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Characterization of [^{3}H]-CGP54626A binding to heterodimeric $GABA_B$ receptors stably expressed in mammalian cells

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1 Functional human $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ receptors have been stably expressed in mammalian CHO K1 cells.

2 Detailed characterization of $GABA_B$ ligand binding at each of the receptors has been compared using [^{3}H]-CGP54626A. In cell membranes fractions, [^{3}H]-CGP54626A bound to a single site with a K_D of 1.51 ± 1.12 nM, B_{max} of 2.02 ± 0.17 pmoles mg protein^{-1} and 0.86 ± 0.20 nM, B_{max} of 5.19 ± 0.57 pmoles mg protein^{-1} for $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ respectively.

3 In competition binding assays the rank order was identical for both $GABA_B$ receptors. For known $GABA_B$ agonists the rank order was CGP27492 > SKF97541 = CGP46381 > GABA > Baclofen and for $GABA_B$ antagonists the rank order was CGP54262A > CGP55845 > CGP52432 > SCH 50911 > CGP51176 > CGP36742 = CGP35348 $\geqslant$ 2-OH Saclofen $\geqslant$ ABPA.

4 The allosteric effect of calcium cations was also investigated. The effect of removal of $CaCl_2$ from the binding assay conditions was ligand dependent to either cause a decrease in ligand affinity or to have no significant effect. However, these effects were similar for both $GABA_B$ receptors.

5 A whole cell, scintillation proximity binding assay was used to determine agonist affinity at exclusively heterodimeric $GABA_B$ receptors. In competition assays, the rank order was the same for both $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ and consistent with that seen with cell membrane fractions.

6 These data suggest that, in terms of ligand binding, the currently identified isoforms of the $GABA_B$ receptor are pharmacologically indistinguishable.

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Abbreviations: CaSR, Calcium sensing receptor; CHO, Chinese hamster ovary; GABA, γ-amino butyric acid; GPCR, G protein coupled receptor; mGluR, metabotropic glutamate receptor; SPA, scintillation proximity assay

Introduction

The metabotropic $GABA_B$ receptor, which mediates slow, inhibitory actions of the neurotransmitter γ-aminobutyric acid (GABA), was described pharmacologically almost 20 years ago allowing it to be distinguished from the ionotropic $GABA_A$ binding site (Hill & Bowery, 1981). The regulation of synaptic transmission *via* the $GABA_B$ receptor has since been shown to be mediated *via* the $G_{i/o}$ family of G proteins (Campbell *et al*., 1993; Menon-Johansson & Dolphin, 1993). However, the molecular nature of the $GABA_B$ receptor has only been revealed very recently. Initially, expression cloning identified a receptor, termed $GABA_{B(1)}$, with homology to other family C G protein coupled receptors (GPCRs), which displayed many of the antagonist binding characteristics of the endogenous rat brain receptor (Kaupmann *et al*., 1997), and which was expressed in two N terminal isoforms. However, this receptor failed to bind agonists with high affinity and failed to couple efficiently to second messenger pathways when expressed recombinantly. It has since been demonstrated that coexpression of a second homologous 7 transmembrane (7TM) protein, $GABA_{B(2)}$, is required to create a fully functional, high affinity $GABA_B$ receptor (for review see Marshall *et al*., 1999).

The identification of the two main isoforms of $GABA_{B(1a)}$, $GABA_{B(1b)}$ and its partner $GABA_{B(2)}$ (IUPHAR Nomenclature) has allowed the $GABA_B$ receptor to be studied in heterologous expression systems and has permitted further pharmacological characterization. For example, it has recently been demonstrated that the binding affinity of antagonist ligands to the large N terminal extracellular domain of $GABA_{B(1)}$ (Galvez *et al*., 1999; Malitschek *et al*., 1999) is relatively unaffected by coexpression of $GABA_{B(2)}$ (Kaupmann, *et al*., 1998; Galvez, *et al*., 2000). In contrast, agonist affinity for $GABA_{B(1)}$ is 10–100 fold lower when expressed alone compared with natively expressed heterodimeric receptors (Kaupmann *et al*., 1997). There have been a number of reports demonstrating that the $GABA_{B(2)}$ subunit does not respond to GABA agonists and fails to bind antagonist ligands (Jones *et al*., 1998; White *et al*., 1998; Kaupmann *et al*., 1998), however it is worth noting that Martin *et al*. (1999) showed that Chinese hamster ovary (CHO) cells expressing $GABA_{B(2)}$ alone could be activated weakly by GABA. Furthermore, it appears that generating a

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heterodimeric receptor, with 1 : 1 stoichiometric expression of each of the subunits, is required to reconstitute a recombinant $GABA_B$ receptor with similar agonist affinity relative to its wild-type counterpart in rat brain membrane preparations (White *et al*., 1998).

In addition to the structural homology to other family C GPCRs, it has been demonstrated that the $GABA_B$ receptor, like the Calcium sensing receptor (CaSR) and metabotropic glutamate receptor 1 (mGluR1) is also sensitive to Ca^{2+} ions. Both Wise *et al*. (1999) and Galvez *et al*. (2000) demonstrated that the receptor was allosterically modified by calcium at physiologically relevant concentrations of this cation which lead to an increase of agonist potency. Using site directed mutagenesis Galvez *et al*. (2000) identified a serine amino acid residue, in the N terminus of $GABA_{B(1)}$ that is critical for the calcium sensitivity of the $GABA_B$ receptor and its allosteric effect on ligand binding.

The $GABA_B$ receptor complex is widely distributed throughout the central nervous system (CNS) and mRNA for $GABA_{B(1)}$ has also been identified in several peripheral tissues (Isomoto *et al*., 1998). However, expression of $GABA_{B(2)}$ appears to be restricted to the CNS where its distribution almost entirely parallels that of $GABA_{B(1)}$ (Clark *et al*., 2000; Jones *et al*., 1998). A distribution pattern for each of the $GABA_{B(1)}$ receptor isoforms is now emerging and *in situ* hybridization studies have suggested associations of $GABA_{B(1a)}$ and $GABA_{B(1b)}$ isoforms with pre- and post-synaptic elements, respectively (Billinton *et al*., 1999).

