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The human $GABA_{B1b}$ and $GABA_{B2}$ heterodimeric recombinant receptor shows low sensitivity to phaclofen and saclofen

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1 The aim of this study was to characterize the pharmacological profile of the $GABA_{B1}/GABA_{B2}$ heterodimeric receptor expressed in Chinese hamster ovary (CHO) cells. We have compared receptor binding affinity and functional activity for a series of agonists and antagonists.

2 The chimeric G-protein, G_{qi5}, was used to couple receptor activation to increases in intracellular calcium for functional studies on the Fluorimetric Imaging Plate Reader (FLIPR), using a stable $GABA_{B1}/GABA_{B2}/G_{qi5}$ CHO cell line. [^{3}H]-CGP-54626 was used in radioligand binding studies in membranes prepared from the same cell line.

3 The pharmacological profile of the recombinant $GABA_{B1/B2}$ receptor was consistent with that of native $GABA_B$ receptors in that it was activated by GABA and baclofen and inhibited by CGP-54626A and SCH 50911.

4 Unlike native receptors, the $GABA_{B1}/GABA_{B2}/G_{qi5}$ response was not inhibited by high microMolar concentration of phaclofen, saclofen or CGP 35348.

5 This raises the possibility that the $GABA_{B1}/GABA_{B2}/G_{qi5}$ recombinant receptor may represent the previously described $GABA_B$ receptor subtype which is relatively resistant to inhibition by phaclofen.

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Abbreviations: CHO, Chinese hamster ovary; FLIPR, fluorimetric imaging plate reader; GABA, γ-aminobutyric acid

Introduction

γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the mammalian nervous system, where it activates ionotropic $GABA_{A/C}$ receptors and metabotropic $GABA_B$ receptors. The $GABA_B$ receptor was first identified pharmacologically (Bowery *et al*., 1980; Hill & Bowery, 1981) and was shown to modulate activity of calcium channels (Takahashi *et al*., 1998), inward rectifying K^+ channels and to cause inhibition of adenylyl cyclase (see Kerr & Ong, 1995). These effects were inhibited by pertussis toxin indicating the involvement of a Go/Gi G-protein (see Kerr & Ong, 1995). The $GABA_B$ receptor is selectively activated by baclofen (Hill & Bowery, 1981) and selectively antagonized by phaclofen (Kerr & Ong, 1995) and, more recently, by highly potent and selective compounds such as CGP 35348 and CGP 62349 (see Kaupmann *et al*., 1997).

The availability of potent and selective antagonists has allowed the cloning of two splice variants of the $GABA_B$ receptor $-GABA_{B1a}$ and $GABA_{B1b}$ (Kaupmann *et al*., 1997). Although these proteins contain a GABA recognition site, as determined in radioligand binding studies, attempts to characterize their functional activation were hampered by apparent poor coupling to both potassium channels and to adenylyl cyclase (Kaupmann *et al*., 1997). Further studies suggested that the lack of functional coupling to a wide variety of effector pathways was due to a lack of cell surface expression of the $GABA_{B1a}$ and $GABA_{B1b}$ proteins (Couve *et al*., 1998).

Database searching and yeast dihybrid screening has recently led to the discovery of a related gene, $GABA_{B2}$, which exhibits 35% sequence homology to the $GABA_{B1}$ subunit (Jones *et al*., 1999; White *et al*., 1999; Kaupmann *et al*., 1999). It was seen that, in brain, the expression pattern of the $GABA_{B2}$ mRNA overlapped with that of the $GABA_{B1}$ transcript suggesting that the proteins may be co-expressed (Jones *et al*., 1999). Using yeast two-hybrid screening, it was found that the $GABA_{B2}$ receptor subunit formed heterodimers with the $GABA_{B1}$ subtype through a coil–coil interaction at their intracellular carboxy-terminal tails (White *et al*., 1999). Furthermore, the $GABA_{B2}$ and $GABA_{B1a/b}$ proteins co-localize and immunoprecipitate together in dendritic spines (Kaupmann *et al*., 1999). All three groups showed that co-expression of the two receptor sub-units produces a fully functional $GABA_B$ receptor at the cell surface. They also suggested that, *in vivo*, functional $GABA_B$ receptors are heterodimeric and may represent the first identified multimeric G-protein-coupled receptor (for review see Marshall *et al*., 1999).

