British Journal of Pharmacology (1997) 122, 1551–1560

Analogues of γ-aminobutyric acid (GABA) and *trans*-4-aminocrotonic acid (TACA) substituted in the 2 position as $GABA_C$ receptor antagonists

[1]Mary Chebib, Robert J. Vandenberg & Graham A.R. Johnston

Adrien Albert Laboratory of Medicinal Chemistry, Department of Pharmacology, D06, The University of Sydney, NSW 2006, Australia

1 γ-Aminobutyric acid (GABA) and *trans*-4-aminocrotonic acid (TACA) have been shown to activate $GABA_C$ receptors. In this study, a range of C2, C3, C4 and N-substituted GABA and TACA analogues were examined for activity at $GABA_C$ receptors.

2 The effects of these compounds were examined by use of electrophysiological recording from *Xenopus* oocytes expressing the human ρ_1 subunit of $GABA_C$ receptors with the two-electrode voltage-clamp method.

3 *trans*-4-Amino-2-fluorobut-2-enoic acid was found to be a potent agonist ($K_D = 2.43$ μM). In contrast, *trans*-4-amino-2-methylbut-2-enoic acid was found to be a moderately potent antagonist ($IC_{50} = 31.0$ μM and $K_B = 45.5$ μM). These observations highlight the possibility that subtle structural substitutions may change an agonist into an antagonist.

4 4-Amino-2-methylbutanoic acid ($K_D = 189$ μM), 4-amino-2-methylenebutanoic acid ($K_D = 182$ μM) and 4-amino-2-chlorobutanoic acid ($K_D = 285$ μM) were weak partial agonists. The intrinsic activities of these compounds were 12.1%, 4.4% and 5.2% of the maximal response of GABA, respectively. These compounds more effectively blocked the effects of the agonist, GABA, giving rise to K_B values of 53 μM and 101 μM, respectively.

5 The sulphinic acid analogue of GABA, homohypotaurine, was found to be a potent partial agonist ($K_D = 4.59$ μM, intrinsic activity 69%).

6 It was concluded that substitution of a methyl or a halo group in the C2 position of GABA or TACA is tolerated at $GABA_C$ receptors. However, there was dramatic loss of activity when these groups were substituted at the C3, C4 and nitrogen positions of GABA and TACA.

7 Molecular modelling studies on a range of active and inactive compounds indicated that the agonist/competitive antagonist binding site of the $GABA_C$ receptor may be smaller than that of the $GABA_A$ and $GABA_B$ receptors. It is suggested that only compounds that can attain relatively flat conformations may bind to the $GABA_C$ receptor agonist/competitive antagonist binding site.

Keywords: $GABA_C$ receptors; γ-aminobutyric acid (GABA); $GABA_A$ agonists; *Xenopus* oocytes; *trans*-4-aminocrotonic acid (TACA)

Introduction

The major inhibitory neurotransmitter, γ-aminobutyric acid (GABA, compound (1) in Figure 1), activates three major classes of GABA receptors, the $GABA_A$, $GABA_B$ and $GABA_C$ receptors. $GABA_A$ receptors are ligand-gated Cl^- channels which are inhibited by the alkaloid, bicuculline (Johnston, 1996a). These receptors are heterooligomeric receptors made up of five subunits. To date, there are 14 known subunits which include α_{1-6}, β_{1-3}, γ_{1-3}, δ and ε subunits (Johnston, 1996a; Davies *et al*., 1997). $GABA_A$ receptors assemble predominantly by combining 2α and 2β subunits, and one other subunit which may consist of either γ, δ or ε subunits (Johnston, 1996a; Davies *et al*., 1997).

$GABA_B$ receptors are seven transmembrane receptors coupled to second messenger systems and Ca^{2+} and K^+ channels via G-proteins. These receptors are not blocked by bicuculline but are activated by (−)-baclofen and blocked by compounds such as phaclofen and saclofen, which are phosphonic and sulphonic analogues of baclofen (Kerr & Ong, 1995). There may be many subtypes of $GABA_B$ receptors, two of which have now been cloned (Kaupmann *et al*., 1997). These receptors have been shown to have structural similarities with metabotropic glutamate receptors.

$GABA_C$ receptors were first proposed when a series of conformationally restricted GABA analogues, including *cis*-4-aminocrotonic acid (CACA), that had a bicuculline-insensitive depressive effect on neuronal activity, showed no affinity for [^{3}H](−)-baclofen binding sites in rat cerebellar membranes (Drew *et al*., 1984). CACA, a conformationally restricted analogue of GABA in a folded conformation, has moderate partial agonist activity at $GABA_C$ receptors ($K_D = 74$ μM) and may be the most selective agonist for this receptor subtype (Johnston, 1996b).

Subsequently, $GABA_C$ receptors were detected electrophysiologically in rod bipolar cells from rat retina. These receptors were insensitive to bicuculline and baclofen but were activated by CACA after co-application of GABA with 100 μM bicuculline to abolish the $GABA_A$ component (Feigenspan *et al*., 1993). It was found that $GABA_C$ receptors were more sensitive to GABA, the channel was open longer with maintained agonist application than $GABA_A$ receptors (Feigenspan & Bormann, 1994).

$GABA_C$ receptors were also detected in white perch retina on rod-driven horizontal cells (H4) but not bipolar cells which were shown to contain $GABA_A$ receptors (Qian & Dowling, 1993). Differences between the two receptor types were detected by applying GABA on both types of cells. Firstly, the bipolar cells showed rapid desensitization, while on rod-driven horizontal cell desensitization was not observed (Qian & Dowling, 1993). Further studies showed that $GABA_C$ recep-

[1] Author for correspondence.