Intriguingly, some groups have suggested subtle pharmacological differences for pre- and post-synaptic $GABA_B$ receptors with both human and rodent preparations (Bonanno *et al*., 1997; Yu *et al*., 1999) and Cunningham & Enna (1996) also proposed that two receptor subtypes were capable of regulating cyclic AMP production in rat brain. In contrast, Seabrook *et al*. (1990) previously concluded that pre- and post-synaptic $GABA_B$ receptors were indistinguishable in rat brain slices. These differences may be attributed to the existence of $GABA_B$ heterodimers composed of either $GABA_{B(1a/2)}$ or $GABA_{B(1b/2)}$ and such phenomenon can now be addressed at the molecular level following heterologous expression of these proteins.

In view of the effects of heterodimerization on ligand binding, we decided that cell lines stably expressing particular $GABA_B$ heterodimeric partners may provide a suitable system from which a thorough and comparable pharmacological characterization of $GABA_B$ receptors could be performed. Hence, we report successful stable expression of $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ in CHO K1 cells and detailed characterization of these receptors using the radio-labelled antagonist [^{3}H]-CGP54626A (Bittiger *et al*., 1992). In addition, the effect of calcium on ligand binding at each of the receptors has also been compared.

Methods

Cloning and expression of $GABA_B$ receptor cDNA

$GABA_{B(1a\ and\ 1b)}$ and $GABA_{B(2)}$ were cloned into pcDNA 3.1 (−) (Invitrogen) as previously described (White *et al*., 1998). For the construction of stable cell lines $GABA_{B(1a\ and\ 1b)}$ and $GABA_{B(2)}$ inserts were subcloned into the mammalian expression vectors pCIN3 (Neomycin selection) and pCIH6 (Hygromycin selection), respectively, (Rees *et al*., 1996).

For generation of stable cell lines, CHO K1 cells were maintained in DMEM F12 Ham containing 9% v v^{-1} heat inactivated foetal calf serum (FCS) and 2 mM glutamine. Cells were seeded in 6 well culture plates and grown to 50% confluency (18–24 h) prior to transfection with vectors containing the relevant cDNA inserts. For transfection, a total of 3 μg DNA/well was mixed with 10 μl of Lipofectamine reagent in 0.2 ml OptiMEM (both Life Technologies Inc.) followed by incubation at room temperature for 30 min prior to the addition of 1.6 ml OptiMEM. Cells were exposed to the transfection mixture for 5 h before 2 ml of 20% v v^{-1} FCS in DMEM F12 Ham media was added. Cells were transfected with 1 μg each of pCIN3 $GABA_{B(1a\ or\ 1b)}$, pCIH6 $GABA_{B(2)}$ and Gal 4 Elk1 Luciferase expression vector (puromycin selection). The latter provides a reporter system for the MAPK pathway but is not employed in the studies reported here. Forty-eight hours post transfection the media was replaced and supplemented with 500 μg ml^{-1} neomycin, 400 μg ml^{-1} Hygromycin and 2.5 μg ml^{-1} puromycin for selection of antibiotic resistant cells. Clonal cell lines were isolated by dilution and tested for functional $GABA_B$ receptors. A cyclic AMP accumulation assay was employed to measure GABA mediated inhibition of a 10 μM forskolin evoked cyclic AMP response. Clones in which 1 mM GABA produced a complete inhibition of the forskolin response were selected for further analysis. Stable expression of $GABA_{B(1a\ and\ 1b)}$ and $GABA_{B(2)}$ in these clones was also confirmed using Western blotting. Expression was visualized with pan-specific $GABA_{B(1)}$ or specific $GABA_{B(2)}$ receptor antibodies following immunoblotting of P2 membrane fractions (White *et al*., 1998).

P2 membrane preparation

Plasma membranes containing P2 particulate fractions were prepared from freshly cultured cells. All procedures were carried out at 4°C. Cell pellets were resuspended in 10 mM Tris-HCl and 0.1 mM EDTA, pH 7.4 (buffer A) followed by homogenization for 20 s with an Ultra Turrax and passed five times through a 25-gauge needle. Cell lysates were centrifuged at $1000 \times g$ for 10 min in a microcentrifuge to pellet the nuclei and unbroken cells and P2 particulate fractions were recovered by microcentrifugation at $16{,}000 \times g$ for 30 min. P2 particulate fractions were resuspended in buffer A and stored at −80°C until required. Protein concentrations were determined using the bicinchoninic acid (BCA) procedure (Smith *et al*., 1985) using BSA as a standard.

[^{3}H]-CGP54626A filter binding assays

For competition experiments CHO-$GABA_B$ membranes were incubated with ~0.5 nM [^{3}H]-CGP5426A in 50 mM Tris HCl, 2 mM $CaCl_2$ pH 7.4 in the presence or absence of competitor compound for 30 min at room temperature. P2 membrane protein fractions (10 μg/well) were used for all competition studies in a total volume of 500 μl. Ten mM GABA was used to define non-specific binding. Assays were terminated by vacuum filtration (using a Brandel cell Harvester) over GF/B filters, pre-soaked in assay buffer and the filters were washed four times with 1 ml ice cold buffer. Bound radioactivity (d.p.m.) was counted by liquid scintillation spectrometry.

For saturation studies, specific binding of [^{3}H]-CGP54626A was determined over a range of radioligand concentrations (0.05–30 nM) in the absence or presence of 10 mM GABA using 10 μg protein/well. For Ca^{2+} free studies, $CaCl_2$ was replaced with 1 mM EGTA. 5′-Guanylyl-

imido-diphosphate (Gpp(NH)p) was included at 30 μM where indicated.

