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Activity of novel 4-PIOL analogues at human $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors—correlation with hydrophobicity

Martin Mortensen [a], Bente Frølund [b], Anne T. Jørgensen [b], Tommy Liljefors [b], Povl Krogsgaard-Larsen [b], Bjarke Ebert [c,*]

[a] *Department of Pharmacology, The Royal Danish School of Pharmacy, 2 Universitetsparken, DK-2100 Copenhagen Ø, Denmark*
[b] *Department of Medicinal Chemistry, The Royal Danish School of Pharmacy, 2 Universitetsparken, DK-2100 Copenhagen Ø, Denmark*
[c] *Department of Molecular Pharmacology, H. Lundbeck A/S, 9 Otilliavej, DK-2500 Valby, Denmark*

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Abstract

A series of novel 5-(4-piperidyl)-3-isoxazolol (4-PIOL) analogues where the 4-position of the 3-isoxazolol ring was substituted with groups of different size, flexibility, and lipophilicity have been characterised. Their activity as agonists and/or antagonists on human $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors expressed in *Xenopus* oocytes was studied using two-electrode voltage clamp electrophysiology. Methyl- and ethyl-substituted 4-PIOL analogues were characterised as partial agonists since weak agonist responses could be potentiated with lorazepam and inhibited by the competitive antagonist 2-(3-carboxypropyl)-3-amino-6-methoxyphenyl-pyradizinum bromide (SR95531). All larger substituents in the 4-position of the 3-isoxazolol ring of 4-PIOL converted the compounds into pure competitive antagonists. Additionally, for GABA, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP), piperidine-4-sulphonic acid (P4S), and 5-(4-piperidyl)-3-isothiazolol (thio-4-PIOL), a negative linear correlation was found between the agonist efficacy of the compound and the ability of lorazepam to potentiate EC_{95} responses. Furthermore, a positive linear correlation between the lipophilicity of the substituents in the 4-position of the 3-isoxazolol ring of 4-PIOL and the antagonist affinity was found. These data suggest that the $GABA_A$ receptor contains a hydrophobic binding pocket at the GABA recognition site and that the binding of the 4-PIOL analogues is largely determined by the transfer from the aqueous phase to the hydrophobic pocket.

Keywords: $GABA_A$ receptor; *Xenopus* oocyte; 4-PIOL (5-(4-piperidyl)-3-isoxazolol); Partial agonist; Structure–activity; Lipophilicity

1. Introduction

γ-Amino butyric acid (GABA), being the major inhibitory neurotransmitter in the vertebrate brain, exerts its effect via ionotropic $GABA_A$ and $GABA_C$ receptors as well as via G-protein coupled $GABA_B$ receptors (Chebib and Johnston, 2000). $GABA_A$ receptors are central in the regulation of signal transmission between neurons, and have been associated with several neurological and psychiatric disorders caused by abnormal $GABA_A$ receptor activity. Hypoactivity seems to be associated with epilepsy, anxiety, pain, insomnia, Huntington's chorea, and tardive dyskinesia, whereas hyperactivity is thought to play a part in schizophrenia symptoms (De Deyn et al., 1990; Smith, 2001; Kontinen et al., 2001; Landolt and Gillin, 2000; Lloyd et al., 1990; Benes et al., 1992). Bringing the activity of the receptor to a normal level with a receptor-specific drug can, at least in theory, treat a disease caused by an abnormal receptor activity. This can often be done using drugs that work as modulators for the receptor in question. However, the use of, for instance, benzodiazepines as positive modulators of $GABA_A$ receptors results in several adverse effects (Barbee, 1993; Little, 1991). If used, full agonists and neutral antagonists will also result in severe side effects in the subject (Salinas and McGaugh, 1995; Lin et al., 1989). Additionally, receptors often desensitise with high concentrations of full agonist, which along with other reasons like internalisation can lead to tolerance and subsequent withdrawal symptoms. On the contrary, partial agonist can only

* Corresponding author. Tel.: +45-2220-0245; fax: +45-3644-0043.
E-mail address: bjeb@lundbeck.com (B. Ebert).

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induce a submaximal activation and, depending on the level of activity, does not desensitise receptors to the same degree as full agonists. Furthermore, partial agonists may, dependent on the efficacy, act as inhibitors under conditions where hyperactivity is a problem. These characteristics of partial agonists make them very interesting in drug research for some of the abovementioned diseases.

4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP), piperidine-4-sulphonic acid (P4S), and 5-(4-piperidyl)-3-isothiazolol (thio-4-PIOL) have previously been characterised as partial agonists on αβγ receptors expressed in oocytes (Ebert et al., 1994; Maksay et al., 2000), whereas 5-(4-piperidyl)-3-isoxazolol (4-PIOL) only has been characterised as a partial agonist in whole cell patch clamping on cerebral cortical neurons and cultured hippocampal neurons (Frølund et al., 1995; Kristiansen et al., 1991). The novel series of 4-PIOL analogues studied in this paper have recently been shown to act as $GABA_A$ receptor antagonists in binding experiments and functional patch clamp experiments (Frølund et al., 2000, 2002).