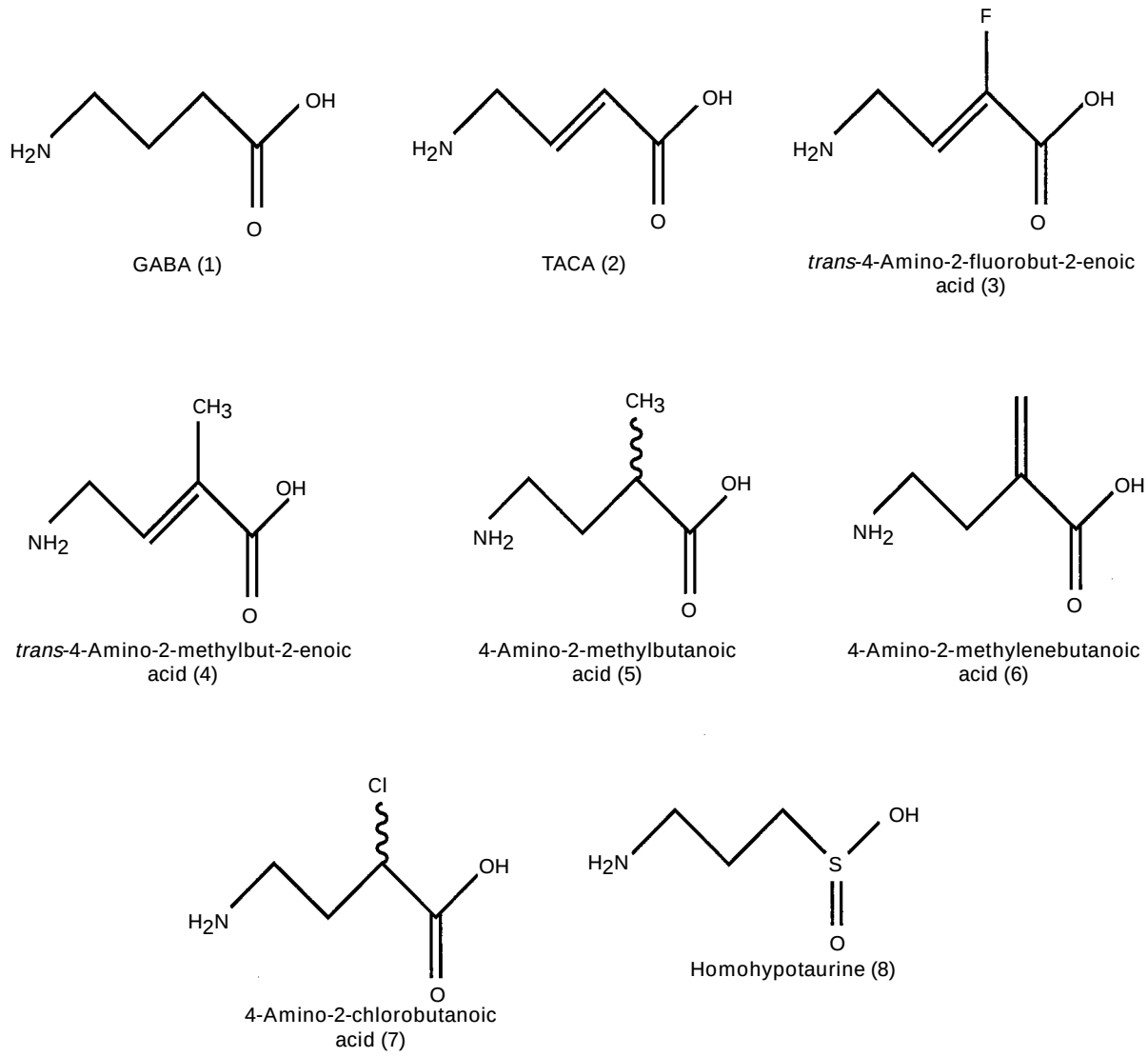

Figure 1 Structures that show agonist or antagonist effects at $GABA_C$ receptors.

tors were found on cone- but not rod-driven horizontal cells that had been isolated from catfish retina (Dong *et al*., 1994). GABA receptors on bipolar terminals in tiger salamander retina were shown to gate Cl^- conductance with similar pharmacology to $GABA_C$ receptors (Lukasiewicz *et al*., 1994).

Expression of poly(A)$^+$ RNA from mammalian retina injected in *Xenopus* oocytes generated two pharmacologically distinct GABA receptors, consisting of a bicuculline-sensitive component ($GABA_A$ component) and a bicuculline-insensitive and (−)-baclofen-insensitive component ($GABA_C$ component) (Woodward *et al*., 1993). The $GABA_A$ component was blocked by bicuculline and the remaining current ($GABA_C$ component) was used to determine a structure-activity profile. The most potent $GABA_C$ receptor agonists in the study were found to be *trans*-4-aminocrotonic acid (TACA, compound (2) in Figure 1, $K_D = 0.6\ \mu M$) and GABA ($K_D = 1.7\ \mu M$) (Woodward *et al*., 1993). These compounds do not selectively activate $GABA_C$ receptors. Instead, $GABA_C$ receptors are selectively antagonized by the compound, (1,2,5,6-tetrahydropyridine-4-yl)methylphosphinic acid (TPMPA; $K_D = 2.1\ \mu M$) (Murata *et al*., 1996; Ragazzoni *et al*., 1996).

Two human and three rat cDNAs that have 30–38% sequence identity with $GABA_A$ receptor subunits have been cloned from retinal mRNA and termed the ρ subunits. Human ρ_1 and ρ_2 have 74% sequence identity (Cutting *et al*., 1991; 1992). These subunits do not combine with any of the $GABA_A$ subunits. Instead the subunits combine to form either homo-oligomeric (made up of either ρ_1 subunits or ρ_2 subunits), or heteroligomeric (made up of ρ_1 and ρ_2 subunits) receptors (Zhang *et al*., 1995). These combinations form intrinsic Cl^- channels when expressed in *Xenopus* oocytes. $GABA_C$ receptors expressed in *Xenopus* oocytes have slower activation times, do not desensitize readily with maintained GABA application, and have slower closing times than the corresponding $GABA_A$ receptor expressed in *Xenopus* oocytes consisting of α_1, β_2 and γ_2 subunits (Amin & Weiss, 1994).

Rat ρ_1 and ρ_2 subunits have 99% and 88% receptor homology to the human ρ_1 and ρ_2 subunits, respectively (Enz *et al*., 1995). The rat ρ_3 subunit has 63% and 61% sequence identity to the rat ρ_1 and ρ_2 subunits, respectively (Ogurusu & Shingai, 1996). The use of reverse transcriptase polymerase chain reaction (PCR) and *in situ* hybridization showed that rat ρ_1, ρ_2 and ρ_3 mRNAs are predominantly in the retina. However, rat ρ_2 mRNA was also found in all brain areas (Enz *et al*., 1995). Although $GABA_A$ and $GABA_C$ receptors are similar in that both are ionotropic receptors that conduct Cl^- ions, the similarities stop there. The physiological and pharmacological differences between the two receptors, discussed above, classify these receptors as $GABA_C$ receptors (Cutting *et al*., 1991; 1992; Polenzani *et al*., 1991; Shimada *et al*., 1992; Kusama *et al*., 1993a,b; Wang *et al*., 1994; Bormann & Feigenspan, 1995; Johnston, 1996b).

In this study, we have investigated a series of alkyl and halo substituted analogues of GABA and TACA to determine the

position(s) on the carbon backbone, where substitution is tolerated for the development of active and selective agonists and antagonists for the $GABA_C$ receptor. We have also investigated replacing the carboxylic acid moiety with a sulphinic acid moiety to help analyse what groups are required for agonist and antagonist activity and build up the structure-activity profile of the $GABA_C$ receptor.

Methods

Electrophysiological recording

Xenopus laevis were anaesthetized with 0.17% 3-aminobenzoic acid ethyl ester and a lobe of the ovaries was removed. The lobe of the ovary was rinsed with OR2 buffer (82.5 mM NaCl, 2 mm KCl, 1 mM $MgCl_2.6H_2O$, 5 mM HEPES, pH 7.5) and treated with Collagenase A (2 mg ml^{-1} in OR2, Boehringer Mannheim) for 2 h. Released oocytes were then rinsed in frog ringer solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2.6H_2O$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5) supplemented with 2.5 mM pyruvate, 0.5 mM theophyline and 50 μg ml^{-1} gentamycin, and stage V–VI oocytes collected.

The human ρ_1 cDNA in pcDNA (Invitrogen, San Diago, CA, U.S.A.) was obtained from Dr George Uhl (National Institute for Drug Abuse, Baltimore). Capped RNA was synthesized from linearized plasmid containing the ρ_1 cDNA and cRNA was made by use of the 'Mmessage Mmachine' kit from Ambion Inc. (Austin, Texas, U.S.A.). cRNA, 10 ng 50 nl^{-1}, was injected into defolliculated Stage V *Xenopus* oocytes. Oocytes were stored at 16°C and two to eight days later, receptor activity was measured by two electrode voltage clamp recording by means of a Geneclamp 500 amplifier (Axon Instruments Inc., Foster City, CA, U.S.A.), a MacLab 2e recorder (ADInstruments, Sydney, NSW, Australia) and Chart version 3.5 programme. Oocytes were voltage clamped at −60 mV and continuously superfused with frog ringer solution. For receptor activation measurements, the indicated concentrations of drug were added to the buffer solution.

Analysis of kinetic data

Current (I) as a function of agonist concentration ([A]) was fitted by least squares to $I = I_{max}[A]^{n_H}/K_D{}^{n_H} + [A]^{n_H}$, where I_{max} is the maximal current, the K_D is the effective dose that activates 50% of the maximal current and n_H is the Hill coefficient. K_D values are expressed as mean ± s.e.mean ($n = 3$–6) and were determined by fitting data from individual oocytes by use of Kaleidagraph 2.1 (1990). Current (I) as a function of antagonist concentration ([Ant]) was fitted by least squares to $I = I_{max} - \{I_{max}[Ant]^{n_H}/(IC_{50}{}^{n_H} + [Ant]^{n_H})\}$, where the IC_{50} is the dose that blocks 50% of the current generated by 1 μM GABA and n_H is the Hill coefficient. IC_{50} values are expressed as mean ± s.e.mean ($n = 3$–6). K_B values are the apparent binding constants for the antagonists and were determined by means of Schild plot analysis (Arunlakshana & Schild, 1959). $-\log K_B$ values were determined from the following equation $\log\{(A)/(A^*) - 1\} = m.\log[Ant] - \log K_B$, where A is the K_D of GABA in the presence of a known antagonist concentration, A* is the K_D of GABA in the absence of the antagonist, [Ant] is the concentration of the antagonist, and 'm' is the slope of the curve. For simple competitive antagonism, 'm' is 1. $-\log K_D$ values were determined by fitting data to the above function by use of Kaleidagraph 2.1 (1990).

