represents the arithmetic mean (± s.e.mean) calculated as a percentage of the response obtained with a maximum concentration of GABA (3 mM) on each cell. The dashed line in each figure represents the maximum GABA response.

Effect of bicuculline, picrotoxin and SR95531 on GABA and pentobarbitone

In oocytes expressing α6β2γ2s, the currents elicited by GABA were completely inhibited by the competitive GABA antagonists, bicuculline (100 μM) and SR95531 (1 μM), and the non-competitive antagonist, picrotoxin (100 μM) (Figure 4). Direct activation of the receptor by pentobarbitone was antagonized by picrotoxin (100 μM) but not by the competitive compounds bicuculline (100 μM) or SR95531 (1 μM) (Figure 4).

Bicuculline at concentrations of 100 μM and 1 mM produced dose-dependent parallel shifts to the right of the GABA concentration-response curves on oocytes expressing human α6β3γ2s receptors (13.3 and 243.9 fold shifts respectively) (Figure 5a and Table 3) confirming it to be a competitive antagonist at the α6β3γ2s receptor subtype. Very small rightward shifts in the concentration-response curves to the direct action of pentobarbitone were observed with bicuculline. These shifts (1.57 with 100 μM bicuculline and 1.43 with 1 mM bicuculline), although statistically significant, were not dose-related (Figure 5b and Table 3).

Discussion

We have systematically investigated the effects of pentobarbitone on receptors containing different α subunits co-expressed with β2 and γ2s, and different β subunits co-expressed with α1 and γ2 or α6 and γ2. Our results show that similar to native $GABA_A$ receptors, the barbiturate pentobarbitone has three actions on $GABA_A$ receptors dependent on increasing concentration, initially a potentiation of the GABA-induced current, followed by a direct activation of the receptor chloride channel, and at millimolar concentrations a blockade of the GABA-induced current.

The EC_{50} for pentobarbitone potentiation of GABA-induced currents was found to be between 20–35 μM on all the receptor combinations tested; however, the maximum degree of potentiation differed, depending on the type of α subunit present, and in α1 containing receptors, the β subunit variant. The most marked differences were found in the direct activation of the receptor by pentobarbitone. On α6βxγ2 (where x is 1, 2 or 3) receptors direct activation by pentobarbitone occurred at lower concentrations, with a much greater efficacy than on receptors containing other α subunits. As measurement of the potentiation included direct activation, this dominated the effects measured for α6βxγ2. Most reported electrophysiological studies have been performed on cell types such as mouse spinal cord neurones (Schulz & Macdonald, 1981) or hippocampal

Table 2 Direct effect of pentobarbitone on oocytes expressing various human $GABA_A$ receptors

Subunit combination	n	*EC_{50} (μM)*	*Maximum response as a % of maximum GABA*	*Slope*
α1β2γ2s	9	314.1 (251.8, 391.7)	65.6 ± 6.9	2.4 ± 0.5
α2β2γ2s	5	138.7 (108.1, 177.8)	82.6 ± 14.8	2.5 ± 0.5
α3β2γ2s	5	262.4 (197.2, 349.1)	67.0 ± 5.2	2.2 ± 0.2
α5β2γ2s	5	528.4 (430.5, 648.6)	45.2 ± 15.8	3.6 ± 0.2
α6β2γ2s	10	57.8 (51.4, 65.0)	168.2 ± 11.3	2.0 ± 0.3
α1β1γ2s	4	539.5 (476.4, 610.9)	33.3 ± 11.8	3.3 ± 0.3
α1β2γ2s	9	314.1 (251.8, 391.7)	65.6 ± 6.9	2.4 ± 0.5
α1β3γ2s	4	189.2 (165.2, 216.8)	75.3 ± 15.7	1.7 ± 0.1
α6β1γ2s	6	77.3 (53.6, 111.4)	154.0 ± 25.4	1.4 ± 0.2
α6β2γ2s	10	57.8 (51.4, 65.0)	168.2 ± 11.3	2.0 ± 0.3
α6β3γ2s	5	52.8 (42.1, 66.4)	159.0 ± 15.5	1.4 ± 0.1

Values for the maximum and the slope are the arithmetic mean (± s.e.mean) and for the EC_{50} are the geometric mean (–s.e.mean, + s.e.mean) from *n* cells.