Although functional activity of the $GABA_{B1}/GABA_{B2}$ heterodimer has been shown, little is known concerning the pharmacological profile of this recombinant receptor. We have therefore studied the pharmacological profile of the $GABA_{B1b}/GABA_{B2}$ heterodimer when both subunits are co-expressed in a stable CHO cell line, which also expresses the adapter G-

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protein, G_{qi5} (Conklin *et al*., 1993). In G_{qi5}, the C-terminal amino acids of the G-protein subunit α_q are replaced with the corresponding amino acids of α_{i2}. This creates an adapter protein which mediates stimulation of phospholipase C by receptors, such as $GABA_B$, which normally couple to inhibition of adenylyl cyclase through Gi. One advantage of this system is that it allows the study of receptors in whole cell based assays in a high throughput system such as the FLIPR (Schroeder & Neagle, 1996; Molecular Devices, CA, U.S.A).

Methods

Materials

For culture and sub-cultivation of CHO cells stably expressing the human $GABA_{B1b}$ and $GABA_{B2}$ subunits, all materials were obtained from Life Technologies. [^{3}H]-CGP-54626-A (specific activity, 40 Ci mmol^{-1}) was from Tocris Cookson. Fluo-3-AM and pluronic acid were obtained from Molecular Probes. Black-walled-clear-bottomed-microplates were obtained from Corning-Costar. NaCl, KCl, HEPES, Glucose, $MgCl_2$, $CaCl_2$, probenecid, pertussis toxin, GABA, uridine triphosphate (UTP) and DMSO were from Sigma. SKF 97541(3-aminopropyl phosphinic acid), phaclofen, saclofen, CGP 35348, CGP 55845, SCH 50911, isoguvacine and TPMPA ((1,2,5,6-tetrahydropyridin-4-yl)methylphosphonic acid) were from Tocris (U.K.). SKF 97530, (3-aminopropyl)-methylphosphinic acid, and CGP 62349 were synthesized in the department of Medicinal Chemistry, SmithKline Beecham. For structures and chemical names of CGP 35348, CGP 55845 and CGP 62349, see Kerr & Ong (1996).

Molecular biology and stable expression of $GAGA_{B1b/2}/G_{qi5}$

$GABA_B$ receptor subunits 1b and 2 were isolated by PCR from a brain cDNA library, confirmed by sequencing on both strands and subcloned into the expression vectors pcDNA3.1 neo and pcDNA3.1 hygromycin respectively. $GABA_{B1b}$ was N-terminally tagged with the c-*myc* epitope inserting the tag between the native signal sequence and mature protein. All commercial expression vectors were purchased from Invitrogen. The chimeric G protein, G_{qi5}, was cloned into the in-house expression vector pCDN (Aiyar *et al*., 1994).

CHO DG44 cells were transfected with $GABA_{B1b}$-*myc*, $GABA_{B2}$ and G_{qi5} in equimolar quantities using Lipofectamine Plus (Life Technologies Inc.) according to the manufacturer's guidelines. Selection was initiated 48 h post transfection, using geneticin 800 μg.ml^{-1} and hygromycin 400 μg.ml^{-1}. Approximately 10 days post-transfection mass cell cultures were tested *via* FLIPR for a functional response. Cell pools that elicited a response were stained with a FITC conjugated anti-*myc* antibody (Santa Cruz) and pools of five cells were sorted from the top 5% fluorescent population by flow cytometry. These pools were then functionally retested and single cell clones isolated from pools that gave the best GABA response. Following expansion of the cells, further testing was carried out to derive the cell line that gave the best functional response.

Cell culture

Cells were grown in alpha-Minimum Essential Media (MEM) with ribonucleosides, 10% foetal bovine serum, 2 mM L-glutamine and the antibiotics hygromycin at 00 μg ml^{-1} and geneticin at 800 μg ml^{-1}. The cells were maintained at 37°C, 5% CO_2, 95% O_2 in a humidified incubator. Cells were subcultured by lifting with trypsin digestion, using serum addition to terminate its enzymic actions. Cells for use in an assay were lifted by Versene and plated into 96-well black-walled-clear-bottomed FLIPR microplates at a density of 40,000 cells per well. The cells were plated out in serum containing medium as above and incubated overnight before assaying. For pertussis toxin treatment, cells were plated out in 96-well plates as above and then incubated with or without the toxin (200 ng ml) for 18–24 h before assaying.