In order to characterise 4-PIOL and these structurally related analogues at a relevant $GABA_A$ receptor combination, we chose the $\alpha_1\beta_2\gamma_{2S}$ combination believed to be the most abundant in the human central nervous system (McKernan and Whiting, 1996). At this receptor combination, we characterised thio-4-PIOL, 4-PIOL, and the 4-PIOL analogues with respect to potency as agonist or antagonist, and in the case of agonist activity, for the maximum response relative to that of GABA (efficacy). Agonist effects were verified by potentiation in the presence of lorazepam, as well as by inhibition in the presence of 2-(3-carboxypropyl)-3-amino-6-methoxyphenyl-pyradizinum bromide (SR95531). GABA, THIP, P4S, and thio-4-PIOL were chosen in these experiments as standard agonists.

2. Materials and methods

2.1. Drug solutions

The partial agonists THIP (**2**), P4S (**3**), thio-4-PIOL (**4**), and 4-PIOL (**5**) characterised in the present study are illustrated in Fig. 1. The synthesis of the novel 4-PIOL analogues (**6a**–**q**) has been described in detail by Frølund et al. (2002). Compounds with low solubility (in particular the 4-PIOL analogues with an aromatic substituent) were solubilised in 100% dimethyl sulfoxide (DMSO) before dilution in modified Barth's saline (MBS buffer). The maximum concentrations obtained for the individual compounds were: >3000 μM for **1**, **2**, **3**, **4**, **5**, and **6a**–**f**; 100 μM for **6g**, **6h**, and **6k**; 50 μM for **6i**, **6l**, **6m**, **6n**, **6o**, and **6q**; and 10 μM for **6j** and **6p**. The final concentration of DMSO was at all times lower than 0.5% in test solutions. This upper limit of DMSO was set in preliminary experiments to insure that DMSO would not affect cell responses (data not shown).

2.2. Expression of $\alpha_1\beta_2\gamma_{2s}$ receptors in Xenopus oocytes

Female *Xenopus laevis* frogs were anaesthetised in 0.1% tricaine (aminobenzoic acid ethyl ester; Sigma); before stages V and VI, oocytes were removed through a small abdominal incision. Following manual isolation with fine forceps, the oocytes were treated with a mild collagenase solution for 6 min (Sigma: type A1 [0.5 mg/ml]). The oocyte nuclei were injected with 18–25 nl of injection buffer (88 mM NaCl, 1 mM KCl, 15 mM HEPES, at pH 7.0, sterile-filtered) containing a combination of human $GABA_A$ α_1, β_2, and γ_{2S} subunit cDNAs engineered into the expression vector pCDM8 or pcDNA1/Amp. DNA was

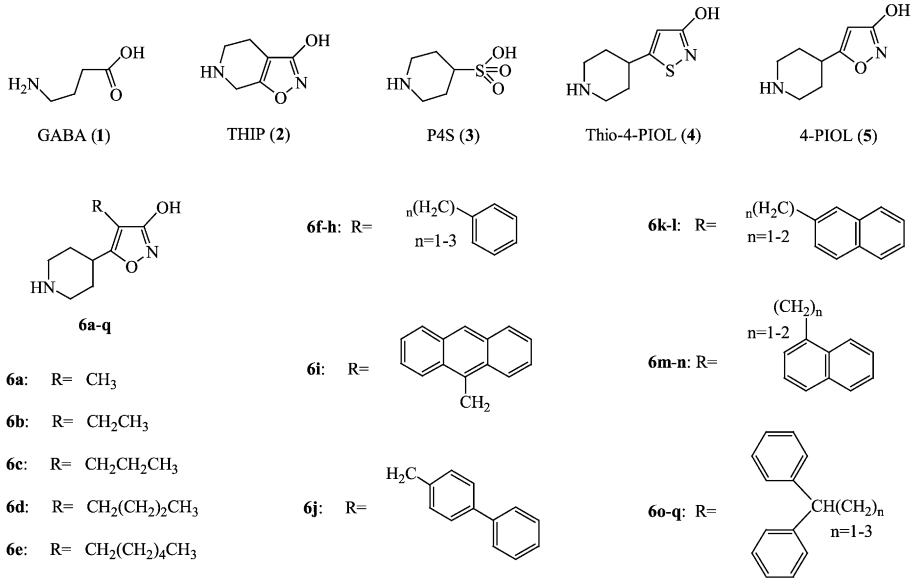

Fig. 1. Structures of GABA (**1**), THIP (**2**), P4S (**3**), thio-4-PIOL (**4**), 4-PIOL (**5**), and the novel 4-PIOL analogues (**6a**–**q**).

a gift from Dr. Paul Whiting, Merck Sharp & Dohme, Terlings Park, Harlow, UK. The isolation and sequencing of α_1, β_2, and γ_{2S} cDNAs have been described elsewhere (Hadingham et al., 1993a,b). The concentration ratio of the cDNA encoding α_1, β_2, and γ_{2S} subunits was 1:1:1 (~ 0.07 ng of each subunit per oocyte). Oocytes were incubated in sterile-filtered MBS buffer (88 mM NaCl, 1 mM KCl, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, 2.4 mM $NaHCO_3$, at pH 7.5) supplemented with penicillin (5000 units/ml), streptomycin (5 mg/ml), gentamycin (10 mg/ml) and pyruvate (final concentration: 2 mM) at 18 °C, and were used in two-electrode voltage clamp experiments 1–7 days after injection.