Table 3 The effect of bicuculline on GABA and pentobarbitone concentration-response curves in oocytes expressing human $\alpha 6\beta 3\gamma 2s$ $GABA_A$ receptors

	n	EC_{50} (μM)
GABA (control)	5	1.5 (1.05, 2.13)
GABA + Bic (100 μM)	4	17.6 (13.3, 23.3)***
GABA + Bic (1 mM)	4	361.4 (252.3, 517.6)***
PB (control)	6	59.8 (47.2, 75.9)
PB + Bic (100 μM)	4	83.9 (71.9, 97.9)**
PB + Bic (1 mM)	4	89.3 (70.1, 113.8)*

Values are the geometric mean (–s.e.mean, +s.e.mean) from n cells. Asterisks show a significant difference from control values (Student's one-tailed t test); *$P<0.05$; **$P<0.01$; ***$P<0.001$.

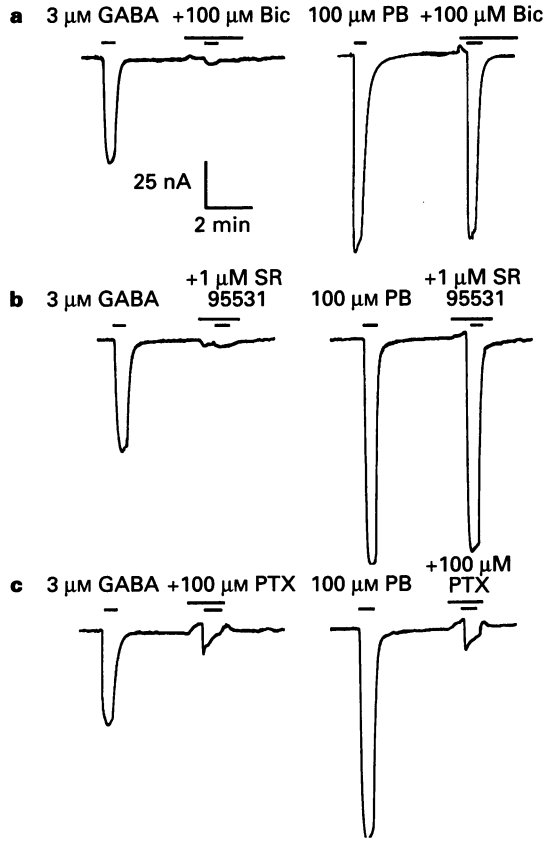

Figure 4 Effects of $GABA_A$ antagonists on currents elicited by GABA and pentobarbitone (PB) on oocytes expressing human $\alpha 6\beta 2\gamma 2s$ $GABA_A$ receptors. Cells were voltage-clamped at −70mV and exposed to agonists and antagonists as illustrated by the bars above each response. Bic = bicuculline; PTX = picrotoxin.