Radioligand binding

$GABA_B$ cells were homogenized in ice-cold Tris-HCl 50 mM, $MgCl_2$ 2.5 mM buffer, pH 7.4 using a Kinematic Ultra-Turrax homogeniser. The homogenates were then centrifuged at 35,000 × *g* for 15 min at 4°C. Membrane pellets were re-suspended in the buffer, re-homogenized and centrifuged as before. The final membrane pellet was re-suspended in buffer and stored at −80°C until required. Binding assays consisted of 50 μl of displacing compound or buffer, 400 μl of membrane suspension (corresponding to approximately 10 μg protein well^{-1}) and 50 μl of [^{3}H]-CGP 54626A (specific activity, 40 Ci mmol^{-1}).

For saturation analysis, membranes were incubated with [^{3}H]-CGP 54626A to give eight final ligand concentrations ranging from approximately 0.3 to 50 nM, for 45 min at 25°C. In competition binding experiments, 10 concentrations of the competing ligands were tested, at a final [^{3}H]-CGP 54626A concentration of 2 nM. Non-specific binding was defined using 1 mM GABA. The experiments were terminated by rapid filtration over Whatman GF/B glass fibre filters, pre-soaked with 0.3% (v : v) polyethyleneimine (PEI), and washed with 6 ml of ice-cold 50 mM Tris-HCl buffer. Radioactivity was determined by liquid scintillation spectrometry using a Packard 2700 liquid scintillation counter.

FLIPR assay

Agonists were prepared typically at 4 mM in Tyrode's buffer (composition (mM)) KCl: 2.5; NaCl: 145; HEPES: 10; glucose: 10; $MgCl_2$:1.2; $CaCl_2$:1.5; probenecid: 2.5, and pH was 7.4). For antagonists, solutions were made at 6 mM in 100% DMSO and were subsequently diluted in Tyrode's buffer. The final concentration of DMSO in the assay was less than 0.3%. Drug dilutions were carried out in 96-well blocks using a Biomek 2000 (Beckman). Phaclofen and saclofen were dissolved by sonication in Tyrode's buffer. Fluo-3AM (1 mg) was dissolved in DMSO (440 μl) and pluronic acid (20% in DMSO, 440 μl) and an aliquot diluted in media to give 20 μM.

The cells were incubated for 1 h at 37°C, 5% CO_2 and 95% O_2 with Fluo-3AM (final concentration 4 μM) in the presence of probenecid (0.8 mM final). Cells were then washed thoroughly using a Denley cell wash system to remove extracellular dye and a residual volume of 125 μl of Tyrode's buffer was left in each well. After washing, 25 μl of antagonists or buffer was added to each well and incubated for 30 min at 37°C, 5% CO_2, 95% O_2. The cell plate was loaded into the FLIPR and a signal test was taken and laser power adjusted to obtain a basal level of ~10 000 Fluorescence Intensity Units (FIU). The cells were then excited at 488 nm using the FLIPR laser and fluorescence

emission determined used a CCD camera with a bandpass interference filter (510–560 nm).

Fluorescence readings were taken at 1 s intervals for 60 s and a further 24 readings were taken at 5 s intervals. Compounds (50 μl) were added after 20 s using the FLIPR. Raw fluorescence data was exported for each well and tabulated versus time within an ASC II file. Data was then imported into Excel and the peak response over basal determined.

Data analysis

In binding studies, K_D and B_{max} values were calculated using Radlig and LIGAND (Biosoft, Cambridge, U.K). IC_{50} was determined as above and pKi values (the negative $\log_{10}$ of the molar K_i) for receptor binding were then calculated from the IC_{50} values as described by Cheng & Prusoff (1973) using the K_D values determined in the saturation binding studies. Data are expressed as the mean ± s.e.mean of at least three separate experiments.

In functional assays, concentration-effect data was analysed by non-linear regression fitted to the 4-parameter logistic equation to provide EC_{50} and maximal response (Bowen & Jerman, 1995). Antagonist data (IC_{50}) were analysed using the same method from inhibition curves using an EC_{80} concentration of GABA (3 μM). Antagonist affinity was defined as apparent pK_B according to Craig (1993). Results are quoted as mean ± s.e.mean from (n) separate experiments.

Statistical significance between data from different experiments was determined following analysis of the variance on the log-transformed data e.g. pKb, which is normally distributed, using the F-test. If this was homogenous, differences were examined using Student's t-test. If the variance was non-homogenous, the Mann–Whitney U-test was used.