2.3. Two-electrode voltage clamping

Oocytes were placed in a 200-μl bath and superfused continuously (6.5 ml/min) with MBS. In two-electrode voltage clamp experiments, oocytes were impaled with two 0.5–1.5 MΩ electrodes containing 2 M KCl and voltage clamped at −50 to −70 mV by a GeneClamp 500B (Axon Instruments, USA).

Drugs were applied in the perfusate until the peak of the response was observed, allowing up to 10 min between each drug application to recover from desensitisation. Current responses were recorded using the Oocyte 3 software from Digitimer (UK).

In concentration response experiments, all agonist res ponses were related to $GABA_{max}$ currents (response to 3 mM GABA) since GABA per definition is considered a full agonist with 100% efficacy.

To estimate the inhibition equilibrium constant values (K_i values) of the 4-PIOL analogues, GABA concentration–response curves were parallel, shifted by fixed concentrations of these ligands. Only one concentration of each antagonist was used.

Preapplications were used in all experiments with the 4-PIOL analogues, the antagonist SR95531 or the modulator lorazepam (5, 5, and 10 s, respectively).

2.4. Data analysis

Concentration–response curves were fitted using the nonlinear equation $I/I_{max} = E_{max}/[1+(EC_{50}/x)^n]$ where I and I_{max} represent the peak currents activated by an agonist concentration, x, and a saturating concentration of a full agonist ($GABA_{max}$). The E_{max} value is the normalized maximum response (%) of an agonist relative to the maximum response obtained with $GABA_{max}$. EC_{50} is the concentration of drug eliciting a half-maximal response and n is the slope factor.

The K_i values, representing the antagonist affinity, were calculated according to the method described by Ebert et al. (1997), using the following equation K_i=[Antagonist]/(Shift − 1), where Shift is the ratio between EC_{50} values with or without antagonist of the concentration [antagonist]. Values presented are mean values ± standard error of mean (S.E.M.) of at least four individual experiments.

The computer program GraFit 4.0.12 (Erithacus Software, Staines, UK) was used to analyse and plot data, and ChemDraw Ultra 5.0 (Cambridge Soft, Cambridge, MA, USA) was used to estimate the logarithm of partition coefficient (*n*-octanol/water) named log *P*, of all substituents of the 4-PIOL analogues using Crippen's fragmentation method (Ghose and Crippen, 1986; Ghose et al., 1988). SigmaStat 2.03 (SPSS, San Rafael, CA, USA) was used for statistical analysis.

3. Results

3.1. Identification of very low efficacy partial agonists and pure antagonists by lorazepam potentiation and SR95531 inhibition

$GABA_A$ receptors containing the $\alpha_1\beta_2\gamma_{2S}$ subunit combination were expressed in *Xenopus* oocytes where GABA-activated maximum currents reached up to 5 μA with a tolerable drop in the voltage clamp potential of less than 1 mV. At a voltage clamp potential of −60 mV, the mean baseline leak current was −32 ± 3.9 nA (n = 12), when superfusing the $\alpha_1\beta_2\gamma_{2S}$ expressing oocytes with ligand-free MBS.

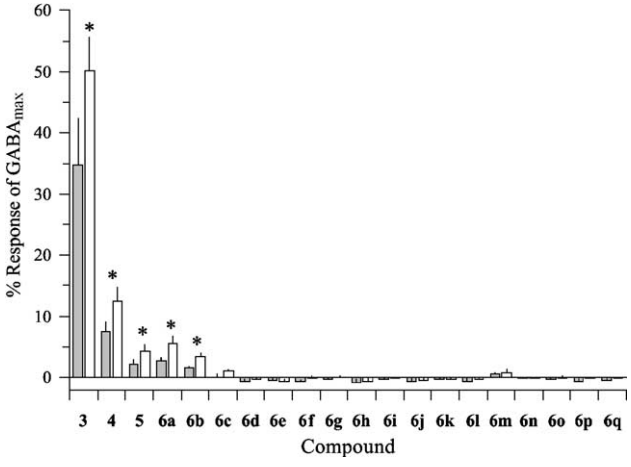

Fig. 2. $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptor responses to P4S (**3**), thio-4-PIOL (**4**), 4-PIOL (**5**), and 4-PIOL analogues (**6a**–**q**) without and with 1 μM lorazepam (light grey columns and white columns, respectively) measured by two-electrode voltage clamping of *Xenopus* oocytes. The concentrations of the individual compounds were: 500 μM for **6c**, **6d**, **6e**, and **6f**; 300 μM for **3**, **4**, **5**, **6a**, and **6b**; 100 μM for **6g**, **6h**, and **6k**; 50 μM for **6i**, **6l**, **6m**, **6n**, **6o**, and **6q**; and 10 μM for **6j** and **6p**. For **6g**–**q**, these were the maximum concentrations obtainable (see Materials and methods). Asterisks indicate a significant difference between responses with and without lorazepam (paired *t*-tests; $P < 0.05$). The responses are expressed as mean percent current with respect to the maximum responses obtained with 3000 μM GABA ± S.E.M. from four to six oocytes.