Molecular modelling

A computer-assisted study was carried out on 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol(THIP), *trans*-4-amino-2-methyl- but-2-enoic acid, 4-amino-3,3-dimethylbutanoic acid, 4-amino-4,4-dimethylbutanoic acid, 2-aminocyclopentylidene acetic acid, gabainic acid and TPMPA by use of Chem-X (Chemical Design Ltd., Oxford UK, 1994) and Chem-3D (Cambridge Scientific Computing, Inc. Cambridge, MA, U.S.A.) to determine the conformation of the ligands at the receptor site. The three-dimensional matrices of the compounds were optimized with the molecular mechanics optimization routines in Chem-X (Chemical Design Ltd., Oxford, UK, 1994). The conformers of each compound were then subjected to conformational search routines about the bonds at which torsional rotations were possible. A search was subsequently undertaken to determine the low energy conformation(s) of the *trans*-4-amino-2-methylbut-2-enoic acid, 4-amino-3,3-dimethylbutanoic acid, 4-amino-4,4-dimethylbutanoic acid, 2-aminocyclopentylidene acetic acid, gabainic acid and TPMPA that were fitted to the conformationally restricted GABA analogue, THIP (Krogsgaard-Larsen *et al*., 1977; 1979). At $GABA_C$ receptors, THIP is a competitive antagonist with a K_B value of 32 μM (Woodward *et al*., 1993). Two fits were considered, a 'GABA' orientation and a 'muscimol' orientation. Muscimol is a potent partial agonist at $GABA_C$ receptors ($K_D = 2.3$ μM) and is a conformationally unrestricted analogue of THIP. These orientations are proposed to be the orientations that each respective compound attains to interact with the agonist/competitive antagonist binding sites on the $GABA_C$ receptor.

Materials

trans-4-Amino-2-fluorobut-2-enoic acid, *trans*-4-amino-2-methylbut-2-enoic acid, 4-amino-2-methylbutanoic acid, 4-amino-2-methylenebutanoic acid, 4-amino-2-chlorobutanoic acid, homohypotaurine, 4-amino-2,2-dimethylbutanoic acid, 4-amino-3,3-dimethylbutanoic acid, 4-amino-4,4-dimethylbutanoic acid, *trans*-4-amino-3-methylbut-2-enoic acid, *trans*-4-amino-pent-2-enoic acid, *trans*-4-aminomethylbut-2-enoic acid, *trans*-4-amino-3-chlorobut-2-enoic acid, 4-amino-2,4-dimethylbutanoic acid, 2-aminocyclopentylidene acetic acid, 2-aminocyclohexylidine acetic acid, *cis*-5-aminocyclohex-3-ene carboxylic acid and gabainic acid were prepared as previously described by Dr Robin D. Allan (Department of Pharmacology, The University of Sydney, Sydney, Australia) and

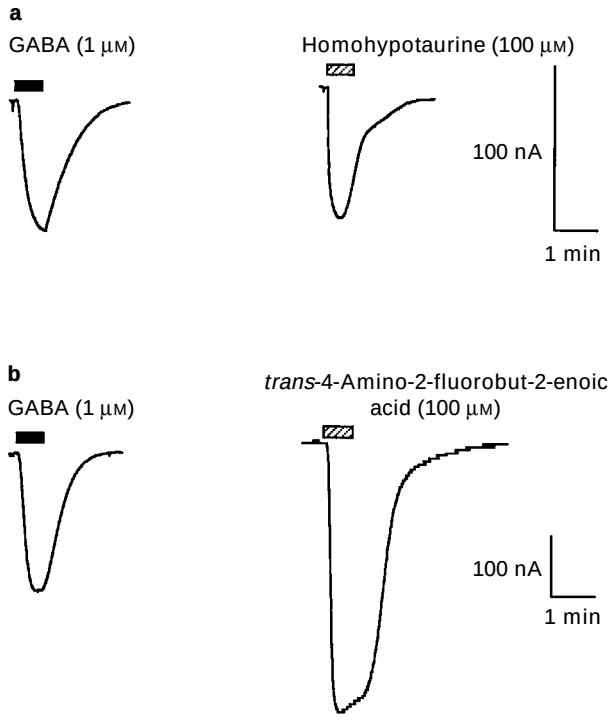

Figure 2 GABA (1 μM) (duration indicated by solid bar) activated inward current in oocytes expressing ρ_1 cRNA and clamped at −60 mV. Agonist activity (duration indicated by hatched bar) of (a) homohypotaurine (100 μM), the sulphinic analogue of GABA and (b) *trans*-4-amino-2-fluorobut-2-enoic acid (100 μM).

Bruce Twitchin (Allan, 1978; 1979; Allan & Twitchin, 1978; Allan *et al*., 1980). TACA was prepared as previously described (Johnston *et al*., 1975) by Dr Ken N. Mewett (Department of Pharmacology, The University of Sydney, Sydney, Australia). GABA was purchased from Sigma Chemical Co (St Louis, MO, U.S.A.).

Results

Expression of the human ρ_1 mRNA in *Xenopus laevis* oocytes generates GABA gated ion channels with a similar pharmacological profile to previously described $GABA_C$ receptors. Figure 1 shows the compounds that were active as either agonists or antagonists at $GABA_C$ receptors. These were *trans*-4-amino-2-fluorobut-2-enoic acid (3), *trans*-4-amino-2-methylbut-2-enoic acid (4), 4-amino-2-methylbutanoic acid (5), 4-amino-2-methylenebutanoic acid (6), 4-amino-2-chlorobutanoic acid (7) and homohypotaurine (8). The compounds were first screened at 100 μM to determine agonist activity, by activation of the receptor (Figure 2), or antagonist activity, by blocking the activation of the receptor by 1 μM GABA (Figure 3). The $GABA_C$ receptor was activated by compounds (3), (5), (6), (7) and (8). While GABA activated receptors could be blocked by compound (4).