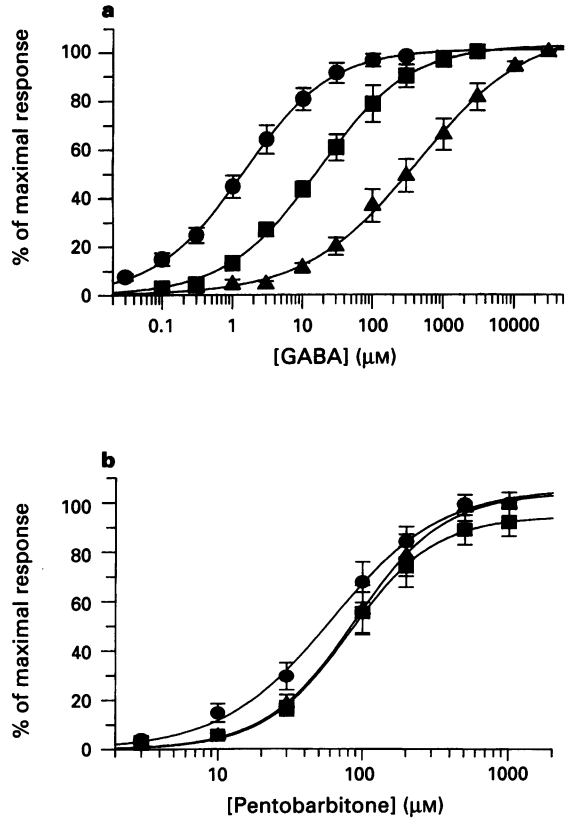

Figure 5 The effect of bicuculline on concentration-response curves to GABA and pentobarbitone on oocytes expressing human $\alpha 6\beta 3\gamma 2s$ $GABA_A$ receptors: (a) (●) control GABA ($n=5$); (■) GABA + bicuculline (100 μM, $n=4$) and (▲) GABA + bicuculline (1 mM, $n=4$); and (b) (●) control pentobarbitone ($n=6$); (■) pentobarbitone + bicuculline (100 μM, $n=4$) and (▲) pentobarbitone + bicuculline (1 mM, $n=4$). Each point represents the arithmetic mean (± s.e.mean) calculated as a percentage of the control GABA or control pentobarbitone maximum.

neurones (Zimmerman *et al.*, 1994) which under normal conditions do not express the $\alpha 6$ subunit (Wisden *et al.*, 1992; Laurie *et al.*, 1992). Most studies report some small degree of direct activation of neurones with concentrations of pentobarbitone over 100 μM (Schulz & Macdonald, 1981; Peters *et al.*, 1988; Robertson, 1989), which would be consistent with receptors containing other α-subunits. As $\alpha 6$ is located primarily on cerebellar granule cells (Baude *et al.*, 1992; Hadingham *et al.*, 1995 unpublished observations), this cell type may show a similar profile to that observed here. $GABA_A$ receptors from whole rat or chick brain mRNA expressed in *Xenopus* oocytes, showed robust potentiation and small direct activation by pentobarbitone (Parker *et al.*, 1986). Previous results with recombinant $GABA_A$ receptors have demonstrated both potentiation and direct activation of receptors by pentobarbitone (Sigel *et al.*, 1990). Unlike benzodiazepine modulation the $\gamma 2$ subunit is not required for potentiation by barbiturates (Schofield *et al.*, 1987; Horne *et al.*, 1993), or direct activation. GABA concentration-response curves were shifted to the left by equal amounts in transfected cells expressing bovine $\alpha 1\beta 1\gamma 2L$ and $\alpha 1\beta 1$; however, a significantly larger effect on the maximum current was observed in the $\alpha 1\beta 1$ combination (Horne *et al.*, 1993). Although until the present study no direct comparison of different subunit combinations has been performed, previous studies have shown several recombinant receptor combinations to be potentiated by pentobarbitone and other anaesthetics (Hadingham *et al.*, 1993b; Lin *et al.*, 1993; Sanna *et al.*, 1995), not requiring the presence of a γ subunit. Other sites on the receptor such as the GABA and benzodiazepine binding sites have been shown to be dependent on more than one subunit. The benzodiazepine site is influenced by both the α and γ subunits (Wafford *et al.*, 1992; 1993). All three subunits can affect the GABA EC_{50} (Ebert *et al.*, 1994), and residues have been identified on the α and β subunits which may form the binding site (Smith & Olsen, 1995). Conversely, the loreclezole site is affected only by the β subunit, being dependent on the presence of $\beta 2$ or $\beta 3$ (Wafford *et al.*, 1994). Other anaesthetic compounds such as propofol, halothane and enflurane as well as the neuroactive steroids such as alphaxalone have been shown to act by enhancing the GABA-induced chloride current (Tanelian *et al.*, 1993; Keane & Biziere, 1987; Wakamori *et al.*, 1991; Lin *et al.*, 1992). Some of these compounds, propofol and neurosteroids for example,

also produce direct activation of the receptor at high concentrations (Tanelian *et al.*, 1983; Keane & Biziere, 1987; Hara *et al.*, 1993). Although the neurosteroid 5α,3α-DHP has been shown not to give large direct currents on α6 containing receptors (Hadingham *et al.*, 1995 unpublished), the subunit dependence of direct activation by propofol has not been studied in detail.