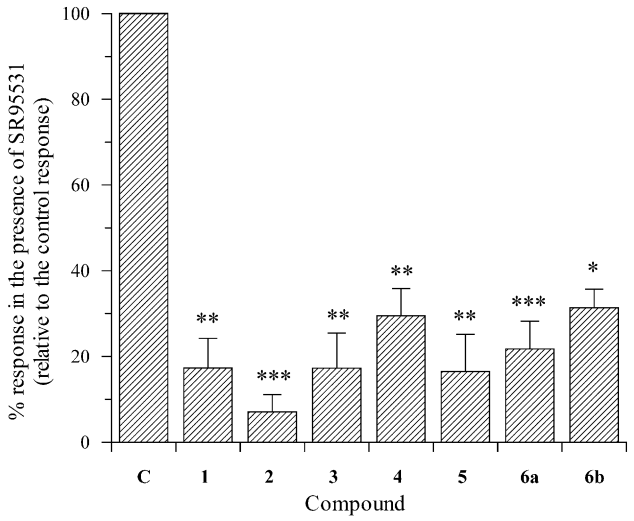

Fig. 3. Inhibition by SR95531 of agonist-mediated currents through $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptor channels expressed in *Xenopus* oocytes. The responses were measured using two-electrode voltage clamping and are expressed as mean percent current in the presence of 10 μM SR95531 relative to the normalized control agonist response (C) ± S.E.M. from four to six oocytes. The following agonist concentrations were used in the experiments (compound, concentration [μM]): **1**, 30; **2**–**3**, 100; and **4**–**6b**, 1000. With respect to the maximum responses obtained in the presence of 1000 μM GABA, the agonist responses of **1**, **2**, **3**, **4**, **5**, **6a**, and **6b** in the used concentrations were 76 ± 7%, 55 ± 5%, 24 ± 3%, 11 ± 0.7%, 3.2 ± 0.2%, 4.1 ± 0.1%, 3.5 ± 0.4%, respectively. Asterisks indicate a significant difference between responses with and without SR95531 (paired *t*-tests; * $P<0.05$, ** $P<0.01$, *** $P<0.001$).

In initial screening for agonist character of thio-4-PIOL, 4-PIOL, and the recently published series of 4-PIOL analogues (Frølund et al., 2002), a week agonistic effect significantly higher than zero was observed with 300 μM thio-4-PIOL (7.5 ± 1.6%, $P<0.001$), 300 μM 4-PIOL (2.2 ± 0.61%, $P<0.001$), 300 μM **6a** (2.6 ± 0.56%, $P<0.001$), 300 μM **6b** (1.65 ± 0.22%, $P<0.001$), 1000 μM **6c** (0.48 ± 0.10%, $P=0.003$), and 300 μM **6m** (0.52 ± 0.24%, $P=0.016$). None of the remaining 4-PIOL analogues (**6d**–**l** and **6n**–**q**) showed any sign of agonism at the concentrations tested.

In order to verify that the agonist activities observed for thio-4-PIOL, 4-PIOL, **6a**, **6b**, **6c**, and **6m**, and in an attempt to see hidden agonist activity in the remaining 4-PIOL analogues, modulation with a benzodiazepine was carried out. A saturating concentration of lorazepam (1 μM) to potentiate the agonist responses was used. P4S which previously has been shown to be a partial agonist at $\alpha_1\beta_2\gamma_{2S}$-containing receptors (Ebert et al., 1994; Krogsgaard-Larsen et al., 1997) was included in this study. As illustrated in Fig. 2, agonist responses by P4S, thio-4-PIOL, 4-PIOL, **6a**, and **6b** were significantly potentiated by 1 μM of lorazepam (paired *t*-test: $P=0.010$, $P=0.032$, $P=0.018$, $P=0.015$, and $P=0.021$, respectively), whereas the remaining 4-PIOL analogues failed to show agonist potentiation, including **6c** and **6m** ($P=0.065$ and $P=0.674$, respectively). To further substantiate that the response of thio-4-PIOL, 4-PIOL, **6a**, and **6b** was indeed $GABA_A$ receptor-mediated responses, the competitive $GABA_A$ receptor antagonist SR95531 was used in inhibition experiments. GABA, THIP, P4S, and thio-4-PIOL were included in these experiments as standard agonists and partial agonists. Fig. 3 illustrates how all agonists, GABA, THIP, P4S, thio-4-PIOL, 4-PIOL, **6a**, and **6b**, were significantly inhibited by 10 μM SR95531 ($P=0.016$, $P<0.001$, $P=0.001$, $P=0.006$, $P=0.007$, $P<0.001$, and $P=0.034$, respectively).