[^{3}H]-CGP54626A whole cell scintillation proximity binding assays

Scintillation proximity assays were carried out under identical buffer conditions to those used in filtration assays. Conditions were optimized with respect to bead and cell density prior to performing characterization studies. Freshly cultured CHO $GABA_B$ cells were harvested with phosphate buffered saline, 5 mM EDTA, pH 7.4 and washed twice in 50 mM Tris Buffer, 2.5 mM $CaCl_2$ pH 7.4. For competition studies, 10^5 cells/well were incubated in white, clear bottom, 96 well plates (Wallac) with 0.5 nM [^{3}H]-CGP54626A in the absence or presence of competitor compound together with 2 mg/well Wheatgerm Agglutinin SPA beads (Amersham) in a total of 200 μl. Binding was allowed to proceed at room temperature for 30 min on an orbital shaker. Plates were then spun at 1500 r.p.m. for 5 min and cell bound radioactivity (corrected counts per min, CCPM) was determined on a Wallac Trilux Microbeta Counter. Specific binding was determined using 10 mM GABA. For saturation studies, specific binding of [^{3}H]-CGP54626A was determined over a range of radioligand concentrations (0.05–30 nM) in the absence or presence of 10 mM GABA.

Data analysis

All experiments were performed a minimum of three times. All saturation studies were performed in duplicate. Single data points were obtained for competition binding with membranes and in duplicate for whole cells. Results are given as means ± s.e.mean.

[^{3}H]-CGP54626A saturation binding data was analysed by a computer based non-linear curve fitting program to obtain K_D and B_{max} values. Competition curves were analysed by use of the ALLFIT model (DeLean *et al*., 1977). IC_{50} values were derived from this analysis and converted to K_i values by use of the Cheng & Prusoff (1973) equation.

Materials

([S-(R*,R)]- [3-[[1- (3,4- Dichlorophenyl)ethyl]amino] -2-hydroxypropyl]([3,4-^{3}H]-cyclohexylmethyl)phosphinic acid ([^{3}H]-CGP54626A) (40 Ci/mmol); GABA, (RS)Baclofen, (+)-2S-5,5-Dimethyl-20-morpholineacetic acid (SCH 50911); (3-Aminopropyl)(methyl)phosphinic acid) (SKF 97541); (RS)-3-Amino-2-(4-chlorophenyl)-2-hydroxypropylsulphonic acid) (2-Hydroxysaclofen); 4-Aminobutylphosphonic acid (ABPA) were all purchased from Tocris Cookson (Bristol, U.K.).

3-Aminopropylphosphinic acid (CGP27492); ((3-Aminopropyl)(diethoxymethyl)phosphinic acid) (CGP35348); [3-[[(3,4 -Dichlorophenyl)methyl]amino]propyl](diethoxymethyl)-phosphinic acid (CGP 52432); [(2S)-3-[[(1S)-1-(3,4-Dichlorophenyl)ethyl]amino]-2-hydroxypropyl](phenylmethyl)phosphinic acid (CGP55845); [S-(R*,R)]-[3-[[1-(3,4-Dichlorophenyl)ethyl]amino]-2-hydroxypropyl](cyclohexylmethyl)phosphinic acid (CGP54626A); ((3-Amino-propyl)(cyclohexylmethyl)-phosphinic acid) (CGP46381); [(2R)-3-Amino-2-hydroxypropyl](cyclohexylmethyl)phosphinic acid (CGP51176) and (3-aminopropyl)butylphosphinic acid (CGP36742) are Novartis compounds but were synthesized in house. Other reagents listed were from the stated suppliers unless from Sigma–Aldrich or Fisher Scientific.

Results

Stable expression of $GABA_B$ receptors in CHO cells

Stable expression of functional $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ receptors was achieved with a CHO K1 host cell line. Employing a cyclic AMP assay, GABA produced an inhibition of forskolin evoked cyclic AMP accumulation in > 75% of the clones tested (data not shown). Six clones each representing $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ expression, in which complete inhibition of forskolin was measured, were selected for immunoblotting of $GABA_B$ receptor proteins. $GABA_{B(2)}$ protein was detected in all of the P2 membrane preparations, appearing as a distinct single band of the expected size with relative molecular mass ~120 K (M_r 120 K). The $GABA_{B(1)}$ subunits were also visualized and appeared as bands of the expected sizes of M_r ~120 K and ~100 K for $GABAB_{(1a)}$ and GABA $B_{(1b)}$, respectively (Figure 1). Unlike the $GABA_{B(2)}$ both of the $GABA_{B(1)}$ proteins appeared to run as doublets. The immunoblotting also revealed small differences in the relative amount of $GABA_B$ protein expression between the clones tested. However, a direct comparison of expression of $GABA_{B(1)}$ relative to $GABA_{B(2)}$ cannot be made due to the different antigenic specificities of antibodies used. In order to provide membrane preparations for binding studies, those clones expressing the highest amounts of $GABA_B$ protein were selected for detailed characterization. Hence, all subsequent data was obtained using $GABA_{B(1a,2)}$ #33 and $GABA_{B(1b,2)}$ #74.

These two clones have also been employed for the functional characterization of $GABA_B$ receptors (Martin *et al*., 2000).

Saturation binding

Saturation analysis of [^{3}H]-CGP54626A binding to both $GABA_B$ receptors was saturable and non linear curve fitting of this data indicated that this binding was to a single site. Affinity estimates, slope and B_{max} values for [^{3}H]-CGP54626A binding are summarized in Table 1. Total binding did not exceed 10% of the amount of [^{3}H]-CGP54626A added and specific binding was > 80% at the K_D value (Figure 2).

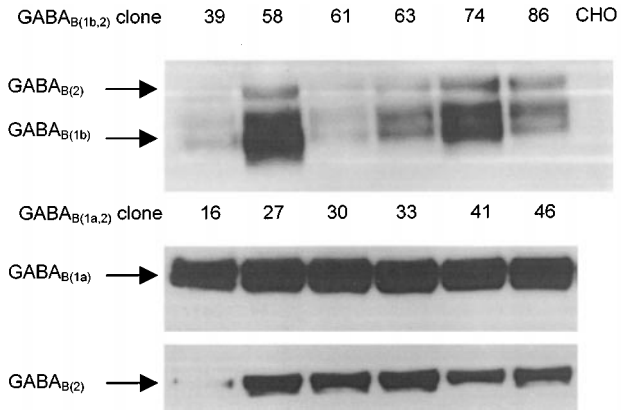

Figure 1 Visualization of $GABA_B$ receptors stably expressed in CHO cell clones by immunoblotting with pan-specific $GABA_{B(1)}$ and specific $GABA_{B(2)}$ antibodies to C terminal epitopes. Western blots reveal relative expression levels of selected clones compared with untransfected CHO K1 cells.