The EC_{50} of pentobarbitone for general anaesthesia is about 50 μM (Franks & Lieb, 1994). From our results we can see that the main effect of pentobarbitone on $GABA_A$ receptors containing α1, α2, α3 and α5 subunits would be potentiation of the GABA response with little or no direct activation. On $GABA_A$ receptors containing α6 subunits, however, pentobarbitone would exert both a direct action and potentiation of the GABA response. On all receptor combinations tested, blockade of the receptor occurred only at extremely high concentrations (1 mM and above), which would be of little relevance at clinically active doses.

Antagonism of receptor activation by pentobarbitone on human α6β2γ2s $GABA_A$ receptors by picrotoxin confirmed that this action was via direct opening of $GABA_A$ receptor chloride channels. Single-channel analysis of this direct activation by pentobarbitone has demonstrated an identical conductance to GABA of 14.7pS, but with an open time five times longer than that of GABA (Mathers & Barker, 1980). This fact together with the steep Hill coefficients produced by pentobarbitone is further evidence for two different binding sites. As the higher affinity, potentiating site is already saturated at high concentrations, this would prolong the channel open time of subsequent pentobarbitone activation of the receptor via the low affinity site. This may explain the long single channel open times observed upon direct activation of $GABA_A$ receptors by pentobarbitone.

The site for direct activation by pentobarbitone was not the GABA binding site since no antagonism was observed with bicuculline or SR95531. This result appears contrary to Robertson (1989) and Nicoll & Wojtowicz (1980) who showed that the direct action of pentobarbitone on mammalian dorsal root ganglion neurones and frog motoneurones respectively was sensitive to block by bicuculline. In our experiments a small but significant effect was observed with bicuculline (see Figure 5b), which was more marked at low pentobarbitone concentrations and unlike GABA antagonism, was not increased with higher concentrations of bicuculline. Trace amounts of contaminating GABA might account for this effect, as blockade of the GABA site would produce an apparently greater block at low pentobarbitone concentrations due to potentiation of the GABA response, and such a small effect would be unaffected by increasing bicuculline concentrations. Recent mutagenesis experiments addressing the GABA binding site demonstrate that mutants with up to 900 fold lower affinity for GABA are unchanged in their sensitivity for direct activation by pentobarbitone (Amin & Weiss, 1993), also suggesting a unique binding site for direct activation by pentobarbitone. This is comparable to another ligand gated ion channel, the nicotinic acetylcholine receptor, which is activated by physostigmine via a site separate from the acetylcholine binding site (Okonjo *et al.*, 1991; Lena & Changeux, 1993).

Krishek & Smart (1995) reported pA_2 values for bicuculline of 5.87, 5.96 and 5.99 on murine α1β1, α1β1γ2s and α1β1γ21 $GABA_A$ receptors respectively. Our experiments indicate that bicuculline is weaker on human α6β3γ2s $GABA_A$ receptors with an approximate pA_2 of 5.0.

Many groups (Akaike *et al.*, 1987; Peters, 1988; Robertson, 1989) have reported on the washout phenomenon observed with high concentrations of pentobarbitone. This effect, which has been termed 'bounce' or 'hump', involves a marked transient increase in current during the washout period of high concentrations of pentobarbitone. We observed this same phenomenon at concentrations of 1 mM and 3 mM pentobarbitone on all the subunit combinations tested. Bounce occurred in both the absence or presence of a GABA EC_{20} concentration. We saw, as did Peters (1988) that this inward current was often of a greater amplitude than the initial response to pentobarbitone. One explanation for this, is rapid open channel blockade of the receptor by high concentrations of pentobarbitone, which is removed more quickly on washout, than it can dissociate from its activating binding site. This would result in reactivation of the channel during the washout period. This effect has also been observed with high concentrations of the anaesthetic, propofol, where a similar mechanism was proposed (Orser *et al.*, 1994).