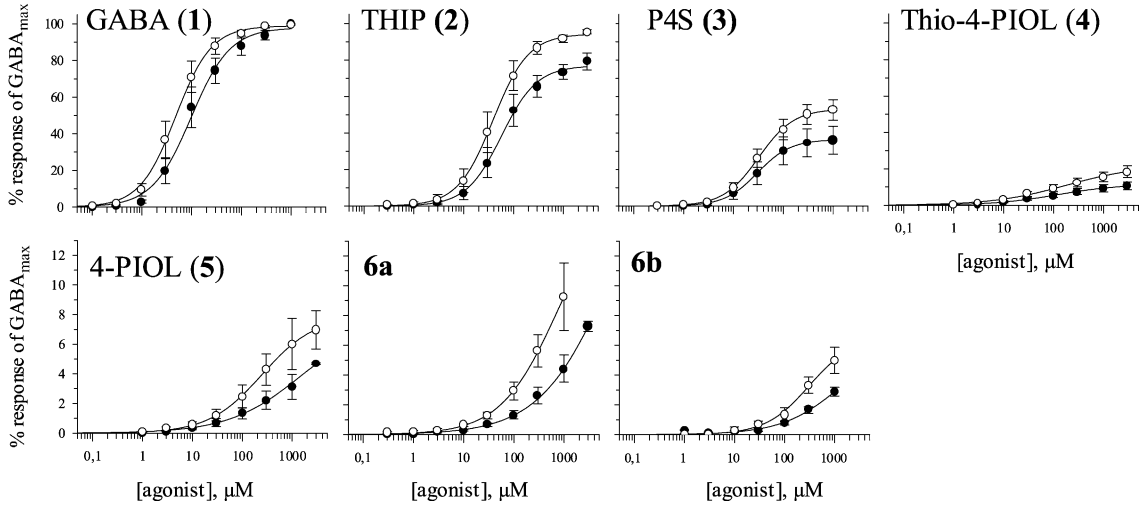

Fig. 4. Effect of lorazepam on agonist concentration–response curves on $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors expressed in oocytes. Agonist currents were shifted by 1 μM lorazepam in two-electrode voltage clamp experiments. Data points are mean values ± S.E.M. from four experiments in which both agonist responses and lorazepam potentiation responses were obtained. Curves were fitted using a nonlinear equation (see Materials and methods). Unmodulated values of maximum responses (%), potency (pEC_{50} or EC_{50}), and slope factor (*n*) were determined for GABA (100%; 5.01 ± 0.18/9.8 μM; 1.31 ± 0.15), THIP (77 ± 2.7%; 4.23 ± 0.18/59 μM; 1.41 ± 0.08), P4S (36 ± 7.7%; 4.46 ± 0.10/34 μM; 1.42 ± 0.06), and thio-4-PIOL (12 ± 2.0%; 3.84 ± 0.13/144 μM; 0.67 ± 0.05), but could not be determined for 4-PIOL, **6a**, and **6b** since their low potency did not allow determination of maximum responses.

3.2. Lorazepam potentiation of $GABA_A$ receptor agonist concentration–response curves

Dose–response curves of the agonists GABA, THIP, P4S, thio-4-PIOL, 4-PIOL, **6a**, and **7b** were all shifted by 1 μM lorazepam (Fig. 4). Due to the low potency of 4-PIOL and **6a**–**b** (EC_{50}>500 μM), we could only determine precise efficacies (% response of $GABA_{max}$) and potencies (pEC_{50}) for GABA (98 ± 0.6%; 5.01 ± 0.18), THIP (78 ± 3.7%; 4.23 ± 0.18), P4S (36 ± 0.10%; 4.46 ± 0.10), and thio-4-PIOL (12 ± 2.0%; 3.84 ± 0.13).

The degree by which lorazepam could potentiate agonists with different efficacies varied at different concentrations. At EC_{20}, there was no significant difference in the level of lorazepam potentiation at GABA (88 ± 2.3%), THIP (89 ± 10.3%), P4S (97 ± 50.5%), and thio-4-PIOL (88 ± 23.7) tested by analysis of variance (P=0.780). However, at EC_{95}, the lorazepam potentiation showed a significant difference (P=0.009) among GABA (2.3 ± 0.5%), THIP (24 ± 5.5%), P4S (56 ± 25.5%), and thio-4-PIOL (81 ± 16.5). A significant linear relationship was observed between the agonist efficacy and the lorazepam potentiation at EC_{95} (Fig. 5; R^2=0.997, P=0.002). On the contrary, no significant linear relationship could be found between the potency and the lorazepam potentiation at EC_{95} (Fig. 5, insert; R^2=0.689, P=0.170).

Significantly higher pEC_{50} values of agonists in the presence of lorazepam, when compared with the nonmodulated pEC_{50} values (see above), were only seen for GABA (5.32 ± 0.04; P=0.004) and THIP (4.42 ± 0.16; P=0.008), whereas it was unaltered for P4S (4.48 ± 0.08; P=0.748) and thio-4-PIOL (3.65 ± 0.24; P=0.393).