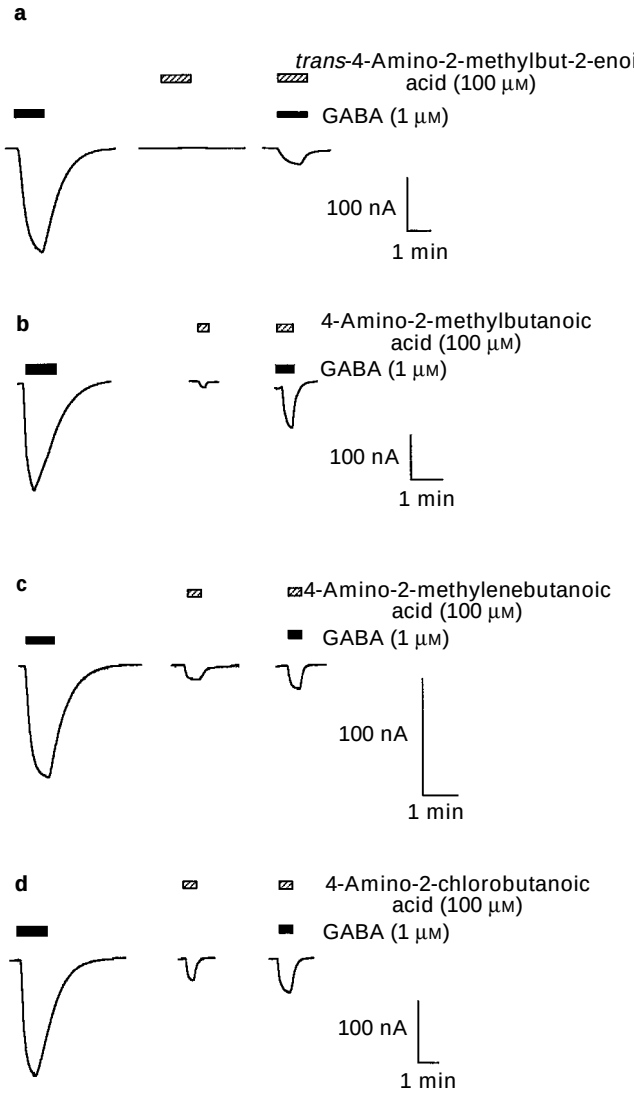

Figure 3 (a) *trans*-4-Amino-2-methylbut-2-enoic acid (100 μM) did not activate the receptor (duration indicated by hatched bar). When compound (100 μM) was co-applied with GABA (1 μM), the GABA response was reduced. (b) 4-Amino-2-methylbutanoic acid (100 μM), (c) 4-amino-2-methylenebutanoic acid (100 μM) and (d) 4-amino-2-chlorobutanoic acid (100 μM) all showed weak activation of the receptor (duration indicated by hatched bar). However, when co-applied with GABA, the response was significantly reduced compared to the application of 1 μM GABA alone.

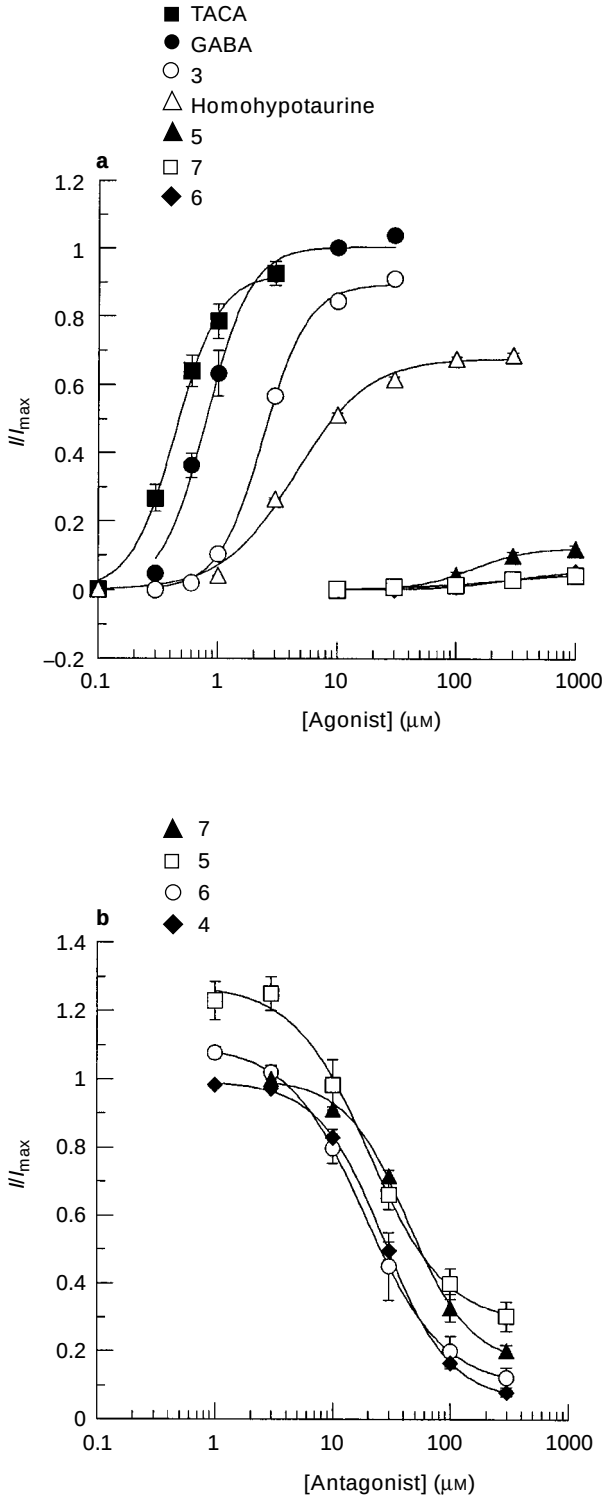

Figure 4 (a) Agonist dose-response curves for TACA, GABA, *trans*-4-amino-2-fluorobut-2-enoic acid (3), homohypotaurine, 4-amino-2-methylbutanoic acid (5), 4-amino-2-chlorobutanoic acid (7) and 4-amino-2-methylenebutanoic acid (6) for *Xenopus* oocytes expressing ρ_1 cRNA. (b) Inhibition curves for antagonists 4-amino-2-chlorobutanoic acid (7), 4-amino-2-methylbutanoic acid (5), 4-amino-2-methylenebutanoic (6) and *trans*-4-amino-2-methylbut-2-enoic acid (4) for *Xenopus* oocytes expressing ρ_1 cRNA. Data are the mean ($n = 3-6$ oocytes); vertical lines show s.e.mean.

Agonist dose-response curves are shown in Figure 4(a). TACA and GABA were the most potent agonists in this study. TACA had a maximal response of 92.6±0.1% of the maximal GABA activated current which was not significantly different from a full agonist response. The next most potent agonist was compound (3) albeit weaker than the parent compound, TACA. The K_D was 2.43±0.15 μM and was almost a full agonist with a maximal dose generating 91.1±0.1% of the maximal GABA activated current. The sulphinic analogue of GABA, compound (8), was found to be a potent partial agonist with a K_D of 4.59±0.64 μM. The maximal response activated by compound (8) was 68.8±0.1% of the maximal GABA activated current. Table 1 shows the Hill coefficients (n_H), intrinsic activity (Im), and K_D values of these compounds.

Compound (4) was found to be a moderately active antagonist with an IC_{50} (ie the concentration that inhibits 50% of the response produced by 1 μM GABA) of 31.0±2.7 μM. Schild analysis showed that *trans*-4-amino-2-methylbut-2-enoic acid was a competitive antagonist at the concentrations tested and had a K_B value (apparent binding constant) of 45.5±5.4 μM (Table 1; Figure 5a).

Compounds (5), (6), (7) were found to be weak agonists with low intrinsic activity. The K_D values were 189±13 μM, 182±8 μM and 285±17 μM, respectively and the maximal responses were 12.1±0.1%, 4.4±0.1%, and 5.2±0.1% of the maximal GABA response, respectively (Table 1; Figure 4a). These compounds were somewhat more potent in blocking the effects of 1 μM GABA than activating the receptor itself suggesting partial agonist properties. Compounds (5), (6), (7) were found to have moderately potent antagonist effects with IC_{50} values of 20.7±0.7 μM, 28.8±4.3 μM and 47.8±4.6 μM, respectively (Table 1). These compounds were found to be competitive antagonists over the concentrations tested. However, the compounds never blocked completely the response to GABA (Figure 4b). From Schild analyses, the binding constants (K_B) for compounds (6) and (7) were obtained and were found to be 53.0±6.0 μM and 101±2 μM, respectively (Table 1; Figure 5b and c). The potencies of the agonists at $GABA_C$ receptors followed the rank order of TACA > GABA > compound (3) > compound (8) > > compound (6) ≈ compound (5) > compound (7).

Analogues of TACA that had substituents in the C3, C4 and nitrogen such as *trans*-4-amino-3-methylbut-2-enoic acid, *trans*-4-aminopent-2-enoic acid, *trans*-4-aminomethylbut-2-enoic acid, and *trans*-4-amino-3-chlorobut-2-enoic acid had no effects as agonists or antagonists when screened at 100 μM. Disubstituted analogues of GABA such as 4-amino-2,2-dimethylbutanoic acid, 4-amino-3,3-dimethylbutanoic acid, 4-amino-4,4-dimethylbutanoic acid and 4-amino-2,4-dimethylbutanoic also had no effect on the $GABA_C$ receptors when tested at 100 μM. Furthermore the cyclic compounds, 2-aminocyclopentylidene acetic acid, 2-aminocyclohexylidine acetic acid, *cis*-5-aminocyclohex-3-ene carboxylic acid and gabainic acid also had no effect on $GABA_C$ receptors when tested at 100 μM.

