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Allosteric modulators affect the efficacy of partial agonists for recombinant $GABA_A$ receptors

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1 Different α subunits of human γ-aminobutyric acid type A ($GABA_A$) receptors were transiently expressed together with β_3 and γ_2 subunits in *Xenopus* oocytes to examine the interactions of various $GABA_A$ agonists and representative allosteric modulators. Chloride currents elicited by agonists were measured using two electrode voltage clamp electrophysiology.

2 Where compounds behaved as full agonists, i.e. GABA on all subtypes and 4,5,6,7-tetrahydroisoxazolo [5,4-c]pyridin-3-ol (THIP) on $\alpha_2\beta_3\gamma_2$ $GABA_A$ receptors, agonist concentration-response curves were shifted to the left by the benzodiazepine full agonist chlordiazepoxide and the anticonvulsant loreclezole, or to the right by the inverse agonist 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylic acid methyl ester (DMCM), with no effect on the maximal currents (I_{max}).

3 In contrast, maximal responses for different partial $GABA_A$ agonists on all benzodiazepine-sensitive $\alpha_x\beta_3\gamma_2$ $GABA_A$ receptors were enhanced by chlordiazepoxide. I_{max} values for piperidine-4-sulphonic acid (P4S) on $\alpha_1\beta_3\gamma_2$, THIP on $\alpha_3\beta_3\gamma_2$, and 5-(4-piperidyl)isothiazol-3-ol (thio-4-PIOL) on $\alpha_2\beta_3\gamma_2$ and $\alpha_5\beta_3\gamma_2$ $GABA_A$ receptors were increased by chlordiazepoxide, while that for P4S on $\alpha_1\beta_3\gamma_2$ receptors was decreased by DMCM.

4 The I_{max} values for partial agonists were also enhanced by pentobarbitone, the neurosteroid allopregnanolone and loreclezole irrespective of receptor subtype or the nature of the partial agonist.

5 In the light of models of ligand-gated ion channel receptor activation we suggest two possible mechanisms of action for the effects of allosteric modulators on partial agonist receptor activation: either selective modulation of agonist affinity for the open/closed state, or direct modulation of the gating process itself.

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Abbreviations: DMCM, 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylic acid methyl ester; P4S, piperidine-4-sulphonic acid; Thio-4-PIOL, 5-(4-piperidyl)isothiazol-3-ol; THIP, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol

Introduction

γ-Aminobutyric acid is the most important inhibitory neurotransmitter in mammalian brain. $GABA_A$ receptors belong to the ligand-gated ion channel superfamily, which include glycine, nicotinic acetylcholine and 5-HT_3 serotonin receptors (Barnard *et al*., 1998). $GABA_A$ receptors are assembled from multiple subunits to form chloride ionophores in a presumed pentameric arrangement (Tretter *et al*., 1997). A great number of different mammalian $GABA_A$ receptor subunits have been cloned including α_{1-6}, β_{1-4}, γ_{1-3}, δ, ε, π, Θ and ρ_{1-3} subunits (reviewed by Barnard *et al*., 1998). It is thought that $GABA_A$ agonists bind at the interface of α and β subunits and critical residues in both these subunits have been shown to be required for agonist-elicited opening of the ionophores (Amin & Weiss, 1993; Sigel & Buhr, 1997). Similarly the type of receptor subunits present also influence the efficacies and EC_{50} values of $GABA_A$ agonists (Ebert *et al*., 1994). The α subunit is a major determinant of the kinetics of ionophore activity and the efficacy of GABA (Lavoie *et al*., 1997) as well as of other $GABA_A$ agonists (Ebert *et al*., 1994). Several conformationally restricted analogues of GABA have been developed with different efficacies such as 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP), piperidine-4-sulphonic acid (P4S) and 5-(4-piperidyl)isothiazol-3-ol (thio-4-PIOL) (Krogsgaard-Larsen *et al*., 1997).

$GABA_A$ receptors have several modulatory sites for a great variety of pharmacologically important agents. The binding sites for anxiolytic benzodiazepines and anxiogenic β-carbolines are probably located at α/γ interfaces as the α_1 subunit is photolabelled by [^{3}H]-flunitrazepam and the γ subunit is essential for bidirectional modulation of channel activity by benzodiazepine ligands (Sigel & Buhr, 1997). Several reports confirm the view that ligands of the benzodiazepine site exert their effects *via* modulating the EC_{50} of the full agonists GABA and muscimol (Sigel & Baur, 1988; Wafford *et al*., 1992). Barbiturates in increasing concentrations have three actions on $GABA_A$ receptors: potentiation of GABA, direct receptor activation, and blockade of the chloride ionophores (Thompson *et al*., 1996) *via* sites distinctly different from that of GABA (Amin & Weiss, 1993). Similar to pentobarbitone, neurosteroids such as allopregnanolone (5α-pregnan-3α-ol-20-one) in increasing concentrations potentiate the effect of GABA, directly activate the receptor and increase the rate of receptor desensitization (Turner & Simmonds, 1989; Puia *et al*., 1990; Woodward *et al*., 1992). The anticonvulsant loreclezole is selective for receptors containing β_2 or β_3 subunits (Wafford *et al*., 1994). Loreclezole decreases the EC_{50} and the apparent maximal response for GABA (Wafford *et al*.,

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1994) and increases the rate of receptor desensitization (Donnelly & Macdonald, 1996).