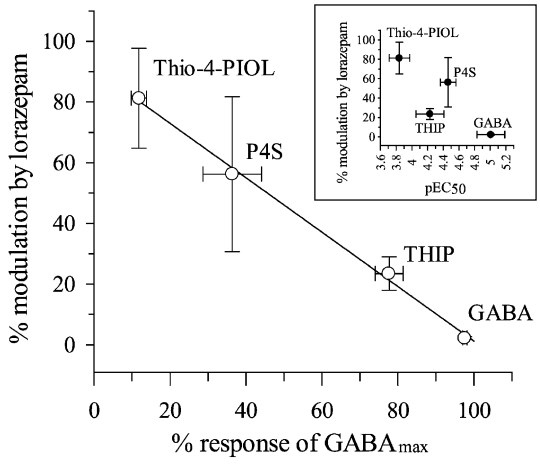

Fig. 5. Correlation between the agonist efficacy and the lorazepam potentiation (1 μM) at EC_{95}. The line shows the significant relationship between the agonist efficacy and the lorazepam potentiation found by linear regression (R^2=0.997, P=0.002). Insert shows the lack of correlation between the agonist potency and the lorazepam potentiation (1 μM) at EC_{95}. Points are means of four experiments and show S.E.M. bars for both efficacy or potency (insert) and the lorazepam potentiation.

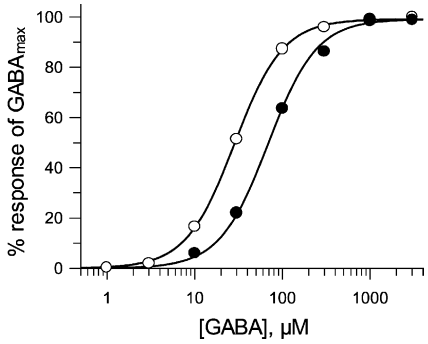

Fig. 6. Representative example of a parallel shift of a GABA concentration–response curve (open circles) in the presence of 10 μM **6b** (filled circles). The K_i value for **6b** was in the present experiment 7.1 ± 0.6 μM. K_i values were determined for **5**–**6q** (summarised in Table 1) in similar experiments where all compounds showed similar competitive antagonist effects.

3.3. Structure–activity relationships for the antagonist character of 4-PIOL analogues

4-PIOL and the related analogues (**6a**–**q**) were characterised as competitive $GABA_A$ receptor antagonists by their ability to shift GABA dose–response curves (illustrated in Fig. 6 and described in Materials and methods). All the characterised compounds could, with varying potencies, induce a parallel shift of GABA curves indicating antagonist character (Fig. 6). These experimentally determined antag-

Table 1
Estimates of the lipophilicity of 4-PIOL analogue substituents (log P) and antagonist potencies (K_i) obtained by two-electrode voltage clamping on $\alpha_1\beta_2\gamma_{2S}$ expressing *Xenopus* oocytes

Compound	log P	K_i, μM (pK_i ± S.E.M.)
5	0	36 (4.449 ± 0.181)
6a	1.09	45 (4.350 ± 0.098)
6b	1.33	10 (4.984 ± 0.059)
6c	1.75	3.7 (5.433 ± 0.073)
6d	2.17	3.9 (5.414 ± 0.111)
6e	3.00	0.66 (6.178 ± 0.081)
6f	2.52	2.8 (5.552 ± 0.102)
6g	2.94	6.2 (5.210 ± 0.158)
6h	3.36	0.80 (6.096 ± 0.150)
6i	4.52	1.5 (5.834 ± 0.077)
6j	4.20	0.31 (6.506 ± 0.046)
6k	3.52	0.099 (7.005 ± 0.132)
6l	3.94	0.77 (6.112 ± 0.062)
6m	3.52	0.62 (6.206 ± 0.073)
6n	3.94	2.3 (5.636 ± 0.027)
6o	4.46	0.42 (6.374 ± 0.038)
6p	4.87	0.026 (7.588 ± 0.087)
6q	5.29	0.12 (6.936 ± 0.043)

The estimates of the logarithm of partition coefficient in *n*-octanol/water (log P) were obtained by use of Crippen's fragmentation method (Ghose and Crippen, 1986; Ghose et al., 1988) in the computer programme ChemDraw Ultra 5.0. The antagonist potencies were determined by shifts of agonist concentration–response curves using two-electrode voltage clamping on $\alpha_1\beta_2\gamma_{2S}$ expressing *Xenopus* oocytes (see Materials and methods). K_i values (μM) are calculated from pK_i values found in brackets. The pK_i values represent mean ± S.E.M. of at least four experiments.

onist potencies (K_i values) can be seen in Table 1, and correspond well with the recent estimates from whole cell patch clamp experiments on cortical neurons (Frølund et al., 2000, 2002).

4-PIOL, **6a**, and **6b** having small alkyl substituents in the 4-position of the 3-isoxazolol ring (a methyl, an ethyl, and a propyl group, respectively) can, in addition to their weak partial agonistic effect, also induce weak antagonistic inhibition of GABA-induced responses. However, compounds with larger substituents, especially aromatic groups as in **6k**, **6p**, and **6q**, show high antagonist potencies against GABA-mediated receptor activation.

Hydrophobicity (log *P*) is related to the desolvation of the ligand, and it is assumed that the desolvation of the ligands in going from water to octanol parallels that of going from water to a cleft of a receptor. Thus, log *P* may be an important term in establishing quantitative relationships between structure and activity (Leo and Hansch, 1999).