Our results describe two independent binding sites for pentobarbitone on human $GABA_A$ receptors. The EC_{50} for barbiturate potentiation of the $GABA_A$ receptor is not dependent on receptor subtype; however, the maximum degree of potentiation is dependent on the α subunit. We have shown direct activation of the receptor on all subunit combinations, which is dependent on both α and β subunits. This site for pentobarbitone activation has a higher affinity and efficacy on α6 containing receptors than any other receptor combination, and suggests pentobarbitone direct activation of $GABA_A$ receptors at clinically relevant doses may be most marked in the cerebellum.

References

AKAIKE, N., MARUYAMA, T. & TOKUTOMI, N. (1987). Kinetic properties of the pentobarbitone-gated chloride current in frog sensory neurones. *J. Physiol.*, **394,** 85–98.

AMIN, J. & WEISS, D.S. (1993). $GABA_A$ receptor needs two homologous domains of the β-subunit for activation by GABA but not by pentobarbital. *Nature*, **366,** 565–569.

BAUDE, A., SEQUIER, J-M., MCKERNAN, R.M., OLIVIER, K.R. & SOMOGYI, P. (1992). Differential subcellular distribution of the α6 subunit versus the α1 and β2/3 subunits of the $GABA_A$/benzodiazepine receptor complex in granule cells of the cerebellar cortex. *Neurosci.*, **51,** 739–748.

EBERT, B., WAFFORD, K.A., WHITING, P.J., KROGSGAARD-LARSEN, P. & KEMP, J.A. (1994). Molecular pharmacology of γ-aminobutyric acid type A receptor agonists and partial agonists in oocytes injected with different α, β and γ receptor subunit combinations. *Mol. Pharmacol.*, **46,** 957–963.

EVANS, R.H. (1979). Potentiation of the effects of GABA by pentobarbitone. *Brain Res.*, **171,** 113–120.

FRANKS, N.P. & LIEB, W.R. (1994). Molecular and cellular mechanisms of general anaesthesia. *Nature*, **367,** 607–614.

HADINGHAM, K.L., WINGROVE, P., LE BOURDELLES, B., PALMER, K.J., RAGAN, C.I. & WHITING, P.J. (1993a). Cloning of cDNA sequences encoding human α2 and α3 γ-aminobutyric $acid_A$ receptor subunits and characterisation of the benzodiazepine pharmacology of recombinant α1-, α2-, α3- and α5-containing human γ-aminobutyric $acid_A$ receptors. *Mol. Pharmacol.*, **43,** 970–975.

HADINGHAM, K.L., WINGROVE, P., WAFFORD, K.A., BAIN, C., KEMP, J.A., PALMER, K.J., WILSON, A.W., WILCOX, A.S., SIKELA, J.M., RAGAN, C.I. & WHITING, P.J. (1993b). Role of the β subunit in determining the pharmacology of human γ-aminobutyric acid type A receptors. *Mol. Pharmacol.*, **44,** 1211–1218.

HARA, M., KAI, Y. & IKEMOTO, Y. (1993). Propofol activates $GABA_A$ receptor-chloride ionophore complex in dissociated hippocampal pyramidal neurons of the rat. *Anesthesiology*, **79,** 781–788.

HORNE, A.L., HARKNESS, P.C., HADINGHAM, K.L., WHITING, P.J. & KEMP, J.A. (1993). The influence of the γ_{2L} subunit on the modulation of responses to $GABA_A$ receptor activation. *Br. J. Pharmacol.*, **108,** 711–716.

JACKSON, M.B., LECAR, H., MATHERS, D.A. & BARKER, J.L. (1982). Single channel currents activated by γ-aminobutyric acid, muscimol, and (−)-pentobarbital in cultured mouse spinal neurones. *J. Neurosci.*, **2**, 889−894.