Molecular modelling

We modelled the conformations of various GABA analogues used in this study in an attempt to determine a model that may explain why some analogues bind and activate $GABA_C$ receptors while other similar structures either block the actions of GABA or have no effect at all. The GABA analogues used in this modelling study were THIP, TPMPA, *trans*-4-amino-2-methylbut-2-enoic acid, 4-amino-3,3-dimethylbutanoic acid, 4-amino-4,4-dimethylbutanoic acid, 2-aminocyclopentylidene acetic acid and gabainic acid. Most of the analogues, with the exception of THIP, TPMPA, 2-aminocyclopentylidene acetic acid and gabainic acid, are flexible molecules and can exist in a large number of low energy conformations. This makes it difficult to determine which conformation is the conformation that binds to $GABA_C$ receptors expressed in *Xenopus* oocytes. THIP is a conformationally restricted analogue of muscimol and a competitive antagonist at $GABA_C$ receptors (Woodward *et al*., 1993). Consequently, THIP reduces considerably the number of conformations that can be considered as interacting with the ligand-recognition site on the receptor. Structurally, THIP is a relatively flat and rigid molecule. In this study we used the conformation of THIP as the basis of our modelling. This compound gives the relative positions of the amino group and the carboxylate group. *trans*-4-Amino-2-methylbut-2-enoic acid, 4-amino-3,3-dimethylbutanoic acid, 4-amino-4,4-dimethylbutanoic acid, 2-aminocyclopentylidene acetic acid, gabainic acid and TPMPA could be superimposed against the amino and carboxylic acid pharmacophores of THIP in two ways and both of these possibilities can be considered as the orientations in which flexible molecules fit into the binding site of the $GABA_C$ receptor. The fits shown in Figures 6 and 7 will be referred to as the 'muscimol' and 'GABA' orientations, respectively.

The first fit, as shown in Figure 6, aligns the carbon backbone of the GABA and TACA analogues against the 'muscimol' moiety of THIP. This resulted in a partial folding of the carbon chain around the C3 and C4 atoms of the TACA and GABA analogues (Figure 6). The TACA analogue, *trans*-4-amino-2-methylbut-2-enoic acid is a moderately potent antagonist. This compound has a methyl group substituted in the C2 position and this group was found to align relatively well against the C2′ carbon of THIP (Figure 6). Like THIP, the compound is relatively flat.

In Figure 6, the double bond of TPMPA aligns against the double bond of THIP. TPMPA, the most selective antagonist at $GABA_C$ receptors is a relatively flat compound and as a result both the amine and methylphosphinic acid moieties fit well against the amine and isoxazole groups of THIP. As the methylphosphinic acid moiety is attached to the ring with a

Table 1 Summary of K_D, IC_{50}, K_B, Hill coefficients and intrinsic activities of various agonists and antagonists at the $GABA_C$ receptor expressed in *Xenopus* oocytes

Compound[a]	K_D (μM)[b]	IC_{50} (μM)[c]	K_B (μM)[d]	n_H[e]	*Im* (%)[f]
GABA (1)	0.82±0.09	–	–	2.6±0.2	100
TACA (2)	0.44±0.02	–	–	2.4±0.2	92.6±0.1
trans-4-Amino-2-fluorobut-2-enoic acid (3)	2.43±0.15	–	–	2.4±0.1	91.1±0.1
Homohypotaurine (8)	4.59±0.64	–	–	1.4±0.1	68.8±0.1
4-Amino-2-methylbutanoic acid (5)	189±13	20.7±0.7	ND	1.3±0.1	12.1±0.1
4-Amino-2-chlorobutanoic acid (7)	285±17	47.8±4.5	101±2	1.9±0.2	5.2±0.1
4-Amino-2-methylenebutanoic acid (6)	182±8	28.8±4.4	53.0±6.0	1.4±0.2	4.4±0.1
trans-4-Amino-2-methylbut-2-enoic acid (4)	–	31.0±2.7	45.5±5.4	1.4±0.1	–

[a]Numbers in parentheses after the compound names refer to the corresponding structures in Figure 1. [b]K_D is the apparent dissociation constant for the agonist at $GABA_C$ receptors expressed in *Xenopus* oocytes. Data are the mean±s.e.mean (n=3–6 oocytes). [c]IC_{50} is the concentration that inhibits 50% of the response produced by 1 μM GABA. Data are the mean±s.e.mean (n=3–6 oocytes). [d]K_B is the apparent binding constant for the antagonist and was determined by means of Schild plot analysis as described in Methods. Data are the mean±s.e.mean (n=3–6 oocytes). [e]n_H is the Hill coefficient. Data are the mean±s.e.mean (n=3–6 oocytes). [f]Intrinsic activity (Im) of the compounds relative to the maximal response of GABA which is assigned as 100%.

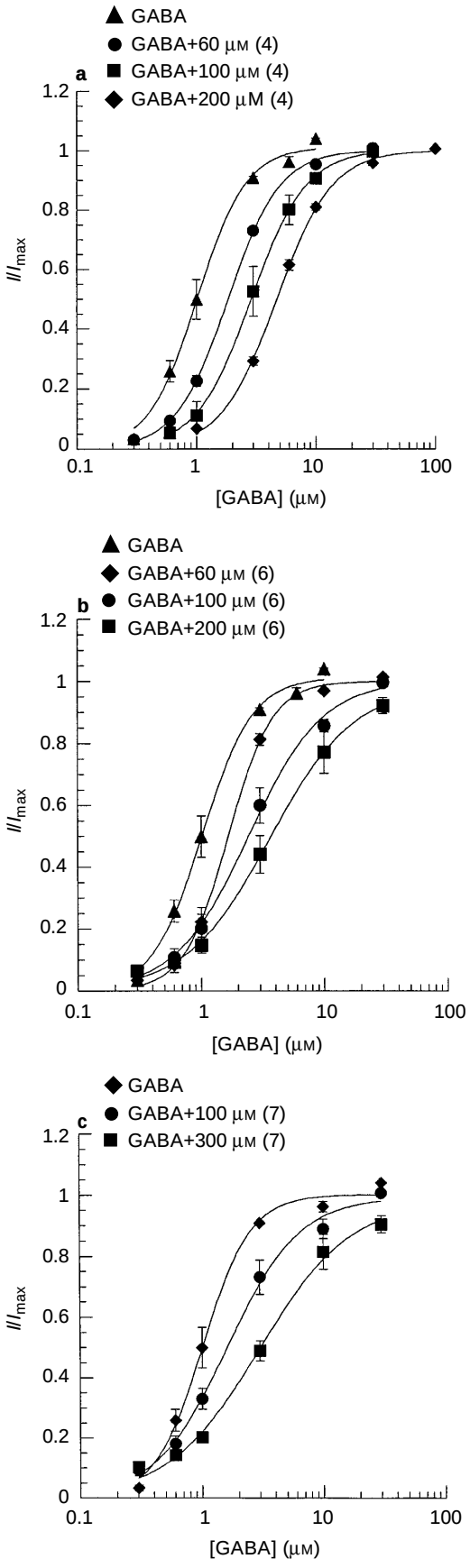

Figure 5 Schild analysis of (a) GABA alone, GABA in the presence of 60 μM, 100 μM and 200 μM *trans*-4-amino-2-methylbut-2-enoic acid (4). (b) GABA alone, GABA in the presence of 60 μM, 100 μM and 200 μM *trans*-4-amino-2-methylenebutanoic (6). (c) GABA alone, GABA in the presence of 100 μM and 300 μM 4-amino-2-chlorobutanoic acid (7).