Most studies dealing with these allosteric agents have been restricted to interactions with full $GABA_A$ agonists such as GABA and muscimol. Here we have used recombinant receptors, different α subunits in combination with β_3 and γ_2 $GABA_A$ subunits, for which the efficacies of different partial agonists have been determined (Ebert *et al*., 1997). We investigated the allosteric modulation of receptors activated by partial $GABA_A$ agonists exerting both high and low efficacies depending on receptor subtype.

Methods

Oocyte expression

Oocytes were removed from anaesthetized *Xenopus laevis* and defolliculated with forceps. After treatment with collagenase (0.5 mg ml^{-1}) for 5 min, a 20 nl aliquot of mixtures of human α_1, α_2, α_3 α_5 or α_6 with β_3 and γ_2 $GABA_A$ receptor subunit cDNAs (20 ng μl^{-1}) were injected into the nuclei of the oocytes. The cDNAs were engineered into the expression vector pCDM8 or pcDNAAmp. The injection buffers contained (mM): NaCl 88, KCl 1, HEPES 15 at pH 7.0. The oocytes were incubated for 1–3 days at 20°C in modified Barth's solution consisting of (mM): NaCl 88, KCl 1, HEPES 10, $MgSO_4$ 0.82, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.91 and $NaHCO_3$ 2.4 at pH 7.

Electrophysiology

Oocytes were placed in a 50 μl chamber and continuously perfused with modified Barth's solution at a rate of 4–6 ml min^{-1}. Cells were impaled with two 1–4 MΩ capillary electrodes containing 2 M KCl and voltage clamped at −70 mV. $GABA_A$ agonists were added to the perfusion medium. All allosteric agents were preapplied for 30 s prior to the coapplication of $GABA_A$ agonists, except for chlordiazepoxide for which preapplication was not necessary. Following the observation of the peak current, cells were washed for a minimum of 3 min after returning to baseline, and for at least 5 min between saturating agonist concentrations. Increasing concentrations of the agonists were followed by the addition of 3 mM GABA. The peak agonist responses were normalized to the maximal response of 3 mM GABA which was reached within 10 s.

Data were fitted *via* the computer program GraphPad Prism 2.0 (San Diego, CA, U.S.A.). Curves were fitted using a non-linear square-fitting program to the equation $f(x) = I_{max}/[1 + (EC_{50}/x)^n]$ where x is the agonist concentration, I_{max} is the maximal current, EC_{50} is the concentration eliciting a half-maximal response and n is the Hill coefficient. Statistical analysis was performed *via* Student's *t*-test for lg EC_{50} and the Mann–Whitney nonparametric test for I_{max} values and considered significant if $P \leqslant 0.05$.

Drugs

Chlordiazepoxide, collagenase, pentobarbitone and allopregnanolone were purchased from Sigma (Poole, U.K.), 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylic acid methyl ester (DMCM) from Research Biochemicals (Natick, MA, U.S.A.), and P4S from Tocris-Cookson (U.K.). Loreclezole was a gift from Janssen, THIP and thio-4-PIOL were gifts from Prof P. Krogsgaard-Larsen (Copenhagen, Denmark).

Results

Modulation via *the benzodiazepine site*

$GABA_A$ agonists elicited inward chloride currents on oocytes expressing $\alpha_x\beta_3\gamma_2$ $GABA_A$ receptors at a holding potential of −70 mV. GABA concentrations that elicited a response equal to 20% of the maximal response for $\alpha_1\beta_3\gamma_2$ $GABA_A$ receptors were combined with different concentrations of chlordiazepoxide, a representative anxiolytic 1,4-benzodiazepine. Figure 1 shows that chlordiazepoxide resulted in concentration dependent potentiation of the current for GABA with an EC_{50} value of 3.1 (2.3, 4.2) μM (mean ± s.e.mean of three experiments). The smaller responses for the partial agonist P4S were similarly potentiated by chlordiazepoxide (Figure 1) with an EC_{50} of 2.0 (1.6,2.4) μM (mean ± s.e.mean of three experiments). However, Figure 1 did not reveal any differences for the allosteric interactions of chlordiazepoxide with the full agonist GABA versus the partial agonist P4S.

Using GABA as an agonist, benzodiazepines have been shown not to enhance the maximum current of $GABA_A$ receptors (Wafford *et al*., 1992; Yakushiji *et al*., 1993), and from the previous experiment the EC_{50} of chlordiazepoxide was independent of the $GABA_A$ agonist. We then studied the effects of modulators on the concentration-response relationship of the partial agonists, particularly the maximal response.

Figure 2a shows the concentration-response curves of P4S at $\alpha_1\beta_3\gamma_2$ $GABA_A$ receptors. A saturating concentration (30 μM) of chlordiazepoxide shifted the curve for P4S to the left, and enhanced the maximal current amplitude (Figure 2a). Table 1 summarizes the I_{max} and EC_{50} values derived from computer fits, demonstrating significant effects on these parameters. Figure 2a also shows that the concentration-response curve for P4S was depressed by DMCM, a representative inverse agonist β-carboline. DMCM decreased the I_{max} values for P4S without significantly affecting its EC_{50} value (Table 1). As a comparison with a full agonist, the allosteric effects on the concentration-response curve of GABA