Results

Radioligand binding

[^{3}H]-CGP 54626A bound to a single high affinity site with an apparent K_D of 4.25 ± 0.45 nM and with a B_{max} of 4.79 ± 1.38 pmol.mg^{-1} protein. Binding was inhibited by $GABA_B$ agonists (Table 1) and by a series of antagonists (Table 2).

Agonist profile in the FLIPR

GABA induced a concentration-dependent, rapid increase in fluorescence which peaked after 5–10 s, subsequently slowly diminished but did not return to basal levels within the time frame of the experiment (up to 3 min; Figure 1). The functional potency for a series of agonists to increase intracellular calcium is shown in Table 1 and illustrated in Figure 2. All of the active agonists tested produced similar maximal increases in fluorescence compared to that seen with GABA (Figure 2). Phaclofen and saclofen had no agonist-like effects at concentrations up to 1 mM. Isoguvacine at concentrations up to 1 mM had no agonist-like effect.

Table 1 Agonist profile at $GABA_{B1b}/GABA_{B2}$ heterodimer co-expressed with G_{qi5} in CHO cells

Compound	*pK_i [^{3}H]-CGP 54626 binding*	*pEC_{50}*
GABA	4.14 ± 0.01	6.80 ± 0.07
Baclofen	4.67 ± 0.06	6.37 ± 0.05
SKF 97530	5.29 ± 0.04	7.32 ± 0.05
SKF 97541	5.14 ± 0.03	7.26 ± 0.06

pK_i values are the mean ± s.e.mean of three separate determinations. pEC_{50} values are the mean ± s.e.mean of 4–5 separate determinations.

Antagonist profile in the FLIPR

The increase in intracellular calcium elicited by GABA was blocked by a series of antagonists (Table 1, Figure 3A). The response to baclofen was also inhibited by these antagonists with similar affinity values (Table 1, Figure 3B). Phaclofen and saclofen had no significant inhibitory effect on the GABA-mediated response at concentrations up to 0.1 mM, inhibition only being seen at higher concentrations. Inhibitory potencies (pIC_{50}) for phaclofen and saclofen were calculated by constraining the minimum of the 4-parameter logistic fit to zero (Table 1, Figure 3a). TPMA up to 100 μM had no significant effect on the GABA response.

Signal transduction studies

The increase in fluorescence induced by GABA was completely inhibited by removal of extracellular calcium in the wash buffer. Under these conditions a response was

Table 2 Antagonist inhibitory potencies at $GABA_{B1b}/GABA_{B2}$ heterodimer co-expressed with G_{qi5} in CHO cells

Compound	*pK_i [^{3}H]-CGP 54626 binding*	*pK_B values GABA as agonist*	*pK_B values Baclofen as agonist*
Phaclofen	3.92 ± 0.03	< 3.85	ND
Saclofen	4.33 ± 0.04	3.85 ± 0.06 (3)	ND
CGP 35348	4.83 ± 0.04	< 4	< 4
CGP 55845	7.85 ± 0.05	8.05 ± 0.10 (3)	8.38 ± 0.16 (3)
CGP 62349	8.66 ± 0.02	8.79 ± 0.08 (3)	8.92 ± 0.07 (3)
CGP 46381	5.33 ± 0.06	4.53 ± 0.11 (3)	4.84 ± 0.40 (3)
CGP 54626	8.09 ± 0.05	8.23 ± 0.08 (3)	8.51 ± 0.04 (3)
SCH 50911	5.46 ± 0.03	6.39 ± 0.21 (4)	6.22 ± 0.20 (4)

pK_i values are the mean ± s.e.mean of three separate determinations. pK_B values are the mean ± s.e.mean from (n) separate determinations. (n shown in parenthesis).

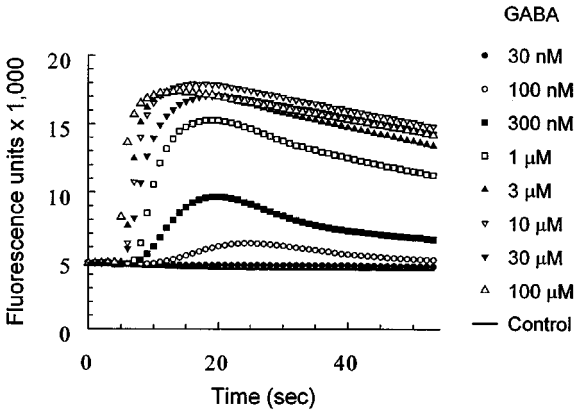

Figure 1 Time response curve for GABA-mediated increase in fluorescence at the $GABA_{B1b/2}/G_{qi5}$ recombinant receptor. Data points represent the signal fluorescence at each concentration of GABA and are representative from a single experiment repeated 3–8 times.