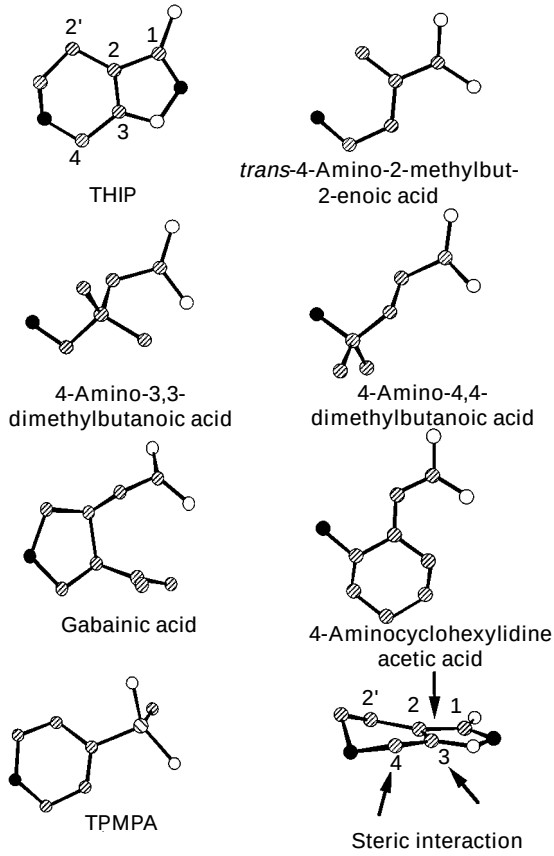

Figure 6 The alignment of GABA and TACA analogues against the 'muscimol' moiety of THIP. This results in a partial folding of the carbon chain around the C3 and C4 atoms of the TACA and GABA analogues. The C2 methyl group of the TACA analogue, *trans*-4-amino-2-methylbut-2-enoic acid, aligned relatively well against the C2′ carbon of THIP. The double bond of TPMPA aligned against the double bond of THIP. TPMPA, the most selective antagonist at $GABA_C$ receptors is a relatively flat compound and as a result both the amine and methylphosphinic acid moieties fitted well against the amine and isoxazole groups of THIP. As the methylphosphinic acid moiety is attached to the ring with a freely rotatable bond, the methyl group on the phosphorous atom can occupy most areas around that radius. Steric interaction around the acidic pharmacaphore appeared to play a lesser part in determining affinity at $GABA_C$ receptors. The other compounds mapped out areas were possible steric interaction may occur between the ligand and the protein. It appeared that compounds need to acquire a relatively flat conformation(s) to bind without steric interaction.

freely rotatable bond, the methyl group on the phosphorous atom can occupy most areas around that radius. Steric interaction around the acidic pharmacaphore appears to play a lesser part in determining affinity at $GABA_C$ receptors.

4-Amino-3,3-dimethylbutanoic acid, 4-amino-4,4-dimethylbutanoic acid, 2-aminocyclopentylidene acetic acid and gabainic acid could be superimposed against THIP. The compounds are not active as agonists or antagonists. However, the methyl substituents on the C3 carbon of 4-amino-3,3-dimethylbutanoic acid and the C4 carbon of 4-amino-4,4-dimethylbutanoic acid may map an area of unfavourable steric interaction between the ligand and the protein. This interaction may be due to a narrow binding site that cannot accommodate the whole of the carbon backbone. Hence it is proposed that any bulk that is located above or below the plane of THIP decreases the affinity of the compound for the receptor site. Similarly, this is observed with 2-aminocyclopentylidene acetic acid and gabainic acid where the substituents and the ring protrude above or below the plane of THIP. Figure 6 shows the area where a possible steric interaction may occur between the ligand and the receptor protein. It appears that compounds need to acquire a relatively flat conforma-

tion(s) to bind without an adverse steric interaction. This appears to be an important feature for the compounds to have in order to possess high affinity for the $GABA_C$ receptors.

The second possible fit of *trans*-4-amino-2-methylbut-2-enoic acid is aligned against THIP in the 'GABA' orientation as shown in Figure 7. This aligns the carbon backbone of the GABA and TACA analogues against the 'GABA' moiety of THIP. The C2 methyl group of *trans*-4-amino-2-methylbut-2-enoic acid was found to align relatively well against the C2′ carbon of THIP (Figure 7). Again the compound aligns in the plane of THIP. In this orientation, TPMPA aligns with the double bond aligning against the C2 and C3 carbons of THIP. Similarly 4-amino-3,3-dimethylbutanoic acid, 4-amino-4,4-dimethylbutanoic acid, 2-aminocyclopentylidene acetic acid and gabainic acid could be superimposed against THIP in this orientation. However, the lack of affinity of these compounds for the receptor indicates that the substitution on the C3 carbon of 4-amino-3,3-dimethylbutanoic acid, the C4 carbon of 4-amino-4,4-dimethylbutanoic acid and the rings and ring substituents 2-aminocyclopentylidene acetic acid and gabainic acid, respectively, map an area of unfavourable steric interaction between the ligand and the receptor protein.

Discussion

In this study, a known series of substituted analogues of GABA and TACA were studied to determine what position(s) of the carbon skeleton subsitution is tolerated for the development of active and selective agonists and antagonists for the $GABA_C$ receptor. Halo- and methyl-substitution on the C2, C3, C4 and nitrogen of TACA, GABA and some cyclic analogues were tested by use of the two-electrode voltage-clamp method with human ρ_1 mRNA expressed in *Xenopus* oocytes. Molecular modelling was also used to map areas of steric interaction and to identify what features a molecule may require to interact with the binding site.

Only the C2 position of TACA and GABA was found to tolerate substitution of either a methyl or a halo group at $GABA_C$ receptors. Substitution may be tolerated in this position because the substituent is able to lie in the plane of the active site. It is proposed that the binding site of $GABA_C$ receptors is smaller than the binding site of $GABA_A$ receptors. Most compounds used in this study have some effect as agonists at the $GABA_A$ receptors but it was only compounds with substituents on the C2 position that had some effect on $GABA_C$ receptors.

In general, antagonists are much larger than agonists. However, at $GABA_C$ receptors only small chemical changes are required at the C2 position to yield full agonists, weak partial agonists with low intrinsic activity and antagonists. This is not unique to GABA receptors but the phenomenon is rare. Both TACA and compound (3) are potent agonists while compound (4) is a moderately potent antagonist at $GABA_C$ receptors. Replacement of the hydrogen from the C2 position of TACA with a fluoro group maintains agonist activity. However, a methyl group in the same position changes the property of the compound from an agonist to an antagonist. It appears that the binding site has an area that tolerates the methyl group. This group lies in the plane with the rest of the molecule.

Compounds (5), (6) and (7) were weak partial agonists with low intrinsic activity. These compounds are flexible compounds and can adopt many more low energy conformations than either compounds (3) and (4). However, when the compounds bind to the active site, they are only able to activate the channel weakly. The substituent in the C2 position may interact sterically to prevent full activation of the channel.

The K_D values of compounds (5), (6) and (7) were larger than the corresponding K_B values for the corresponding partial agonists. The differences between the K_D values were about 3–4 times weaker than the K_B value hence compounds (5), (6) and (7) were less potent as agonists than antagonists. These differences are not as dramatic as those obtained for other compounds. Imidazole-4-acetic acid (I4AA) and (±)-*trans*-2-(aminomethyl)cyclopropane-1-carboxylic acid (TAMP) have differences between the K_D and K_B of the order of 25 and 100 times less potent as agonists than antagonists (Kusama *et al.*, 1993a). The large differences may be mediated by a high affinity binding of the antagonist to the open channel (Kusama *et al.*, 1993a). However, one has also to consider whether one binding event can explain both effects. The possibility that the compounds are mediated by two separate binding interactions may be valid in the case of I4AA and TAMP because the