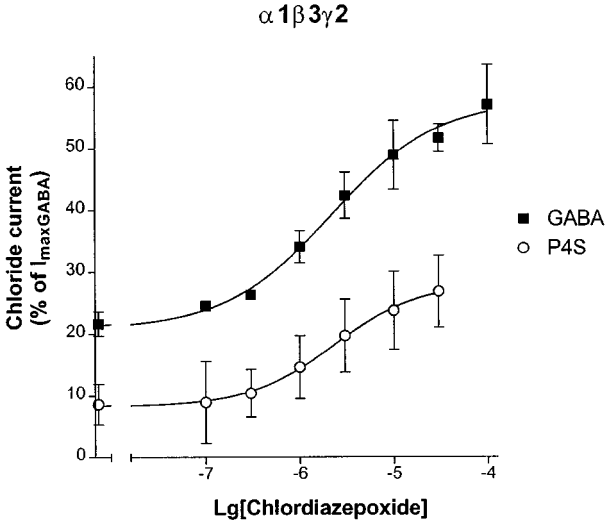

Figure 1 Concentration-response curves of chlordiazepoxide on the chloride currents elicited by GABA and P4S for $\alpha_1\beta_3\gamma_2$ $GABA_A$ receptors. The peaks of the chloride currents were expressed as per cent of the maximal currents by 3 mM GABA ($I_{maxGABA}$). Agonist concentrations were titrated for each oocyte and equivalent to EC_{20} values (0.8 μM GABA and 15 μM P4S). The points are mean ± s.e.mean of three experiments.

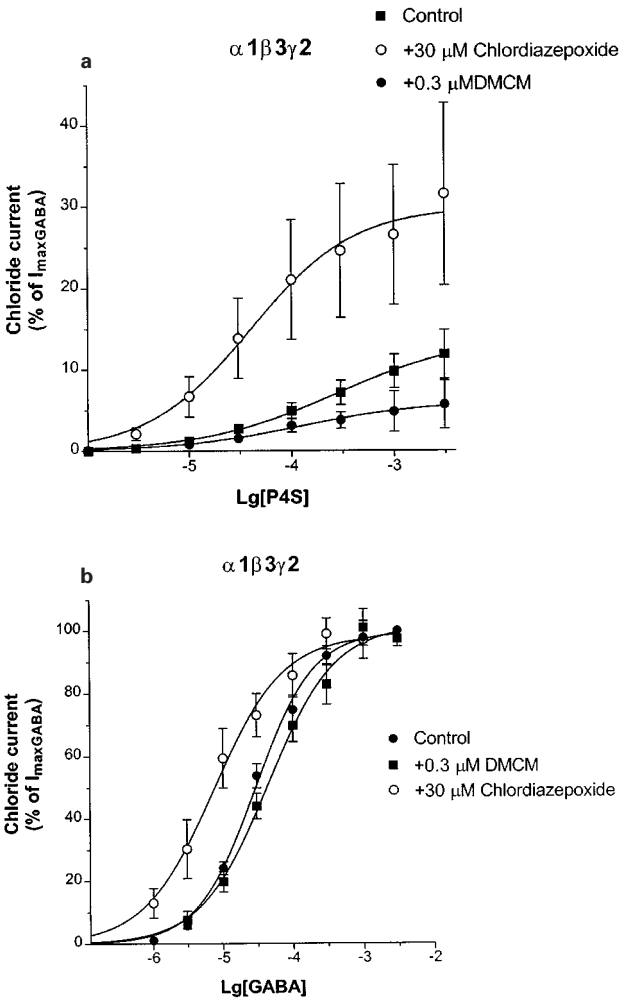

Figure 2 The effects of 30 μM chlordiazepoxide (CDZ) and 0.3 μM DMCM on the concentration-response curves of P4S (a) and GABA (b) for $\alpha_1\beta_3\gamma_2$ GABA$_A$ receptors. Chloride currents are expressed as per cent of the peak current elicited by 3 mM GABA. Data are mean$\pm$s.e.mean of the number of experiments indicated in Table 1. Fitted curves with variable slopes resulted in the I_{max} and EC_{50} values in Table 1.

were also examined for the same $\alpha_1\beta_3\gamma_2$ subunit combination. Figure 2b demonstrates that chlordiazepoxide shifted the response curve of GABA to the left, without affecting the maximum. DMCM resulted in an opposite shift (Figure 2b). Table 1 shows that the bidirectional changes in the EC_{50} value for GABA were statistically significant.

The α_2 subunit enabled us to investigate further partial agonists having either high or low efficacies. THIP has high efficacy at $\alpha_2\beta_3\gamma_2$ GABA$_A$ receptors (Figure 3). Chlordiazepoxide shifted the concentration response curve for THIP to the left (Figure 3). Table 1 shows that chlordiazepoxide did not significantly affect the high maximal response for THIP but decreased its EC_{50} value. In contrast, THIP has low efficacy for $\alpha_3\beta_3\gamma_2$ GABA$_A$ receptors (Figure 4b). Chlordiazepoxide enhanced the maximal current elicited by THIP (Figure 4b) but did not significantly decrease the EC_{50} of THIP for $\alpha_3\beta_3\gamma_2$ receptors (Table 1). The concentration-dependence of chlordiazepoxide was examined against a nearly saturating concentration of THIP (3.3 mM), a concentration which elicited 25% of the maximally activated GABA current on $\alpha_3\beta_3\gamma_2$ receptors (Figure 4c). Chlordiazepoxide enhanced this response with $EC_{50}=6$ (3,11) μM with a Hill slope value of 0.64 (0.57,0.71) (mean$\pm$s.e.mean of five experiments) (Figure 4c).