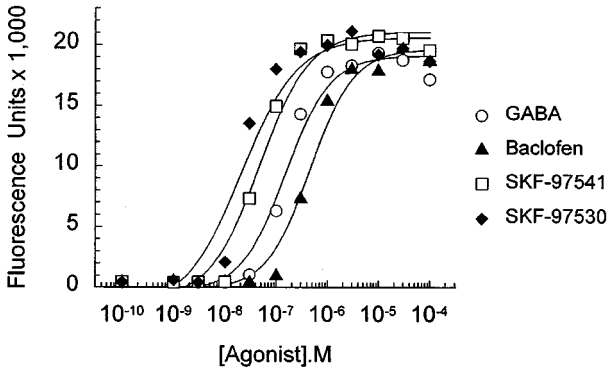

Figure 2 Concentration response curves at the $GABA_{B1b/2}/G_{qi5}$ recombinant receptor. Data points are the peak fluorescent signal obtained at different concentrations of the agonists and are representative from a single experiment with data summarized in Table 1.

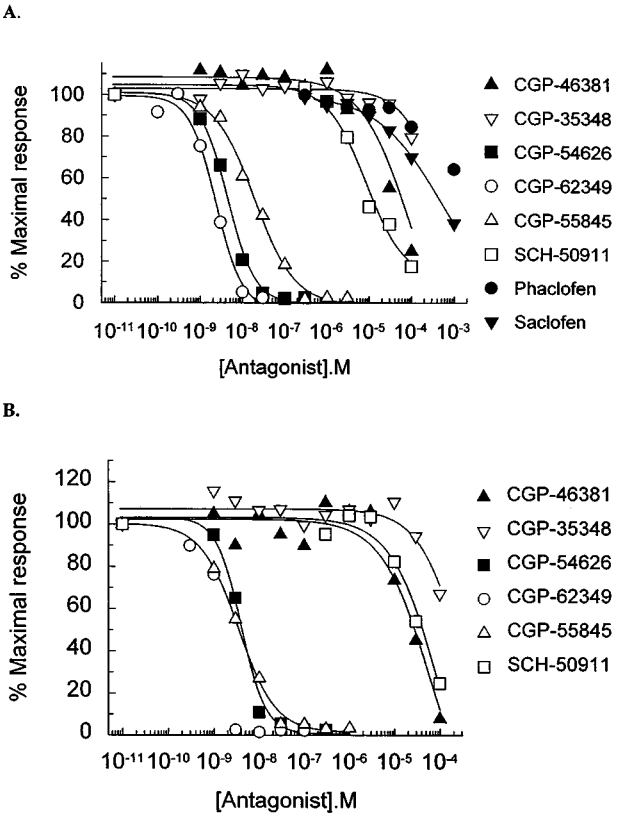

Figure 3 Concentration response curves for inhibition of GABA (A) and baclofen (B) responses at the $GABA_{B1b/2}/G_{qi5}$ recombinant receptor. Data points are the peak fluorescent signal obtained at different concentrations of the antagonists and are representative from a single experiment with data summarized in Table 2.

still seen to UTP (100 μM). Pre-treatment (added as antagonist at cell wash stage, therefore 30 min incubation prior to agonist addition) with either thapsigargin (1 μM) or U73122 (3 μM) also abolished the GABA response. Pertussis toxin pre-treatment produced a small but significant reduction in the functional potency of GABA from pEC_{50} 6.64 ± 0.04 (7) to 6.28 ± 0.07 (7) ($P<0.01$, Student's two-tailed t-test) and reduced the maximal response to GABA to $67.4\% \pm 4.4$ ($P<0.01$, Student's two-tailed t-test). Similarly, pertussis toxin pre-treatment reduced the functional potency of baclofen from pEC_{50} 6.23 ± 0.03 (6) to 5.84 ± 0.06 ($P<0.01$, Student's two-tailed t-test).