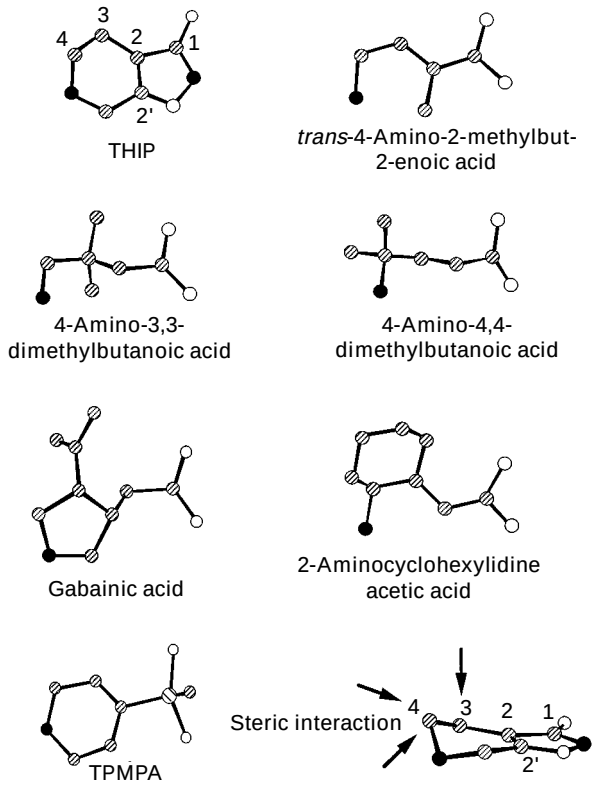

Figure 7 The alignment of GABA and TACA analogues against the 'GABA' moiety of THIP. This resulted in a partial folding of the carbon chain around the C3 and C4 atoms of the TACA and GABA analogues. The C2 methyl group of the TACA analogue, *trans*-4-amino-2-methylbut-2-enoic acid, aligned relatively well against the C2′ carbon of THIP. The double bond of TPMPA aligned against C2 and C3 carbons of THIP. The other compounds mapped out areas were a possible steric interaction may occur between the ligand and the protein. It appeared that compounds need to acquire a relatively flat conformation(s) to bind without steric interaction.

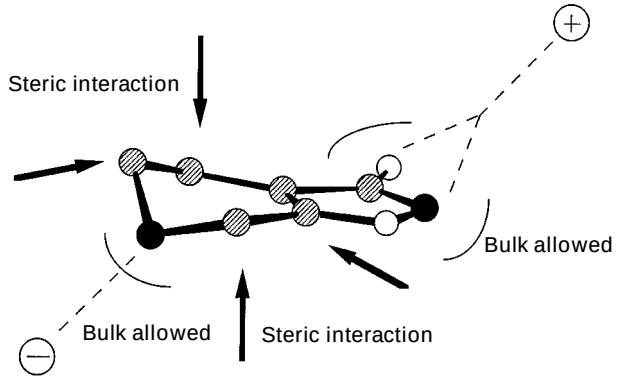

Figure 8 The relatively flat molecule, THIP, is shown the plane of the binding site summarizing the areas of possible steric interaction and areas where proposed bulk may be tolerated.

differences are so much greater than that observed with compounds (5), (6) and (7). I4AA and TAMP may be more active on the open channel than the closed channel as proposed by Kusama *et al*. (1993a). However, with compounds (5), (6) and (7), the differences are small and therefore the proposed mode of binding may be explained from one binding event.

It is proposed that the more flexible the C2 substituted compound is, the higher the intrinsic activity, while the larger the C2 substituent, the lower the intrinsic activity. Compound (6) is more flexible than compound (4) but less flexible than compounds (5) and (7). Consequently the intrinsic activity for compounds (5) and (7) is larger (12.1% and 5.2% of the maximal response of GABA, respectively). Furthermore the methylene group of compound (6) is smaller than the methyl group of compound (4). Thus, for compound (6) the intrinsic activity was higher at 4.4% of the maximal response of GABA whereas compound (4) was an antagonist with no observable effects on its own. The methyl substituent stops the channel from opening and this may be due to steric effects. A fluorine or a hydrogen group is very small and when compounds like TACA and compound (3) are bound to the receptor the transition of the channel from closed to open may be associated with a conformational change of the agonist. In the case of the antagonist, compound (4), steric interaction between the methyl group and the protein may lock the channel shut and conformational changes between the ligand and protein may not easily occur. This may explain the antagonistic effects of the compound.

Substitution in the C3, C4 and nitrogen positions were not tolerated and the compounds were inactive as agonists or antagonists. Disubstituted analogues were also found to be inactive and interact unfavourably at the $GABA_C$ receptor.

trans-4-Aminomethylbut-2-enoic acid has a methyl substituent on the amine moiety. This compound had no effect on the $GABA_C$ receptor. However, substitution on the nitrogen is not totally unfavourable. Compounds such as THIP, 2-(3-carboxypropyl)-3-amino-6-(4-methoxyphenyl)pyridazinium bromide (SR95331) and I4AA are competitive antagonists (Woodward *et al*., 1993). The nitrogen in these compounds is relatively planer and in the cases of SR95331 and I4AA the positive charge is delocalized. The substituents are relatively planer or may protrude away from the binding site. In *trans*-4-aminomethylbut-2-enoic acid, the nitrogen has a tetrahedral shape. The methyl group changes the electrical charge of the nitrogen and protrudes out of the plane of the compound. This may cause an unfavourable steric interaction with the protein. Figure 8 shows THIP, a relatively flat molecule, in the plane of the binding site. The figure also marks the areas of possible steric interaction and areas where proposed bulk may be tolerated.

At $GABA_C$ receptors, replacement of the carboxylic acid group with a phosphonic acid, phosphinic or sulphonic acid group produce potent antagonists (Woodward *et al*., 1993). In this study, compound (8), the sulphinic analogue of GABA, was found to be a potent partial agonist. The phosphinic, phosphonic, sulphonic and sulphinic acids are larger and have a different charge on the acid moiety than the corresponding carboxylic acids. The different electrical charge of these groups determines the type of interaction that may occur. The electrical charge of the phosphonic (Woodward *et al*., 1993), sulphonic (Woodward *et al*., 1993) and phosphinic acids (Woodward *et al*., 1993; Chebib *et al*., 1997) interact with the receptor to block the site rather than activate the channel. Steric effects appear to be less of a problem around the acidic group because the binding site on the $GABA_C$ receptor can accommodate the larger phosphinic, phosphonic, sulphonic and sulphinic acids.

Structure activity studies with a series of GABA agonists and antagonists that are both active and inactive aid in developing a model of the binding site at $GABA_C$ receptors. THIP, a conformationally restricted analogue of muscimol and a competitive antagonist at $GABA_C$ receptors was used as the basis of the modelling. Molecular modelling was performed on active and inactive compounds against THIP in two different orientations. The inactive compounds map areas where unfavourable steric interaction may occur.

It is difficult to determine which orientation represents the way GABA and TACA analogues interact with the binding site. Synthesis and analysis of substituted THIP analogues may provide a better understanding of where an unfavourable steric interaction occurs. Nevertheless, this modelling study may define several features of the $GABA_C$ receptor binding site. We propose that for compounds to have an effect as agonists or antagonists at $GABA_C$ receptors, compounds need to attain relatively flat conformations. TPMPA, the most selective antagonist at $GABA_C$ receptors, fits this model as it is relatively flat with the exception of the methylphosphinic acid moiety. Compounds substituted in the C2 position are tolerated by the receptor because the substituent does not protrude above or below the plane of the binding site. Steric interaction between the ligand and protein may occur in one or both positions around the C3 and C4 carbons of GABA and these are shown in Figures 6 and 7.

From this study, several properties of the binding site are proposed. The active site of the $GABA_C$ receptor appears to be a narrow cavity that occupies the carbon backbone. Any chemical feature that protrudes excessively above or below the plane, as shown in Figure 8, may interact unfavourably with the protein. This results in a loss of affinity of the compounds for the receptor. Consequently, GABA and TACA analogues that were substituted in the C3 and C4 positions were inactive at the $GABA_C$ receptor. Similarly with 2-aminocyclopentylidene acetic acid, 2-aminocyclohexylidine acetic acid, *cis*-5-aminocyclohex-3-ene carboxylic acid and gabainic acid, the substituents or the ring protrudes out of the plane, interacting unfavourably with the receptor protein.