Table 1 The effects of allosteric agents on the concentration-response curves of GABA$_A$ agonists for recombinant $\alpha_x\beta_3\gamma_2$ GABA$_A$ receptors

Drugs	I_{max} (%)[a]	EC_{50} (μM)
	$\alpha_1\beta_3\gamma_2$	
P4S		
Control (20)	10$\pm$1	125 (103,152)
+30 μM Chlordiazepoxide (5)	25$\pm$7↑	↓24 (18,32)
+0.3 μM DMCM (5)	5$\pm$2↓	68 (43,107)
+50 μM Pentobarbitone (9)	44$\pm$5↑	155 (124,194)
+0.1 μM Allopregnanolone (8)	16$\pm$2↑	↓18 (13,25)
+10 μM Loreclezole (4)	33$\pm$2↑	↓9 (7,10)
GABA		
Control (8)	101$\pm$1	30 (25,36)
+30 μM Chlordiazepoxide (5)	97$\pm$6	↓8 (5,13)
+0.3 μM DMCM (7)	98$\pm$5	↑68 (46,100)
	$\alpha_2\beta_3\gamma_2$	
THIP		
Control (4)	83$\pm$9	203 (149,277)
+30 μM Chlordiazepoxide (4)	91$\pm$4	↓88 (58,132)
Thio-4-PIOL		
Control (5)	4$\pm$1	16 (11,22)
+30 μM Chlordiazepoxide (7)	13$\pm$3↑	19 (16,21)
P4S		
Control (3)	88$\pm$1	12 (11,13)
+10 μM Loreclezole (4)	88$\pm$1	↓2.0 (1.8,2.2)
	$\alpha_3\beta_3\gamma_2$	
THIP		
Control (6)	37$\pm$7	265 (232,302)
+30 μM Chlordiazepoxide (3)	62$\pm$10↑	201 (164,248)
+0.1 μM Allopregnanolone (3)	81$\pm$3↑	↓85 (79,91)
+50 μM Pentobarbitone (3)	95$\pm$1↑	↓33 (27,41)
	$\alpha_5\beta_3\gamma_2$	
Thio-4-PIOL		
Control (4)	5$\pm$1.6	48 (40,59)
+30 μM Chlordiazepoxide (7)	13$\pm$2↑	↓27 (24,31)
	$\alpha_6\beta_3\gamma_2$	
P4S		
Control	30$\pm$2	33 (26,43)
+50 μM Pentobarbitone (4)	121$\pm$7↑	↓1.5 (1,2)
+0.1 μM Allopregnanolone (4)	50$\pm$8↑	↓14 (12,16)

Data for I_{max} are the arithmetic mean$\pm$s.e.mean and for the EC_{50} values are the geometric mean (−s.e.mean, + s.e. mean). The number of experiments is indicated in parentheses. [a]Expressed in per cent of the response for 3 mM GABA. ↑/↓: Significantly different ($P<0.05$) from control in Mann–Whitney's nonparametric test for I_{max} values and in Student's t-test for lg EC_{50} values.

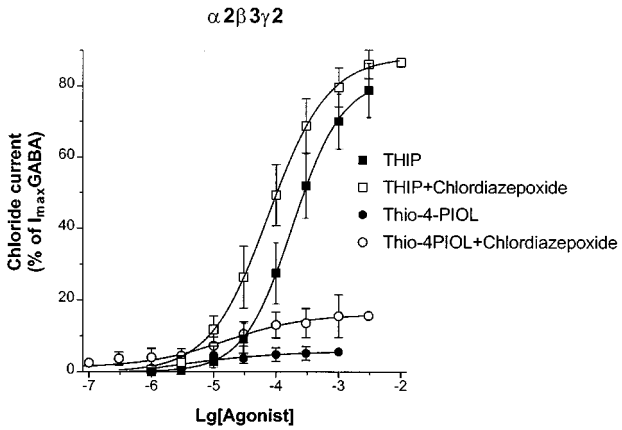

Figure 3 The effects of 30 μM chlordiazepoxide on the concentration-response curves of THIP and thio-4-PIOL for $\alpha_2\beta_3\gamma_2$ GABA$_A$ receptors. Chloride currents are expressed as per cent of the peak current elicited by 3 mM GABA. Data are mean$\pm$s.e.mean of four experiments.