Discussion

The present study reports the pharmacological characterization of the cloned human $GABA_{B1b/2}$ heterodimeric receptor. Although it displays much of the expected pharmacological profile as seen in native receptors, there is a difference in that the GABA-mediated increase in intracellular calcium was not attenuated by phaclofen at concentrations up to 0.1 mM. The pharmacological profile of the recombinant $GABA_{B1b/2}$ heterodimeric receptor was consistent with that of native $GABA_B$ receptors in that it was activated by GABA and baclofen, inhibited by CGP 54626A and SCH 50911, was not activated by the $GABA_A$ agonist isoguvacine (which, at high concentrations, also activates $GABA_C$ receptors) and was not blocked by the $GABA_C$ antagonist TPMPA (Raggozzino *et al*., 1996).

It has been suggested that the $GABA_B$ receptor consists of a heterodimer of two related 7-transmembrane subunits, $GABA_{B1}$ and $GABA_{B2}$. Although it is clear that formation of the heterodimer is required for functional activity, there are regions in the brain, such as caudate/putamen, where $GABA_{B1}$ mRNA is abundant and $GABA_{B2}$ mRNA is low or undetectable (Ng *et al*., 1999). This raises the possibility that either subunit may function as homodimeric receptors or associate with other proteins to form functional receptors. In support of this, pharmacological studies have suggested the presence of $GABA_B$ subtypes (for review see Bonanno & Raiteri, 1993). In these studies, phaclofen reversed the baclofen-mediated inhibition of GABA release, but was ineffective in reversing the baclofen-mediated inhibition of glutamate release (Bonanno & Raiteri, 1992). In the present study, the baclofen- and GABA-mediated increases in intracellular calcium were antagonized by CGP 55845 and CGP 62349, but were only antagonized by high concentrations of phaclofen and saclofen. Phaclofen is a relatively weak antagonist (IC_{50} 100 μM; Bowery, 1989) and it is conceivable that this weak activity is difficult to detect. However, saclofen is more potent as an antagonist (IC_{50} 8 μM; Bowery, 1989) and further, the structurally related compound, 2-OH-saclofen, has been shown to inhibit the $GABA_{B1/2}$ response when co-expressed with rat Kir3 inwardly rectifying potassium channels (Lingenhoehl *et al*., 1999). Efficient functional coupling of the $GABA_{B1/2}$ heterodimer to G_{qi5} has been demonstrated, although small differences in functional potency between chimeric G-proteins were seen (Franek *et al*., 1999). This raises the possibility that differences in antagonist profile may depend on the coupling pathway used in a manner analogous to agonist trafficking and suggests a unique mechanism for receptor signalling regulation. Further work comparing the heterodimeric receptor in different functional assays with different signal transduction mechanisms would be required to confirm this hypothesis. It should be noted that a phaclofen-resistant $GABA_B$ receptor has also been reported in human neocortex (Bonanno *et al*., 1997) suggesting that this does not represent species differences. In the present study, the GABA-mediated response was also insensitive to the antagonist CGP 35348. A CGP 35348-insensitive $GABA_B$ receptor has also been described by Bonanno & Raiteri (1992).

For agonists, although there was a good rank order of potency correlation between inhibition of [^{3}H]-CGP 54626A binding and inhibition of GABA-mediated increases in intracellular calcium, all agonists were significantly more potent on function. This probably reflects a large receptor reserve, which is consistent with a high level of expression, which was reflected in the high B_{max} value for [^{3}H]-CGP 54626A. It should be noted that the binding affinities reported here are lower than that seen in native tissue (see

Bowery, 1989). The reason for this is unclear. It has been suggested that GABA and baclofen interact at different recognition sites on the $GABA_B$ receptor as calcium allosterically enhanced the action of GABA but not that of baclofen (Wise *et al*., 1999). No evidence for this in terms of differential antagonist affinity was seen in the present study.