Table 1 Summary of saturation studies of [^{3}H]-CGP54626A binding to $GABAB_{(1a,2)}$ and $GABA_{B(1b,2)}$ receptors

	$GABA_{B(1a,2)}$			$GABA_{B(1b,2)}$		
	K_d (nM)	*Slope* (nH)	B_{max} (pmoles mg protein^{-1})	K_d (nM)	*Slope* (nH)	B_{max} (pmoles mg protein^{-1})
Normal (2.5 mM $CaCl_2$)	1.51 (1.12)	1.14 (0.12)	2.02 (0.17)	0.86 (0.20)	1.16 (0.13)	5.19 (0.57)
Normal (+30 μM Gpp(NH)p)	1.69 (1.44)	0.92 (0.22)	2.86 (0.66)	1.23 (0.56)	0.89 (0.13)	5.62 (0.73)
$-CaCl_2$ (+1 mM EGTA)	7.90 (3.00)	1.24 (0.19)	1.58 (0.55)	4.58 (1.33)*	1.29 (0.17)	1.93 (0.17)*

Data are mean ± s.e.mean from $n=3$ experiments. nH is the Hill Coeffficient. *Indicates $P<0.05$ compared with normal conditions.

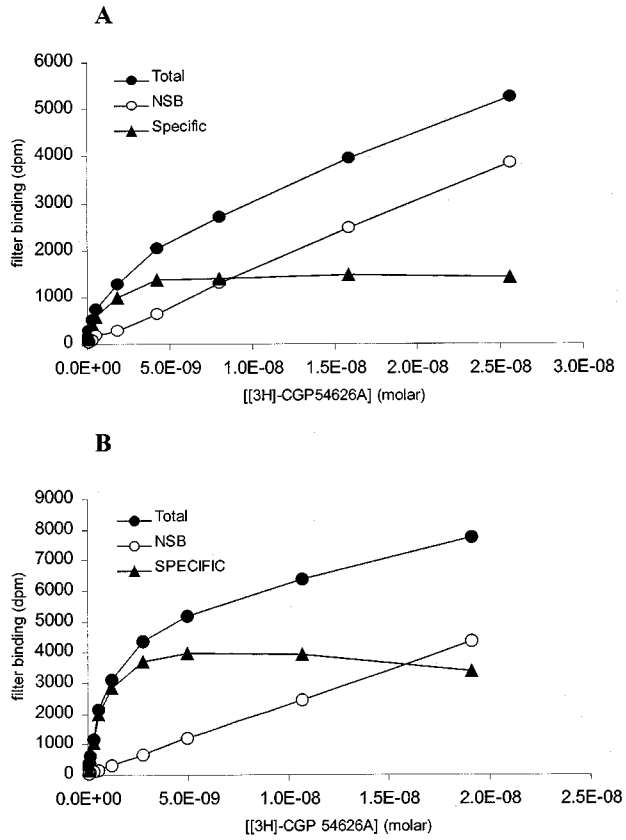

Figure 2 Representative saturation curve data for [3H]-CGP54626A binding to $GABA_{B(1a,2)}$ (A) and $GABA_{B(1b,2)}$ (B). Each point represents mean of duplicate determinations from a single experiment.

Effect of Gpp(NH)p and Ca^{2+} removal on saturation binding

Saturation binding was repeated in the absence of calcium cations and in the presence of Gpp(NH)p. Their effects on the binding of the antagonist ligand to $GABA_B$ receptors are summarized in Table 1. For both $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ the removal of calcium from the assay caused an increase in K_D value of [^{3}H]-CGP54626A without affecting slope values but produced a reduction in calculated B_{max} values. The inclusion of 1 mM EGTA, to ensure complete chelation of Ca^{2+} ions from the assay, caused a decrease in both total and non-specific binding in all assays when included and may be attributable to non receptor mediated effects. The addition of 30 μM Gpp(NH)p had no significant effect on the estimated affinity of [^{3}H]-CGP54626A for both $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$. However, for both receptors B_{max} values were slightly increased relative to control conditions.

Competition studies

A panel of $GABA_B$ ligands was used to displace [^{3}H]-CGP54626A binding to both $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ receptors. All of the compounds tested produced complete inhibition of specific binding with a range of calculated affinity estimates that are summarized in Table 2. Inhibition curves, under normal conditions with $CaCl_2$ included in the assay buffer, are shown in Figures 3 and 4 for displacement of binding to $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ receptors, respectively. The rank order for ligand displacement was identical for binding at both $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$. For $GABA_B$ agonists tested the rank order was CGP27492 > SKF97541 = CGP46381 > GABA > Baclofen. For $GABA_B$ antagonists the rank order was CGP54262A > CGP 55845 > CGP52432 > SCH50911 > CGP51176 > CGP36742 = CGP35348 ≥ 2-OH Saclofen ≥ ABPA.

In the absence of $CaCl_2$, a complete displacement of [^{3}H]-CGP54626A binding was observed with all of the $GABA_B$ ligands. However, the effect of removal of $CaCl_2$ from such assays was ligand dependent and resulted in either no marked effect or a decrease in ligand affinity. These effects are also summarized in Table 2. We also derived a relative molar ratio for ligand affinity from the K_i values obtained in the absence and presence of calcium and indicate that similar effects were observed for both of the $GABA_B$ receptors. GABA, SKF 97541, CGP27492, ABPA and CGP52432 were most sensitive to the removal of calcium which lead to at least a 10 fold decrease in affinity for both receptors. In contrast, the affinity of 2-OH Saclofen, CGP51176 and CGP35348 was only decreased by a maximum of 3 fold at both $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$.