KEANE, P.E. & BIZIERE, K. (1987). The effects of general anaesthetics on gabaergic synaptic transmission. *Life Sci.*, **41**, 1437−1448.

KRISHEK, B.J. & SMART, T.G. (1995). A functional comparison of the antagonists bicuculline and picrotoxin at recombinant murine $GABA_A$ receptors. *Br. J. Pharmacol.*, **114**, 291P.

LAURIE, D.J., SEEBURG, P.H. & WISDEN, W. (1992). The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. II. Olfactory bulb and cerebellum. *J. Neurosci.*, **12**, 1063−1076.

LENA, C. & CHANGEUX, J-P. (1993). Allosteric modulations of the nicotinic acetylcholine receptor. *Trends Neurosci.*, **16**, 181−186.

LIN, L-H., CHEN, L.L., ZIRROLLI, J.A. & HARRIS, R.A. (1992). General anesthetics potentiate γ-aminobutyric acid actions on γ-aminobutyric $acid_A$ receptors expressed by *Xenopus* oocytes: lack of involvement of intracellular calcium. *J. Pharmacol. Exp. Ther.*, **263**, 569−578.

LIN, L-H., WHITING, P. & HARRIS, R.A. (1993). Molecular determinants of general anesthic action: Role of $GABA_A$ receptor structure. *J. Neurochem.*, **60**, 1548−1553.

MACDONALD, R.L., ROGERS, C.L. & TWYMAN, R.E. (1989). Barbiturate regulation of kinetic properties of the $GABA_A$ receptor channel of mouse spinal neurones in culture. *J. Physiol.*, **417**, 483−500.

MATHERS, D.A. & BARKER, J.L. (1980). (−)Pentobarbital opens ion channels of long duration in cultured mouse spinal neurons. *Science*, **209**, 507−509.

NICOLL, R.A. & WOJTOWICZ, J.M. (1980). The effects of pentobarbital and related compounds on frog motoneurons. *Brain Res.*, **191**, 225−237.

OKONJO, K.O., KUHLMANN, J. & MAELICKE, A. (1991). A second pathway of activation of the *Torpedo* acetylcholine receptor channel. *Eur. J. Biochem.*, **200**, 671−677.

OLSEN, R.W. (1988). Barbiturates. *Int. Anesthesiol. Clin.*, **26**, 254−261.

ORSER, B.A., WANG, L-Y., PENNEFATHER, P.S. & MACDONALD, J.F. (1994). Propofol modulates activation and desensitization of $GABA_A$ receptors in cultured murine hippocampal neurons. *J. Neurosci.*, **14**, 7747−7760.

PARKER, I., GUNDERSEN, C.B. & MILEDI, R. (1986). Actions of pentobarbital on rat brain receptors expressed in *Xenopus* oocytes. *J. Neurosci.*, **6**, 2290−2297.

PETERS, J.A., KIRKNESS, E.F., CALLACHAN, H., LAMBERT, J.J. & TURNER, A.J. (1988). Modulation of the $GABA_A$ receptor by depressant barbiturates and pregnane steroids. *Br. J. Pharmacol.*, **94**, 1257−1269.

ROBERTSON, B. (1989). Actions of anaesthetics and avermectin on $GABA_A$ chloride channels in mammalian dorsal root ganglion neurones. *Br. J. Pharmacol.*, **98**, 167−176.

ROGERS, C.J., TWYMAN, R.E. & MACDONALD, R.L. (1994). Benzodiazepine and β-carboline regulation of single $GABA_A$ receptor channels of mouse spinal neurones in culture. *J. Physiol.*, **475**, 69−82.

SANNA, E., GARAU, F. & HARRIS, R.A. (1995). Novel properties of homomeric β1 γ-aminobutyric acid type A receptors: actions of the anesthetics propofol and pentobarbital. *Mol. Pharmacol.*, **47**, 213−217.