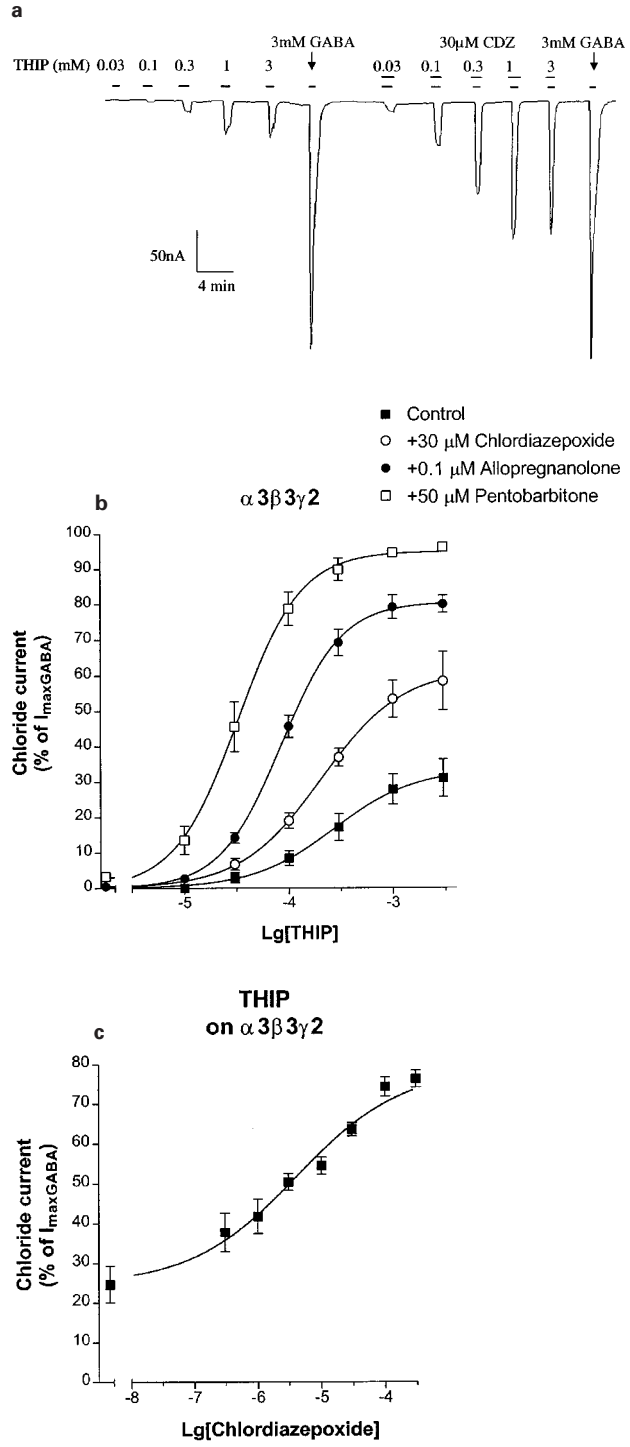

Figure 4 The effects of chlordiazepoxide, allopregnanolone and pentobarbitone on the concentration-response curves of THIP for $\alpha_3\beta_3\gamma_2$ $GABA_A$ receptors. (a) Example recording of currents in response to THIP in the absence and presence of 30 μM chlordiazepoxide, showing control responses to a saturating concentration of GABA (3 mM) after each concentration-response curve. Drugs were applied as indicated by the bars. (b) Mean concentration-response curves to THIP in the absence and presence of 30 μM chlordiazepoxide, 100 nM allopregnanolone and 50 μM pentobarbitone. Chloride currents are expressed as per cent of the peak current elicited by 3 mM GABA. Data are mean$\pm$s.e.mean of 3–6 experiments. (c) The effects of chlordiazepoxide on the chloride currents elicited by a maximally effective concentration (3.3 mM) of THIP for $\alpha_3\beta_3\gamma_2$ $GABA_A$ receptors. Data are mean$\pm$s.e.mean of five experiments. Chloride currents are expressed as per cent of the peak current elicited by 3 mM GABA. 3.3 mM THIP alone elicited 25$\pm$5% of the current peak for 3 mM GABA. It was enhanced by chlordiazepoxide with an EC_{50} value of 6 (3,11) μM and a Hill slope value of 0.64 (0.57, 0.71).

The low slope value and the responses beyond 50% in Figure 4c might indicate an additional response to chlordiazepoxide with potency above 10 μM.

Thio-4-PIOL has been characterized as a low potency $GABA_A$ antagonist with no efficacy of its own for most recombinant $GABA_A$ receptors (Ebert *et al.*, 1997). Thio-4-PIOL was able to elicit extremely small currents on $\alpha_2\beta_3\gamma_2$ $GABA_A$ receptors but its efficacy was very low (Figure 3), reaching a maximum of 4$\pm$1% of a 3 mM GABA current (Table 1). However, chlordiazepoxide strongly enhanced the currents for thio-4-PIOL (Figure 3) by increasing its efficacy without significantly affecting its potency (Table 1).

Thio-4-PIOL elicited very small currents for $\alpha_5\beta_3\gamma_2$ $GABA_A$ receptors, too (Figure 5). Chlordiazepoxide strongly enhanced the currents for thio-4-PIOL by increasing not only its efficacy but in this case also its potency (Table 1).

Allosteric potentiating effects of a barbiturate, a neurosteroid and loreclezole

Pentobarbitone, allopregnanolone and loreclezole were applied in concentrations that did not elicit significant direct currents for $\alpha_1\beta_3\gamma_2$ $GABA_A$ receptors but strongly potentiated the currents for P4S (see the effects of allopregnanolone in Figure 6a). Pentobarbitone at 50 μM resulted in a great enhancement of the maximal response to P4S (Figure 6b). Table 1 reveals that pentobarbitone did not affect the EC_{50} of P4S. The neurosteroid allopregnanolone (0.1 μM) produced a large decrease in the EC_{50} of P4S, as well as significantly increased its efficacy at $\alpha_1\beta_3\gamma_2$ $GABA_A$ receptors (Figure 6b and Table 1).

Loreclezole (10 μM) produced a much greater increase in maximal response for P4S on $\alpha_1\beta_3\gamma_2$ $GABA_A$ receptors than the neurosteroid (Figure 6b). Table 1 shows that loreclezole enhanced both the efficacy and potency of P4S. For comparison, the effects of loreclezole were also examined on $\alpha_2\beta_3\gamma_2$ $GABA_A$ receptors for which P4S has higher potency and almost full efficacy (Table 1). In this case loreclezole did not change the efficacy of P4S but further enhanced its high potency (Table 1).