The calcium mobilizing effects of GABA were blocked by thapsigargin and U73122, consistent with a phospholipase C mediated release of calcium from intracellular stores. It was therefore surprising that the GABA response was abolished by removal of extracellular calcium, suggesting the increase in intracellular calcium was a result of influx through cation channels. Similar results have been described for metabotropic glutamate receptors (Toms & Roberts, 1999) and are consistent with a proposed direct effect of phospholipase C or G-protein $\beta\gamma$ subunits on calcium channels (Recasens & Vignes, 1995). Pertussis toxin (PTX) pre-treatment produce only a modest attenuation of the GABA- and baclofen-mediated responses. PTX is known to specifically inhibit the activation of Gαi by ADP ribosylation of a cysteine residue at −4, which is present in the chimeric G_{qi5} (Conklin *et al*., 1993). Although PTX did affect the G_{qi4} chimera, this effect was also modest (Conklin *et al*., 1993). Interestingly, using a larger segment of the Gi in the chimeric G-protein (G_{qi9}), a marked effect was seen with PTX (Franek *et al*., 1999) suggesting that although the cysteine residue at −4 may be the site of ADP ribosylation, other downstream residues are involved in the recognition and binding of PTX.

In conclusion, we have shown that the pharmacological profile of the $GABA_{B1/2}$ heterodimer is consistent with the proposed phaclofen-insensitive subtype of the $GABA_B$ receptor (Bonanno & Raiteri, 1993). Whether this is a consequence of the signal transduction process employed in the current study, or indicates the presence of other $GABA_B$ subunits or chaperones, remains to be determined.

References

AIYAR, N., BAKER, E., WU, H.-L.E., NAMBI, P., EDWARDS, R.M., TRILL, J.J., ELLIS, C. & BERGSMA, D. (1994). Human AT1 receptor is a single copy gene: characterization in a stable cell line. *Mol. Cell. Biochem.,* **131,** 75–86.

BONANNO, G., FASSIO, A., SCHMID, G., SEVERI, P., SALA, R. & RAITERI, M. (1997). Pharmacologically distinct GABA(B) receptors that mediate inhibition of GABA and glutamate release in human neocortex. *Br. J. Pharmacol.,* **120,** 60–64.

BONANNO, G. & RAITERI, M. (1992). Functional evidence for multiple γ-aminobutyric $acid_B$ receptor subtypes in the rat cerebral cortex. *Eur. J. Pharmacol.,* **262,** 114–118.

BONANNO, G. & RAITERI, M. (1993). Multiple $GABA_B$ receptors. *Trends Pharmacol. Sci.,* **14,** 259–261.

BOWEN, W.P. & JERMAN, J.C. (1995). Nonlinear regression using spreadsheets. *Trends Pharmacol. Sci.,* **16,** 413–417.

BOWERY, N.G. (1989). $GABA_B$ receptors and their significance in mammalian pharmacology. *Trends Pharmacol. Sci.,* **10,** 401–407.

BOWERY, N.G., HILL, D.R., HUDSON, A., DOBLE, A., MIDDLEMISS, D.N., SHAW, J. & TURNBULL, M. (1980). (−)Baclofen decreases neurotransmitter release in the mammalian CNS by an action at a novel GABA receptor. *Nature,* **283,** 92–94.

CHENG, Y.C. & PRUSOFF, W.H. (1973). Relationship between inhibition constant (Ki) and the concentration of inhibitor which causes 50% inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.,* **92,** 888–894.

CONKLIN, B.R., FARFEL, Z., LUSTIG, K.D., JULIUS, D. & BOURNE, H.R. (1993). Substitution of three amino acids switches receptor specificity of $G_q\alpha$ to that of $G_i\alpha$. *Nature,* **363,** 274–276.

COUVE, A., FILIPPOV, A.K., CONNOLLY, C.N., BETTLER, B., BROWN, D.A. & MOSS, S.J. (1998). Intracellular retention of recombinant $GABA_B$ receptors. *J. Biol. Chem.,* **41,** 26361–26367.

CRAIG, D.A. (1993). The Cheng-Prusoff relationship: something lost in the translation. *Trends Pharamcol. Sci.,* **14,** 89–91.

FRANEK, M., PAGANO, A., KAUPMAN, K., BETTLER, B., PIN, J.-P. & BLAHOS, J. (1999). The heteromeric GABA-B receptor recognises G-protein a-subunit C-termini. *Neuropharmacol.,* **38,** 1657–1666.

HILL, D.R. & BOWERY, N.G. (1981). ^{3}H-Baclofen and ^{3}H-GABA bind to bicuculline-insensitive $GABA_B$ sites in rat brain. *Nature,* **290,** 149–152.