[^{3}H]-CGP54626A whole cell scintillation proximity binding assays

A whole cell binding assay was established to determine ligand binding at $GABA_B$ receptors expressed at the cell surface. This characterization was performed under normal $GABA_B$ binding conditions with calcium included in the buffer. Saturation studies were performed on both cell lines and analysis of [^{3}H]-CGP54626A binding to both $GABA_B$ receptors was saturable and non-linear curve fitting of this data indicated that this binding was to a single site. Affinity estimates and slope values for [^{3}H]-CGP54626A binding are summarized in Table 3. Typical saturation data from whole cell binding is shown in Figure 5. As scintillation proximity assays do not permit accurate determination of DPM values, B_{max} values cannot be quoted. However, specific whole cell binding was higher with the $GABA_{B(1b,2)}$ cells relative to $GABA_{B(1a,2)}$ cells, which was consistent with the expression levels calculated in the P2 membrane filter binding assays.

In competition studies all of the $GABA_B$ agonists tested fully displaced the [^{3}H]-CGP54626A whole cell binding. Displacement of binding to both $GABA_{B(1a,2)}$ and

Table 2 Summary of affinity values for the displacement of [^{3}H]-CGP54626A binding to $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ receptors

	$GABA_{B(1a,2)}$ pK_i (±s.e.mean)					$GABA_{B(1b,2)}$ pK_i (±s.e.mean)				
	Ca^{2+}	*nH*	*EGTA*	*nH*	K_i *ratio*	Ca^{2+}	*nH*	*EGTA*	*nH*	K_i *ratio*
GABA	5.56 (0.10)	0.7	3.41 (0.13)	1.0	139.67	5.11 (0.04)	0.7	3.19 (0.04)	0.9	84.07
Baclofen	5.13 (0.05)	0.8	3.92 (0.11)	1.3	15.93	4.57 (0.03)	0.6	3.53 (0.03)	1.0	10.96
SKF 97541	6.08 (0.15)	0.6	3.56 (0.26)	0.8	316.84	5.43 (0.02)	0.7	3.69 (0.06)	0.8	53.69
CGP46381	5.93 (0.01)	1.0	5.20 (0.02)	1.2	5.30	5.67 (0.02)	0.9	5.39 (0.14)*	1.0	1.93
CGP27492	6.21 (0.11)	0.8	3.84 (0.20)	1.4	230.31	6.08 (0.14)	0.8	3.97 (0.04)	0.9	129.61
SCH 50911	6.57 (0.10)	1.0	5.09 (0.09)	1.1	30.38	6.42 (0.07)	1.0	5.04 (0.09)	0.8	24.08
2OH-Saclofen	4.66 (0.03)	1.1	4.80 (0.22)*	0.8	0.71	4.50 (0.04)	0.7	4.08 (0.44)*	0.8	2.32
ABPA	4.59 (0.13)	1.1	2.91 (0.21)	1.1	46.65	4.39 (0.09)	0.8	2.40 (0.03)	0.7	96.54
CGP51176	5.97 (0.06)	1.0	5.54 (0.15)*	1.4	2.65	5.89 (0.06)	1.0	5.65 (0.02)$^{\#}$	0.8	1.72
CGP54626	8.86 (0.10)	1.1	7.67 (0.10)	1.3	15.58	8.85 (0.02)	1.0	7.83 (0.06)	0.8	10.41
CGP52432	7.51 (0.10)	1.0	5.79 (0.11)	1.1	51.82	7.63 (0.14)	0.5	5.77 (0.04)	0.9	72.56
CGP55845	8.53 (0.02)	1.0	7.70 (0.09)	0.8	6.87	8.57 (0.03)	0.8	7.36 (0.11)	1.1	16.18
CGP36742	4.84 (0.01)	1.1	4.50 (0.07)	1.2	2.18	4.81 (0.07)	1.1	4.14 (0.18)$^{\#}$	1.1	4.68
CGP35348	4.92 (0.02)	1.1	4.90 (0.14)*	1.1	1.02	4.97 (0.06)	1.0	4.68 (0.03)$^{\#}$	1.2	1.94

Data are expressed as pK_i values (the negative logarithm of molar concentration of competing ligand to displace 50% of 0.5 nM [^{3}H]-CGP54626A binding), nH is the Hill Coefficient. Data are mean of $n=3$ experiments. K_i ratios indicate relative molar ratio of each competing ligand derived from affinity values in absence and presence of $CaCl_2$. Student's *t*-test was used to compare pK_i values in absence and presence of $CaCl_2$. Except where indicated $P<0.01$. *Denotes $P>0.05$, $^{\#}$denotes $P>0.01$.