On $\alpha_6\beta_3\gamma_2$ $GABA_A$ receptors pentobarbitone at 50 μM exerted significant currents in the absence of GABA agonists (Figure 7). However, pentobarbitone resulted in a huge

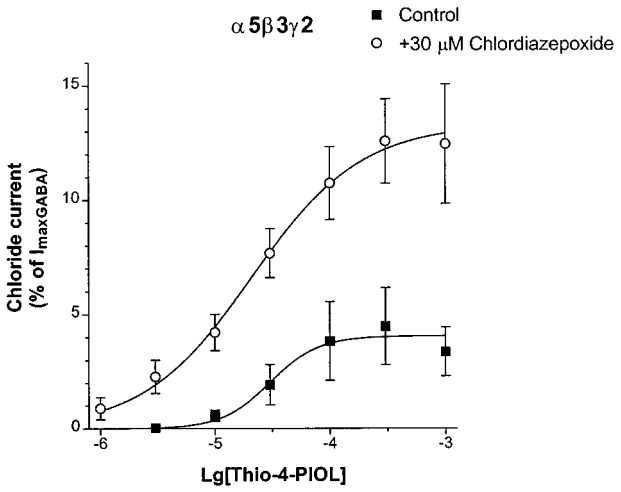

Figure 5 The effects of 30 μM chlordiazepoxide on the concentration-response curves of thio-4-PIOL for $\alpha_5\beta_3\gamma_2$ $GABA_A$ receptors. Chloride currents are expressed as per cent of the peak current elicited by 3 mM GABA. Data are mean$\pm$s.e.mean of the number of experiments indicated in Table 1.

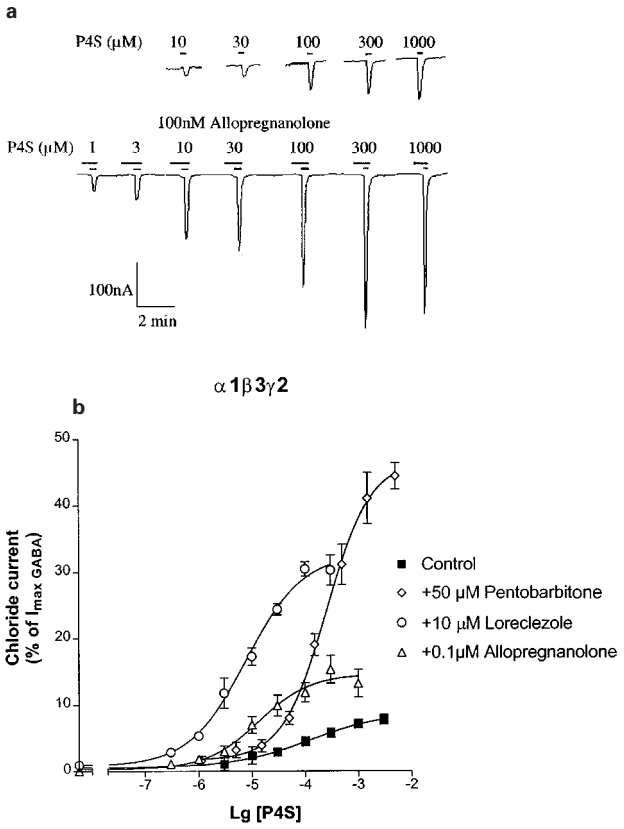

Figure 6 The effects of pentobarbitone, allopregnanolone and loreclezole on the concentration-response curves of P4S for $\alpha_1\beta_3\gamma_2$ GABA$_A$ receptors. (a) Example traces of a concentration-response curve to P4S on a cell expressing $\alpha_1\beta_3\gamma_2$ GABA$_A$ receptors in the absence and presence of 100 nM allopregnanolone. Drugs were applied as indicated by the bars. The current to 1 mM P4S was equivalent to 10% of that to 3 mM GABA (not indicated). (b) The effects of 50 μM pentobarbitone, 0.1 μM allopregnanolone and 10 μM loreclezole on the concentration-response curves of P4S for $\alpha_1\beta_3\gamma_2$ GABA$_A$ receptors. Chloride currents are expressed as per cent of the peak current elicited by 3 mM GABA. Data are mean$\pm$s.e.mean of the number of experiments indicated in Table 1.

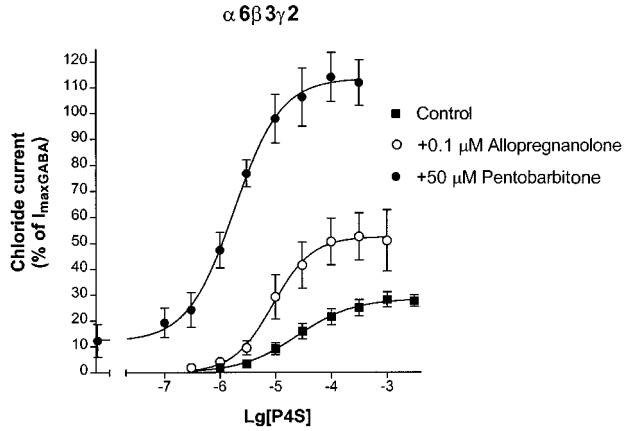

Figure 7 The effects of 100 nM allopregnanolone and 50 μM pentobarbitone on the concentration-response curves of P4S for $\alpha_6\beta_3\gamma_2$ GABA$_A$ receptors. Chloride currents are expressed as per cent of the peak current elicited by 3 mM GABA. Data are mean$\pm$ s.e.mean of 3–4 experiments.

enhancement of the maximal current and potency of P4S (Figure 7), the maximal response surpassing that for 3 mM GABA alone (Figure 7). Allopregnanolone at 100 nM did not elicit chloride currents but also enhanced strongly the efficacy and potency of P4S on $\alpha_6\beta_3\gamma_2$ GABA$_A$ receptors (Figure 7).