In order to obtain an estimate of the lipophilicity of the compounds, log *P* values for the substituents in the 4-position, being the variable component of the 4-PIOL lead structure, were calculated (see Materials and methods). These log *P* values are listed in Table 1.

As shown in Fig. 7, a linear correlation between pK_i and log *P* values was obtained for the complete data set ($R^2 = 0.725$, $P < 0.001$; stippled line; slope of 0.510). Apparently, the dataset splits up into three subsets consisting of a large group of compounds showing an excellent correlation ($R^2 = 0.925$, $P < 0.001$; unbroken line; slope of 0.501), a group of compounds (**6k** and **6p**) showing higher affinity, and a group of compounds (**6g**, **6i**, and **6n**) showing lower affinity than can be explained by log *P*. For the two latter groups of compounds, additional interactions in the receptor binding site may be of importance (see Discussion).

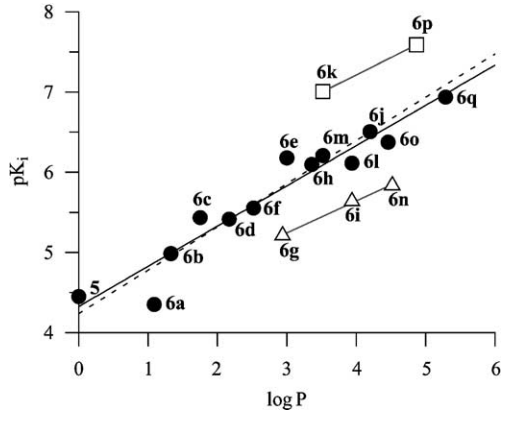

Fig. 7. Correlation between the mean antagonist affinity (pK_i) and the lipophilicity (log *P*) of the substituent of the 4-PIOL analogues. The stippled line shows the correlation for all compounds, the two grey lines connect the high affinity compounds (open squares) and the low-affinity compounds (open triangles), respectively, and the black line shows the correlation between the remaining compounds (black circles).

4. Discussion

Quantitative structure–activity studies are crucial for the understanding of molecular determinants for receptor binding and activation. In a number of molecular biological studies, some of the determinants for the $GABA_A$ receptor protein have been elucidated. However, in order to further understand receptor–ligand interactions in details, systematic variation in the structure of the ligand is important. In the present study, a series of structural analogues of the low efficacy partial agonist 4-PIOL have been characterised at human $\alpha_1\beta_2\gamma_{2S}$-containing $GABA_A$ receptors expressed in *Xenopus* oocytes.

As illustrated in Fig. 1, the 4-PIOL analogues in which the substituent in the 4-position of the 3-isoxazolol ring varies from a hydrogen (4-PIOL) to a tricyclic aromatic ring system connected to the 3-isoxazolol ring via a methylene unit cover a range of lipophilicities and sizes, thereby allowing analysis of some of the molecular determinants of activity.

The antagonist activities of the 4-PIOL analogues determined at recombinant human $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors expressed in oocytes are in agreement with functional data obtained from cultured cerebral cortical neurons from rat (Frølund et al., 2000, 2002). This shows, first of all, the high correlation in pharmacology between reconstituted receptors and native receptors expressed in neurons, and secondly, that the differences in the antagonist pharmacology on these $GABA_A$ receptors from humans versus rats are negligible. Although several different $GABA_A$ receptor combinations exist in cortical neurons, the functional consequences of this heterogeneity are, in terms of antagonist affinity, diminutive, whereas the converse is the case for agonists (Ebert et al., 2001). The reason for this is most likely that whereas agonist activity is dependent on both binding and the conformational change leading to gating, antagonist activity is only dependent on binding (Colquhoun, 1998).

In agreement with the data published by Frølund et al. (2000, 2002), our data clearly indicate that the modification of the 4-position of the 3-isoxazolol ring of 4-PIOL contributes to the receptor–ligand interaction of the characterised compounds. Thus, increasing size and lipophilicity of the 4-substituent increase the affinity of the compound suggesting that the receptor contains a relatively large lipophilic cavity.

4-PIOL and thio-4-PIOL have previously been characterised as low efficacy partial agonists at cerebral and hippocampal neurons (Frølund et al., 1995; Kristiansen et al., 1991). Recently, it has been shown that thio-4-PIOL acts as a weak partial agonist on recombinant $\alpha_2\beta_3\gamma_2$ and $\alpha_5\beta_3\gamma_2$ $GABA_A$ receptors expressed in *Xenopus* oocytes (Maksay et al., 2000). However, on $\alpha_1\beta_2\gamma_{2S}$ receptors expressed in *Xenopus* oocytes, only the antagonist effects of thio-4-PIOL have been reported (Ebert et al., 1997). In the present study, having optimised the signal to noise ratio,