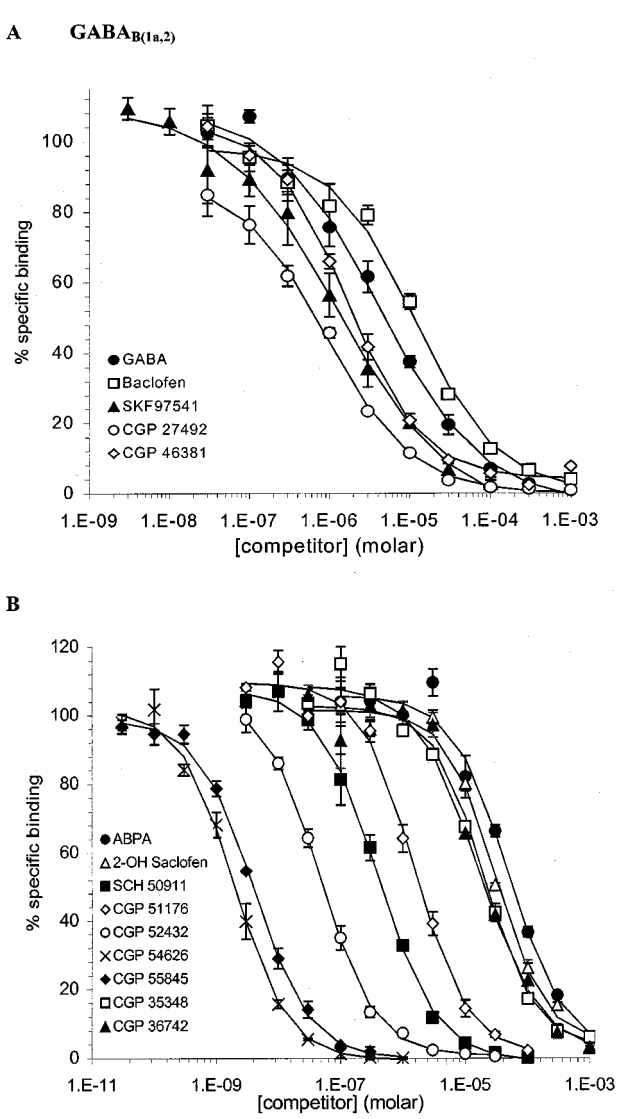

Figure 3 Competition studies of [^{3}H]-CGP54626A binding to $GABA_{B(1a,2)}$ receptors. Displacement of [^{3}H]-CGP546262A binding by $GABA_B$ agonists (A) and antagonists (B) is represented as per cent specific binding. Each curve represents mean±s.e.mean from $n=3$ experiments.

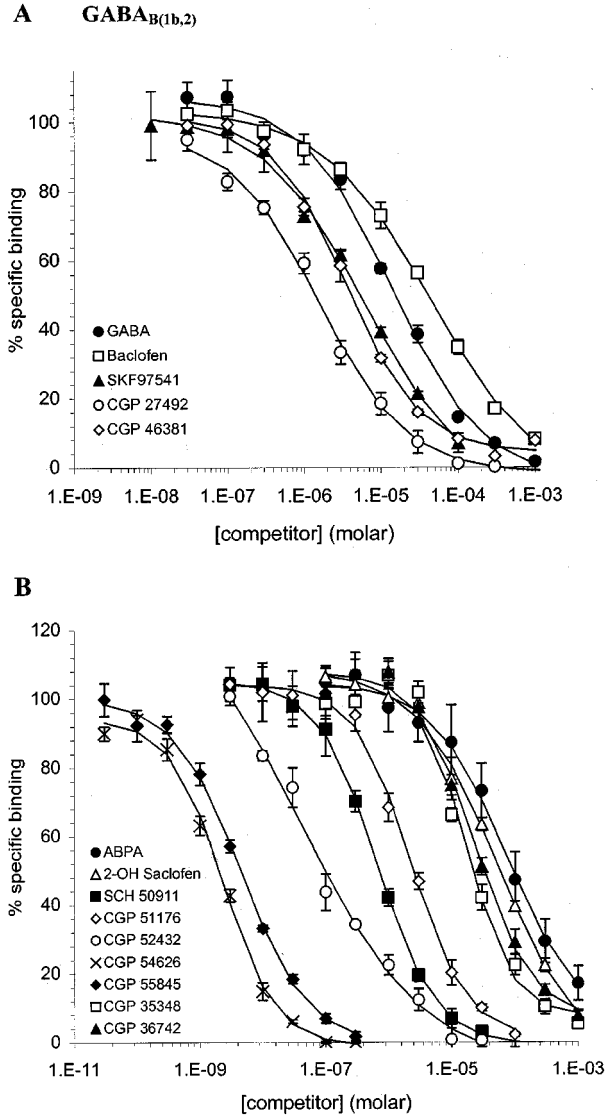

Figure 4 Competition studies of [^{3}H]-CGP54626A binding to $GABA_{B(1b,2)}$ receptors. Displacement of [^{3}H]-CGP546262A binding by $GABA_B$ agonists (A) and antagonists (B) is represented as per cent specific binding. Each curve represents mean±s.e.mean from $n=3$ experiments.

Table 3 Summary of whole cell binding saturation studies of [^{3}H]-CGP54626A binding to $GABAB_{(1a,2)}$ and $GABA_{B(1b,2)}$ receptors

	K_d (nM) (±s.e.mean)	*Slope* (nH) (±s.e.mean)
$GABA_{B(1a,2)}$	2.12 (0.35)	1.24 (0.04)
$GABA_{B(1b,2)}$	4.72 (0.57)	1.23 (0.09)

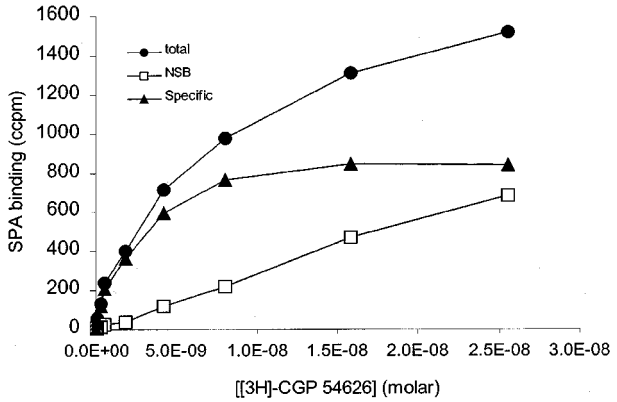

Figure 5 Representative saturation curve data for [^{3}H]-CGP54626A binding to $GABA_{B(1a,2)}$ Each point represents the mean of duplicate determinations from a single experiment.

$GABA_{B(1b,2)}$ are summarized in Figure 6. The rank order for displacement of whole cell binding was identical for both receptors: CGP27492 > SKF97541 ⩾ CGP46381 ⩾ GABA > Baclofen which was in good agreement with that obtained from filtration binding to P2 membrane fractions.

Discussion

There have been several reports describing the pharmacology of the $GABA_B$ receptor since it was first cloned (for review see Bowery & Enna, 2000). These studies have relied primarily on the use of transiently expressed $GABA_B$ subunits individually or as pairs to reconstitute a heterodimeric receptor. This approach has permitted the functional characterization of receptor behaviour and provided further insight into the molecular nature of the receptor such as allosteric modulation of the receptor by calcium cations (Wise *et al*., 1999; Galvez *et al*., 2000). Such experiments have also highlighted the importance of heterodimerization of $GABA_B$ receptor subunits with respect to ligand affinity at the receptor.