Discussion

Benzodiazepine site ligands affect the efficacy and potency of partial and full GABA$_A$ agonists, respectively

Analysis of the allosteric modulation of GABA$_A$ receptor-ionophore function has primarily been carried out using GABA (Lavoie *et al*., 1997) and another full agonist muscimol (Macdonald & Twyman, 1992). The allosteric effects of the ligands of the benzodiazepine site or other allosteric binding sites, and ionophore function of GABA$_A$ receptors have been very thoroughly studied because of their pharmacological importance. Several studies support the view that benzodiazepine agonists facilitate, while inverse agonists inhibit GABAergic neurotransmission, *via* mutual allosteric interactions to modulate the affinity of GABA binding rather than subsequent gating of the channel (Macdonald & Twyman, 1992; Serfozo & Cash, 1992). Recent evidence however suggests that benzodiazepines may be more closely linked to the ion channel itself, and can modulate spontaneously open channels in the absence of GABA (Thompson *et al*., 1999).

The effects of chlordiazepoxide and DMCM on GABA for $\alpha_1\beta_3\gamma_3$ and on THIP for $\alpha_2\beta_3\gamma_2$ GABA$_A$ receptors are in agreement with previous reports which suggest a shift in apparent agonist potency but no effect on maximum response when full agonists are employed to activate the receptor complex (Sigel & Baur, 1988; Wafford *et al*., 1992). Similar studies for partial GABA$_A$ agonists have not been reported previously. Chlordiazepoxide exerted 2–3 fold enhancements of the maximal responses of three partial agonists for $\alpha_x\beta_3\gamma_2$ GABA$_A$ receptors containing all types of α subunits apart from the benzodiazepine-insensitive α_4 and α_6 subtypes. This included thio-4-PIOL, which had a maximum efficacy of only 4–5% on $\alpha_2\beta_3\gamma_2$ and $\alpha_5\beta_3\gamma_2$ receptors, and has previously been described as an antagonist (Ebert *et al*., 1997). This allosteric modulation of the efficacies of partial agonists therefore seems to be a general phenomenon, valid for different structures of both agonists and GABA$_A$ receptor subtypes. The concentration-response curves of chlordiazepoxide resulted in similar EC$_{50}$ values (2–6 μM), using both different agonist concentrations (EC$_{20}$ or maximally effective concentration) and different chemical structures of GABA$_A$ agonists (GABA, P4S and THIP). Its effects are mediated by common benzodiazepine binding sites on the GABA$_A$ receptor complex, whose behaviour appears not to depend on the choice of the GABA agonist applied. The effects on efficacy of partial agonists seems also to be the case of inverse agonists, as the low efficacy of P4S on $\alpha_1\beta_3\gamma_2$ was further decreased by DMCM, a β-carboline inverse agonist.

The effects of the ligands of the benzodiazepine sites on the EC$_{50}$ values of partial agonists were not so consistent. Chlordiazepoxide increased the potencies of P4S for $\alpha_1\beta_3\gamma_2$ and of thio-4-PIOL for $\alpha_5\beta_3\gamma_2$ receptors but its effects on thio-4-PIOL for $\alpha_2\beta_3\gamma_2$ as well as on THIP for $\alpha_3\beta_3\gamma_2$ GABA$_A$ receptors were not significant. As the effects of benzodiazepines on GABA EC$_{50}$ are 2–3 fold at maximum, it is likely that the small shifts in EC$_{50}$ produced by the benzodiazepine and β-carboline, some of which do not reach significance, are overwhelmed by the larger effects observed on the efficacy of partial agonists.

The effects of a barbiturate, a neurosteroid and loreclezole

Low concentrations of pentobarbitone up to 50 μM facilitate the effect of GABA and enhance the potencies rather than the

efficacies of $GABA_A$ agonists (Akaike *et al*., 1985; Zhang & Simmonds, 1997). In contrast, 50 μM pentobarbitone did not affect the potency of the partial agonist P4S but strongly enhanced its efficacy for $\alpha_1\beta_3\gamma_2$ $GABA_A$ receptors.

Pentobarbitone increased the maximal responses for the low efficacy agonists THIP on $\alpha_3\beta_3\gamma_2$ and P4S on $\alpha_6\beta_3\gamma_2$ receptors close to the maximal level of GABA. The enhancement of the maximal response of P4S on $\alpha_6\beta_3\gamma_2$ receptors beyond 100% was unusual. Maximal responses beyond 100% for THIP (Wafford *et al*., 1996) and pentobarbitone on $\alpha_6\beta_3\gamma_2$ receptors have been reported previously (Thompson *et al*., 1996). It is possible that the rapid desensitization of $\alpha_6\beta_3\gamma_2$ receptors when activated by GABA results in a significant reduction in the observed peak response, which may be different when activated by P4S which elicits less desensitization. It is unlikely however that this could account for the large changes in efficacy observed here as shown by compounds such as THIP which can behave as a full or partial agonist. Pentobarbitone also enhanced the potencies of THIP and P4S by about an order of magnitude. It is interesting however, that the low EC_{50} value of P4S on $\alpha_6\beta_3\gamma_2$ receptors was strongly decreased by pentobarbitone while the high EC_{50} value on $\alpha_1\beta_3\gamma_2$ receptors was not affected by it.