we have been able to detect agonist responses down to approximately 1% of the maximum response to GABA. In our *Xenopus* oocyte expression system, both 4-PIOL and thio-4-PIOL showed weak agonist effects on $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors, the former being the lowest with only 2.2% of the maximum GABA currents. As illustrated in Fig. 2, increasing size of the substituent in the 4-position of the 3-isoxazolol ring of 4-PIOL gradually reduced the agonist activity, indicating that large substituents increase the affinity of the compounds, but at the same time, hamper the conversion of binding into a functional response. Electron microscopic analysis of the nicotinic acetylcholine receptor has indicated that the mechanism of channel opening involves rotations of subunits implicated in receptor binding (Unwin, 1995). Since the nicotinic acetylcholine receptors and the $GABA_A$ receptors belong to the same family of ligand-gated ion channels, it is likely that the $GABA_A$ receptors might employ similar rotations of subunits during gating. Since the GABA binding site is believed to be located on the interface between α and β subunits (Smith and Olsen, 1995; Amin and Weiss, 1993; Sigel et al., 1992), it has been speculated that 4-PIOL analogues with large substituents may obstruct these rotations therefore appearing as competitive antagonists (Frølund et al., 2002).

The very weak agonist activities of 4-PIOL, **6a**, and **6b** were confirmed to be mediated via the $GABA_A$ receptor agonist site since these responses were not only potentiated by the $GABA_A$ receptor benzodiazepine, lorazepam, but also inhibited by the competitive $GABA_A$ receptor antagonist SR95531. The remaining 4-PIOL analogues, **6c**–**q**, all failed the lorazepam potentiation test for agonist character and were therefore defined as $GABA_A$ receptor antagonists. This antagonist behaviour was of competitive nature, since **6c**–**q**, all shifted GABA concentration–response curves rightward without suppressing the maximum responses (data not shown).

The experimentally determined efficacies of GABA, THIP, P4S, and thio-4-PIOL showed in linear regression a significant correlation to the degree by which lorazepam could potentiate responses at EC_{95}. These observations indicate that the maximum response of a receptor population is saturable. We speculate that GABA is a true full agonist on $\alpha_1\beta_2\gamma_{2S}$ receptors since GABA I_{max} currents could not be exceeded by the lorazepam modulation. However, it has recently been shown that the maximum responses of THIP can surpass that of GABA at $\alpha_4\beta_3\delta$-containing receptors, indicating that GABA may not be a true full agonist at all $GABA_A$ receptor combinations (Adkins et al., 2001). However, we suggest that GABA, being the endogenous neurotransmitter, should always be designated a full agonist on all $GABA_A$ receptor combinations, whereas agonist with higher efficacies, like THIP on α_4-containing receptors, should be designated super agonists. Our data also indicates that the potency of an agonist is independent of the degree by which the agonist response can be modulated (Fig. 5, insert). This is in agreement with previous studies where we similarly failed to correlate potency with agonist efficacy (Ebert et al., 1997).

The study by Lavoie and Twyman (1996) indicates that diazepam enhances submaximal GABA receptor currents by accelerating GABA association to its receptor and can therefore explain the leftward shift of the agonist concentration–response curve of GABA. However, this does not explain the elevated maximum current and lack of consistent potency shift that we have observed for partial agonists in the presence of lorazepam. These aspects of apparent agonist efficacy and potency of especially partial agonists have to be investigated further and emphasises the need for systematic studies of these factors.

Based on the analysis of correlation between the lipophilic character of the substituent of the 4-PIOL analogues and of the antagonist affinity, we propose a division of the compounds in three different groups with different binding characteristics to the 4-PIOL binding site. We hypothesize that the first and largest group of compounds giving rise to a highly significant correlation between affinity and log *P* are ligands with a common binding mechanism of entering the hydrophobic binding site from an aqueous environment. The correlation between increasing affinity and increasing lipophilicity of the compounds is therefore interpreted as a transfer of the molecules from the aqueous phase to the receptor phase without significant specific interactions with amino acid residues in the binding site. The fact that the line correlating affinity with lipophilicity of these compounds has a slope of 0.501 indicates only partial desolvation of the compounds, or in other words, that the 4-substituents of these ligands when located in the lipophilic binding site are still partly exposed to water (Leo and Hansch, 1999). This indication of aqueous exposure to these large compounds when bound in the binding site supports the previously stated idea of a significant agonist binding pocket extending into the aqueous phase outside the $GABA_A$ receptor (Frølund et al., 2002). This might not be too surprising considering the location of the agonist binding site on the interface between the α and β subunits, which might contain extensive aqueous cavities.

We propose that the second group of compounds, **6g**, **6i**, and **6n**, that all have lower affinity for the binding site than expected according to their log *P* values are compounds with structures that impede the normal binding mechanism. For example, the low affinity of the anthracene ring structure of **6i** has been interpreted in terms of an electrostatic conflict with the receptor binding site proposed to be interacting with the ammonium ion of the ligand (Frølund et al., 2002). The third and final group, **6k** and **6p**, are compounds that have structures favorable for additional binding interactions in the lipophilic binding pocket, which is reflected in affinities higher than expected according to their log *P* values. Which components of the ligands that might be involved in such additional binding interactions will have to be investigated further. This group of high affinity compounds may therefore serve as future lead

compounds in the search for more potent and more selective $GABA_A$ receptor ligands.

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