In this paper we have described the assembly and characterization of $GABA_B$ receptor cell lines stably expressing particular pairs of proteins that represent the two major isoforms of the $GABA_B$ receptor complex that have been identified in distribution studies, that is $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$.

Clones to be used for detailed characterization were initially selected by their ability to respond to $GABA_B$ agonists in a functional assay, such as the inhibition of cyclic AMP accumulation. Expression of $GABA_{B(1)}$ and $GABA_{B(2)}$ proteins in these functional clones was then confirmed by immunoblotting with specific protein antibodies (Figure 1). Whilst this information alone cannot determine the stoichiometry of subunit expression in these cell lines, we have extended our ligand binding studies to include a whole cell (SPA) binding assay to assess agonist affinity at the cell surface and therefore at exclusively heterodimeric receptors. As agonist affinity has been shown to be lower at $GABA_{B(1)}$ expressed alone, if the pharmacology of agonists in the whole cell binding assay is comparable with that using membranes preparations containing total expressed protein, it may suggest that the clones are expressing amounts of $GABA_{B(2)}$ proteins that are in excess of $GABA_{B(1)}$. Therefore, any characterization of these cell lines may be expected to be representative of the native receptor pharmacology.

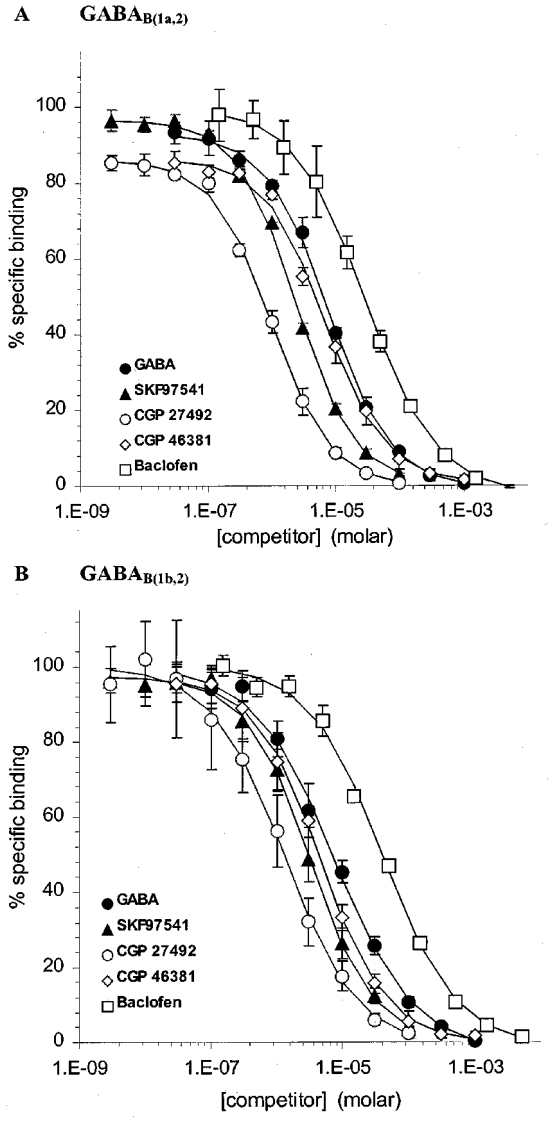

Figure 6 Competition studies of [^{3}H]-CGP54626A binding to $GABA_B$ receptors. Displacement of [^{3}H]-CGP546262A whole cell binding at $GABA_{B(1a,2)}$ (A) and $GABA_{B(1b,2)}$ (B) is represented as per cent specific binding. Each curve represents mean ± s.e.mean from $n = 3$ experiments.

We have compared the pharmacology of $GABA_B$ receptors using the tritiated, high affinity antagonist ligand CGP54626A. In saturation studies with membrane preparations, under standard buffer conditions, that included calcium cations, we obtained K_D values that were consistent with previously published data using rat brain membranes. These were 1.51 nM and 0.86 nM for $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ respectively, compared with 1.48 nM from rat brain membranes that contain a heterologous population of $GABA_B$ receptors (Bittiger *et al*., 1992). Similar values were also

obtained in saturation studies using the whole cell binding assay (Tables 1 and 3).

To further investigate the G protein coupling of these receptors we next examined the effects of the guanylylnucleotide analogue Gpp(NH)p. In paired, saturation studies, the K_D was unaffected by the presence of Gpp(NH)p whilst we observed a trend towards an increase in the calculated B_{max} value. However, statistical analysis indicated that this effect was not significant due to experimental variation (Table 1). For most G protein coupled receptors the addition of such analogues results in a shift in the equilibrium from high to low affinity states. Where certain ligands can discriminate between high and low affinity receptor states this would alter the number of sites labelled. For example, Marshall *et al.* (1997) demonstrated that an agonist ligand for prostaglandin EP_4 receptors only labelled high affinity state receptors and a similar observation was obtained by Clark & Hill (1995) for [^{3}H]-N$^\alpha$-methyl-histamine binding to histamine H_3 receptors. Our studies may indicate that this ligand discriminates for low affinity state receptors only. Although further studies are required to investigate this suggestion in greater detail, our observations with Gpp(NH)p combined with the functional data to select clones, indicate that the stably expressed receptors are G protein coupled.

Several groups have suggested the existence of $GABA_B$ receptor subtypes on the basis of subtle pharmacological differences in functional studies using *in vitro* and *in vivo* preparations. In contrast, binding studies have not revealed significant pharmacological differences of antagonist affinity between $GABA_{B(1a)}$ and $GABA_{B(1b)}$ receptors expressed alone (Kaupmann *et al.*, 1997) compared with native receptors or in combination with $GABA_{B(2)}$ (Kaupmann *et al.*, 1998). Similarly, functional assays using transiently transfected mammalian cells to express heterodimeric receptors, did not suggest pharmacological differences using a limited number of ligands (Brauner-Osborne & Krogskard-Larsen, 1999). Clearly, it is not possible to confirm the existence of $GABA_B$ receptor subtypes with these current data.