SCHOFIELD, P.R., DARLISON, M.G., FUJITA, N., BURT, D.R., STEPHENSON, F.A., RODRIGUEZ, H., RHEE, L.M., RAMACHANDRAN, J., REALE, V., GLENCORSE, T.A., SEEBURG, P.H. & BARNARD, E.A. (1987). Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor super-family. *Nature*, **328**, 221−227.

SCHULZ, D.W. & MACDONALD, R.L. (1981). Barbiturate enhancement of GABA-mediated inhibition and activation of chloride ion conductance: correlation with anticonvulsant and anesthetic actions. *Brain Res.*, **209**, 177−188.

SCHWARTZ, R.D., SUZDAK, P.D. & PAUL, S.M. (1986). γ-Aminobutyric acid (GABA)- and barbiturate-mediated ^{36}Cl uptake in rat brain synaptoneurosomes: evidence for rapid desensitization of the GABA receptor-coupled chloride ion channel. *Mol. Pharmacol.*, **30**, 419−426.

SIGEL, E., BAUR, R., TRUBE, G., MOHLER, H. & MALHERBE, P. (1990). The effect of subunit composition of rat brain $GABA_A$ receptors on channel function. *Neuron*, **5**, 703−711.

SMITH, G.B. & OLSEN, R.W. (1995). Functional domains of the $GABA_A$ receptor. *Trends Pharmacol. Sci.*, **16**, 162−168.

STUDY, R.E. & BARKER, J.L. (1981). Diazepam and (−)-pentobarbital: fluctuation analysis reveals different mechanisms for potentiation of γ-aminobutyric acid responses in cultured neurones. *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 7180−7184.

TANELIAN, D.L., KOSEK, P., MODY, I. & MACLVER, M.B. (1993). The role of the $GABA_A$ receptor/chloride channel complex in anesthesia. *Anesthesiology*, **78**, 757−776.

THOMPSON, S.A., WHITING, P.J. & WAFFORD, K.A. (1995). Alpha subunits influence the action of pentobarbital on recombinant $GABA_A$ receptors. *Br. J. Pharmacol.*, **114**, 296P.

WAFFORD, K.A., BAIN, C.J., QUIRK, K., MCKERNAN, R.M., WINGROVE, P.B., WHITING, P.J. & KEMP, J.A. (1994). A novel allosteric modulatory site on the $GABA_A$ receptor β subunit. *Neuron*, **12**, 775−782.

WAFFORD, K.A., BAIN, C.J., WHITING, P.J. & KEMP, J.A. (1993). Functional comparison of the role of γ-subunits in recombinant human γ-aminobutyric acid A/benzodiazepine receptors. *Mol. Pharmacol.*, **44**, 437−442.

WAFFORD, K.A., WHITING, P.J. & KEMP, J.A. (1992). Differences in affinity and efficacy of benzodiazepine receptor ligands on recombinant $GABA_A$ receptor subtypes. *Mol. Pharmacol.*, **43**, 240−244.

WAKAMORI, M., IKEMOTO, Y. & AKAIKE, N. (1991). Effects of two volatile anesthetics and a volatile convulsant on the excitatory and inhibitory amino acid responses in dissociated CNS neurons of the rat. *J. Neurophysiol.*, **66**, 2014−2021.

WHITING, P.J., MCKERNAN, R.M. & WAFFORD, K.A. (1995). Structure and pharmacology of vertebrate $GABA_A$ receptor subtypes. *Int. Rev. Neurobiol.* (in press).

WISDEN, W., LAURIE, D.J., MONYER, H. & SEEBERG, P.H. (1992). The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. *J. Neurosci.*, **12**, 1040−1062.

ZIMMERMAN, S.A., JONES, M.V. & HARRISON, N.L. (1994). Potentiation of γ-aminobutyric $acid_A$ receptor Cl current correlates with *in vivo* anesthetic potency. *J. Pharmacol. Exp. Ther.*, **270**, 987−991.

(Received July 3, 1995
Revised September 7, 1995
Accepted September 28, 1995)