JONES, K.A., BOROWSKY, B., TAMM, J.A., CRAIG, D.A., DURKIN, M.M., DAI, M., YAO, W.-J., JOHNSON, M., GUNWALDSEN, C., HUANG, L.Y., TANG, C., SHEN, Q., SALON, J.A., MORSE, K., LAZ, T., SMITH, K.E., NAGARATHNAM, D., NAOBLE, S.A., BRANCHEK, T.A. & GERALD, C. (1999). $GABA_B$ receptors function as a heteromeric assembly of the subunits $GABA_B$R1 and $GABA_B$R2. *Nature,* **396,** 674–679.

KAUPMANN, K., HUGGEL, K., HEID, J., FLOR, P., BISCHOFF, S., MICKEL, S.J., McMASTER, G., ANGST, C., BITTIGER, H., FROESTL, W. & BETTLER, B. (1997). Expression cloning of $GABA_B$ receptors uncovers similarity to metabotropic glutamate receptors. *Nature,* **20,** 239–246.

KAUPMANN, K., MALITSCHEK, B., SCHULER, V., HEID, J., FROESTL, W., BECK, P., MOSBACHER, J., BISCHOFF, S., KULIK, A., SHIGEMOTO, R., KARSCHIN, A. & BETTLER, B. (1999). $GABA_B$-receptor subtypes assemble into functional heteromeric complexes. *Nature,* **396,** 683–687.

KERR, D.I.B. & ONG, J. (1995). $GABA_B$ receptors: targets for drug development. *Drug Develop. Today,* **9,** 371–379.

LINGENHOEHL, K., BROM, R., HEID, J., BECK, P., FROESTL, W., KAUPMANN, K., BETTLER, B. & MOSBACHER, J. (1999). γ-Hydroxybutyrate is a weak agonist at recombinant GABAB receptors. *Neuropharmacol.,* **38,** 1667–1673.

MARSHALL, F.H., JONES, K.A., KAUPMANN, K. & BETTLER, B. (1999). $GABA_B$ receptors - the first 7TM heterodimers. *Trends in Pharmacol. Sci.,* **20,** 396–399.

NG, G.Y.K., CLARK, J., COULOMBE, N., ETHIER, N., HEBERT, T.E., SULLIVAN, R., KARGMAN, S., CHATEAUNEUF, A., TSUKAMOTO, N., McDONALD, T., WHITING, P., MEZEY, E., JOHNSON, M.P., LIU, Q., KOLAKOWSKI, L.F., EVANS, J.F., BONNER, T.I. & O'NEILL, G.P. (1999). Identification of a $GABA_B$ receptor subunit, gb2, required for functional $GABA_B$ receptor activity. *J. Biol. Chem.,* **274,** 7607–7610.

RAGOZZINO, D., WOODWARD, R.M., MURATA, Y., EUSIBI, F., OVERMAN, L.E. & MILEDI, R. (1996). Design and In Vitro pharmacology of a selective γ-aminobutyric $acid_c$ receptor antagonist. *Mol. Pharmacol.,* **50,** 1024–1030.

RECASENS, M. & VIGNES, M. (1995). Excitatory amino acid metabotropic receptor subtypes and calcium regulation. *Ann. NY Acad. Sci.,* **757,** 418–429.

SCHROEDER, K.S. & NEAGLE, B.D. (1996). FLIPR: A new instrument for accurate, high throughput optical screening. *J. Biomol. Screening,* **2,** 75–80.

TAKAHASHI, T., KAJIKAWA, Y. & TSUJIMOTO, T. (1998). G-protein-coupled modulation of presynaptic calcium currents and transmitter release by a $GABA_B$ receptor. *J. Neurosci.,* **18,** 3138–3146.

TOMS, N.J. & ROBERTS, P.J. (1999). Group 1 mGlu receptors elevate $[Ca^{2+}]$i in rat cultured cortical type 2 astrocytes: $[Ca^{2+}]$i synergy with adenosine A_1 receptors. *Neuropharmacology,* **38,** 1511–1517.

WHITE, J.H., WISE, A., MAIN, M.J., GREEN, A., FRASER, N.J., DISNEY, G.H., BARNES, A.A., EMSON, P., FOORD, S.M. & MARSHALL, F.H. (1999). Heterodimerization is required for the formation of a functional $GABA_B$ receptor. *Nature,* **396,** 679–682.

WISE, A., GREEN, A., MAIN, M.J., WILSON, R., FRASER, N. & MARSHALL, F.H. (1999). Calcium sensing properties of the $GABA_B$ receptor. *Neuropharmacol.,* **38,** 1647–1656.

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