In these competition studies, under normal conditions, the rank order for displacement of [^{3}H]-CGP546262A binding by agonists and antagonists was identical for both $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ receptors (Figures 3 and 4). The rank order for $GABA_B$ agonists correlates well with the literature in which the phosphinic acids are shown to be the most potent $GABA_B$ agonists. The highest affinity agonists were CGP27492 (3-aminopropyl) phosphinic acid and its methyl analogue, SKF97541. CGP46381 ((3-Amino-propyl)(cyclohexylmethyl)phosphinic acid), which we have described as a partial agonist (unpublished observation), also produced complete displacement of binding. The rank order for $GABA_B$ antagonists is also in accordance with published data with the phosphinic analogues showing the highest affinity. Interestingly, the rank order generated with these heterodimeric receptors is in good agreement with rank orders obtained with binding studies at the $GABA_{B(1)}$ subunit expressed alone. This further supports the growing evidence in favour of $GABA_B$ ligands exclusively binding to the $GABA_{B(1)}$ subunit. Additionally, in all studies calculated Hill coefficients were close to unity suggestive of ligand binding to a single site. Clearly, the $GABA_{B(2)}$ contributes to formation of the heterodimer and ligand affinity but there is a lack of evidence to date in support of $GABA_{B(2)}$ binding ligands directly.

We have established a whole cell binding assay to further study the affinity of $GABA_B$ agonists at the $GABA_B$ receptor. This robust assay format uses a solid bead-based scintillation counting method for detecting receptor-ligand binding interactions. SPA beads only emit a light response when ligand is in close proximity to receptor and since they cannot penetrate the plasma membrane of intact cells, this technique permits examination of ligand binding to exclusively cell surface receptors. In the case of $GABA_B$, this permits the study of a pure population of heterodimeric receptors without any interference from non-dimerized $GABA_B$ receptor proteins that could exist in membrane preparations. This approach is particularly pertinent to the characterization of $GABA_B$ receptor agonists since their true affinity (or that which is comparable to native receptors) is only revealed when the receptor is heterodimeric with 1 : 1 stoichiometry of receptor subunit proteins (White *et al.*, 1998).

In saturation studies with whole cells, the affinity of [^{3}H]-CGP54626A was marginally lower, but still comparable to that observed with membrane fractions. Furthermore, in competition studies the rank order for agonist displacement also closely resembled that previously determined in the filter binding assay with membrane fractions. This rank order was once again identical for both $GABA_B$ receptor isoforms. As the rank order is the same, an interpretation of this is that these stable cell lines are expressing $GABA_{B(2)}$ proteins in excess of $GABA_{B(1)}$ hence the latter will be binding ligand in its heterodimeric form.

It was revealed early in the initial characterization of $GABA_B$ receptors that physiological concentrations of calcium are required to promote [^{3}H]-GABA binding (Hill and Bowery, 1981; Bowery *et al.*, 1983). More recent studies have revealed that this phenomenon may be attributed to the allosteric modulation of the $GABA_B$ receptor by calcium. Regulation of receptor function has been shown to modulate ligand potency and affinity (Wise *et al.*, 1999; Galvez *et al.*, 2000; Martin *et al.*, 2000).

We have also compared the effects of calcium cations on ligand binding to a range of agonists and antagonists at both of the $GABA_B$ receptor subtypes with competition binding assays. These data also indicate that the two, heterodimeric isoform pairs behave identically to one another in the absence of calcium cations. The rank order for ligands is altered from that determined in the presence of calcium but still remains equal for each of the receptor isoforms examined. We have calculated relative molar ratios derived from the K_i values obtained in the presence and absence of calcium. This helps to illustrate which ligands are affected by the allosteric regulation of the receptor and a good correlation is apparent between the values obtained at each of the receptor isoforms (Table 2).

The molecular mode of action of calcium cations on receptor-ligand binding is not yet fully understood. However, structural similarity exists between $GABA_B$ receptors and other members of the family C GPCRs such as the Ca sensing receptor and mGluR1, which are also influenced by calcium cations (Hammerland *et al.*, 1999; Kubo *et al.*, 1998). A comparison of these receptors at amino acid sequence level prompted subsequent site directed mutation studies and led to Galvez *et al.* (2000) elucidating key serine residues that are critical for the allosteric modulation of the $GABA_B$ receptor. These residues are thought to be in close proximity to other amino acids associated with $GABA_B$ ligand binding (Galvez *et al.*, 1999), hence, the allosteric modulation of the $GABA_B$ receptor by calcium is likely to exert significant effects on ligand affinity and potency. The data presented herein exemplify that numerous ligands are sensitive to this

allosterism, further supporting the probable overlap in the ligand and calcium binding domains of the receptor. It has previously been illustrated that baclofen is less sensitive to this allosterism and it has been suggested that its molecular structure may sterically hinder the Ca^{2+} allosterism. We have obtained similar observations in these studies and have also shown that other agonist and antagonist ligands, in particular 2-OH Saclofen and CGP35348, are less susceptible to the effects of calcium. In light of this data, the calcium sensitivity of potential therapeutic agents at the $GABA_B$ receptor should be an important consideration. We have also attempted to use the calcium sensitivity data in parallel with three-dimensional modelling of such ligand structures to predict which pharmacophores are most likely to be influenced by calcium. However, at present we still have insufficient data or enough chemical diversity to draw any conclusions.

In summary, using ligand binding studies we have performed an extensive characterization of the two main isoforms of the $GABA_B$ receptor. Our data strongly support other published observations suggesting that in terms of ligand binding, using currently available ligand tools, these heterodimeric proteins do not represent any pharmacological subtypes. However, the existence of receptor subtypes cannot be disregarded. Further characterization should give consideration to the distribution of particular $GABA_B$ receptor proteins and their relative expression levels as this could modify receptor-ligand interactions. The functionality of these and other, as yet unidentified $GABA_B$ receptor proteins, in respect to G protein coupling, their second messenger and effector systems could also be responsible for possible existence of novel receptor subtypes.

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