The effects of the neurosteroid allopregnanolone up to 100 nM are also restricted to potentiation of GABA (Turner & Simmonds, 1989; Puia *et al*., 1990), i.e. to decrease the EC_{50} value of the full agonists (Woodward *et al*., 1992). In contrast, 100 nM allopregnanolone enhanced not only the potencies of P4S on $\alpha_1\beta_3\gamma_2$ and $\alpha_6\beta_3\gamma_2$ receptors and THIP on $\alpha_3\beta_3\gamma_2$ receptors but also their maximal responses.

The anticonvulsant loreclezole (10 μM) enhanced the affinity of GABA (Wafford *et al*., 1994), muscimol (Zhang & Simmonds, 1997) and P4S (this study). Further, it resulted in an apparent decrease in the maximal response for GABA on recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors (Wafford *et al*., 1994) which might be attributed to the enhancement of desensitization (Donnelly & Macdonald, 1996). In contrast, the high efficacy of P4S (88%) for $\alpha_2\beta_3\gamma_2$ $GABA_A$ receptors was not affected by 10 μM loreclezole. Consequently, loreclezole does not necessarily decrease the maximal response for full agonists. This may be related to receptor subtypes and distinct agonists differing in their rates of desensitization. In contrast, for $\alpha_1\beta_3\gamma_2$ receptors at which P4S is an agonist with low efficacy, 10 μM loreclezole greatly enhanced the maximal response to P4S. In conclusion, the effects of the representative barbiturate, neurosteroid and loreclezole in GABA-facilitating concentrations on partial $GABA_A$ agonists are different from their effects on full agonists.

Allosteric model of receptor activation

Electrophysiological and $^{36}Cl^-$ flux data on $GABA_A$ receptor-ionophores have been analysed in terms of a sequential model (Macdonald & Twyman, 1992; Serfozo & Cash, 1992; Lavoie *et al*., 1997) or a 'bifurcation' model of channel activation (Johnes *et al*., 1998). Spontaneous openings of nicotinic acetylcholine (Galzi *et al*., 1996) and $GABA_A$ receptor channels (Neelands *et al*., 1999) and changes in their pharmacological profiles cannot be reconciled with these sequential models. Recent findings for the phenotypes of neurotransmitter receptor-ionophores have been better explained within the framework of the Monod–Wyman–Changeux allosteric receptor model (Galzi *et al*., 1996). The simplest two-state model is characterized by an isomerization equilibrium between an inactive close state B and an active open state A of the ionophore. Ligands can be differentiated by the ratio of distinct dissociation constants to these states: $C = K_A/K_B$. This model can also be applied for $GABA_A$ receptors. Full agonists are supposed to bind preferentially to the open state. Partial $GABA_A$ agonists such as P4S and THIP might then result in lower selectivities of binding to the open state. This seems to be in agreement with shorter channel opening duration for THIP and P4S in comparison to GABA and muscimol as concluded from the fluctuation analysis of cultured mouse spinal neurons (Barker & Mathers, 1981). The antagonism of thio-4-PIOL for all recombinant $\alpha_x\beta_3\gamma_2$ $GABA_A$ receptors (Ebert *et al*., 1997) can be reconciled with preferential binding to the closed state. Benzodiazepine agonists such as chlordiazepoxide and other allosteric modulators are likely to increase, while the inverse agonist DMCM decreases the receptor's ability to isomerize to the open state (the E value or efficacy in del-Castillo & Katz's two-state model) (Colquhoun, 1998). This is supported by evidence showing modulation of constitutive $GABA_A$ receptor activity by benzodiazepines in the absence of a $GABA_A$ agonist (Thompson *et al*., 1999).

If allosteric agents affect the affinities of the agonist for both states A and B and the ratio $c = K_A/K_B$ remains constant, the concentration-response curves of the agonists are shifted horizontally in a parallel manner (Galzi *et al*., 1996). This seems to be valid for the bidirectional effects of benzodiazepine site ligands on full $GABA_A$ agonists. However, chlordiazepoxide resulted in nonparallel shifts of the concentration-response curves for partial agonists, suggesting that benzodiazepine potentiation is either being mediated *via* a change in gating, or the K_A/K_B ratio is not constant. Modulation of spontaneous activity by allosteric modulators suggests the mechanism to be due to direct changes in channel gating (Thompson *et al*., 1999; Neelands *et al*., 1999).

As to other neurotransmitter receptor-ionophores, a similar effect has been reported for α7 neuronal nicotinic acetylcholine receptors (Krause *et al*., 1998). Ivermectin enhanced the potency and efficacy of the partial agonist 1,1-dimethyl-4-phenylpiperazinium. It has been attributed to an allosteric effect on the isomerization from a closed to open state of the ionophore (Krause *et al*., 1998).

In conclusion, several allosteric agents affect differently the response curves of partial versus full $GABA_A$ agonists suggesting the mechanism to be more than just a shift in agonist affinity. The differential effects appear to be independent of the type of α_7 subunit and are not compound specific. Single channel analysis will hopefully elucidate further the differences in molecular mechanism of the interaction between $GABA_A$ agonists and allosteric agents.

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