Structure activity studies with a series of GABA agonists have been well documented for $GABA_A$ receptors (Krogsgaard-Larsen & Falch, 1981; Allan & Johnston, 1983; Krogsgaard-Larsen *et al*., 1983; 1994). Methyl substituted GABA analogues result in a drop of affinity but not a loss of affinity at the $GABA_A$ receptor and substitution in the C4 position is most tolerated (Allan & Johnston, 1983). Two or more methyl groups substituted on GABA results in a further drop in affinity. Halogenation of GABA and TACA at the C2 and C3 positions retains affinity at $GABA_A$ receptors (Johnston *et al*., 1978; Allan & Johnston, 1983). This is in contrast to $GABA_C$ receptors where only C2 substituted analogues have any effect on the receptor.

Replacement of the carboxylic acid group with a sulphonic or sulphinic acid group results in potent agonists at the $GABA_A$ receptor. However, replacement of the carboxylic acid group with a phosphinic or methylphosphinic acid group results in a drop of affinity for the $GABA_A$ receptor. In general, methylphosphinic acids have weaker effects than phosphinic acids on $GABA_A$ receptors. At $GABA_C$ receptors, this was different. Most methylphosphinic acids are relatively potent antagonists and are somewhat more potent than the corresponding phosphinic acids (Woodward *et al*., 1993; Chebib *et al*., 1997). Nitrogen substituted compounds are more tolerated at $GABA_A$ receptors than at $GABA_C$ receptors.

At $GABA_B$ receptors, substitution on the C3 position of GABA are tolerated. These include compounds such as (−)-baclofen which is an active $GABA_B$ receptor agonist (Kerr & Ong, 1995). Furthermore, $GABA_B$ receptors tolerate alkyl substituents on the C2 position of GABA. Substituting a hydrogen for a butyl or decyl group on the C2 position of GABA produced potent $GABA_B$ receptor antagonists (Kerr & Ong, 1995). This modification is similar to that found in $GABA_C$ receptors. However, a butyl or a decyl group is much larger than a methyl group. Therefore at $GABA_B$ receptors a larger alkyl group is required before antagonistic effects are obtained, while at $GABA_C$ receptors the presence of only a small alkyl group is required to obtain antagonistic effects.

In summary, the agonist/competitive antagonist binding site for $GABA_C$ receptors appears to be smaller than both $GABA_A$ and $GABA_B$ receptors. The site appears to be narrow such that

only relatively flat conformations can occupy the binding site. Compounds that have substituents that protrude above or below the plane may interact unfavourably with the receptor protein resulting in loss of affinity. Substitution on the C2 carbon of GABA and TACA are the only analogues that maintain affinity and these compounds can attain conformations whereby the substituent lies in the plane and, hence, are tolerated by the receptor, while substitution in any other position is not. Steric interactions around the C2 substituent may occur as a result of channel opening, hence compounds substituted in this position appear to be weak partial agonists with low intrinsic activity or antagonists.

The authors wish to thank Dr George Uhl, National Institute for Drug Abuse, Baltimore, for a gift of the ρ_1 cDNA, Dr Robin Allan, Dr Ken N. Mewett and Bruce Twitchin for the preparation of the compounds, Ann Mitrovic and Kong Li for their excellent technical assistance, and the National Health and Medical Research Council of Australia for financial support.

References

ALLAN, R.D. (1978). The synthesis of a decarboxylated derivative of the neurotoxin kainic acid. *Tetrahedron Lett.*, **19,** 2199 – 2200.

ALLAN, R.D. (1979). Synthesis of analogues of GABA. II 4-Alkyl-4-aminobut-2-enoic acid and a new synthesis of some vinyl α-amino acids. *Aust. J. Chem.*, **32,** 2507 – 2516.

ALLAN, R.D. & JOHNSTON, G.A.R. (1983). Synthetic analogs for the study of GABA as a neurotransmitter. *Med. Res. Rev.*, **3,** 91 – 118.

ALLAN, R.D., JOHNSTON, G.A.R. & TWITCHIN, B. (1980). Synthesis of analogues of GABA. V. *trans* and *cis* isomers of some 4-amino-3-halogenbut-2-enoic acids. *Aust. J. Chem.*, **33,** 1115 – 1122.

ALLAN, R.D. & TWITCHIN, B. (1978). Synthesis of some substituted 4-aminobut-2-enoic acids as analogues of the neurotransmitter GABA. *Aust. J. Chem.*, **31,** 2283 – 2289.

AMIN, J. & WEISS, D.S. (1994). Homomeric rho 1 GABA channels: activation properties and domains. *Receptors Channels*, **2,** 227 – 236.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol.*, **14,** 48 – 58.

BORMANN, J. & FEIGENSPAN, A. (1995). $GABA_C$ receptors. *Trends Neurosci.*, **18,** 515 – 519.

CHEBIB, M., VANDENBERG, R.J., FROESTL, W. & JOHNSTON, G.A.R. (1997). Unsaturated phosphinic analogues of γ-aminobutyric acid as $GABA_C$ receptor antagonists. *Eur. J. Pharmacol.*, **329,** 223 – 229.

CUTTING, G.R., CURRISTIN, S., ZOGHBI, H., O'HARA, B., SELDIN, M.F. & UHL G.R. (1992). Identification of a putative GABA ρ_2 receptor subunit cDNA and co-localization of the genes encoding ρ_2 and ρ_1 to human chromosome 6q14-q21 and the mouse chromosome 4. *Genomics*, **12,** 801 – 806.

CUTTING, G.R., LU, L., O'HARA, B., KASCH, L.M., DONOVAN, D., SHIMADA, S., ANTONARAKIS, S.E., GUGGINO, W.B., UHL, G.R. & KAZAZIAN, H.H. (1991). Cloning of the GABA ρ_1 cDNA: a novel GABA subunit highly expressed in the retina. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 2673 – 2677.

DAVIES, P.A., HANNA, M.C., HALES, T.G. & KIRKNESS, E.F. (1997). Insensitivity to anaesthetic agents conferred by class of $GABA_A$ receptor subunit. *Nature*, **385,** 820 – 823.

DONG, C.-J., PICAUD, S.A. & WEBLIN, F.S. (1994). GABA transporters and $GABA_C$-like receptors on catfish cone- but not rod-driven horizontal cells. *J. Neurosci.*, **14,** 2648 – 2658.

DREW, C.A., JOHNSTON, G.A.R. & WEATHERBY, R.P. (1984). Bicuculline-insensitive GABA receptors: studies on the binding of (−)-baclofen to rat cerebellar membranes. *Neurosci. Lett.*, **52,** 317 – 321.

ENZ, R., BRANDSTATTER, J.H., HARTVEIT, E., WASSLE, H. & BORMANN, J. (1995). Expression of GABA receptor rho1 and rho2 subunits in the retina and brain of rat. *Eur. J. Neurosci.*, **7,** 1495 – 1501.

FEIGENSPAN, A. & BORMANN, J. (1994). Differential pharmacology of $GABA_A$ and $GABA_C$ receptors on rat retinal bipolar cells. *Eur. J. Pharmacol - Mol. Pharmacol. Sect.*, **288,** 97 – 104.

FEIGENSPAN, A., WÖSSLE, H. & BORMANN, J. (1993). Pharmacology of GABA receptor Cl^- channels in rat retinal bipolar cells. *Nature*, **361,** 159 – 162.

JOHNSTON, G.A.R. (1996a). $GABA_A$ receptor pharmacology. *Pharmacol. Ther.*, **69,** 173 – 198.

JOHNSTON, G.A.R. (1996b). $GABA_C$ receptors relatively simple transmitter-gated ion channels? *Trends Pharmacol. Sci.*, **17,** 319 – 323.

JOHNSTON, G.A.R., ALLAN, R.D., KENNEDY, S.M.E. & TWITCHIN, B. (1978). Systematic study of GABA analogues of restricted conformation. In *GABA-Neurotransmitters*. Alfred Benzon Symposium 12, pp. 149 – 164. Copenhagen, Denmark: Munksgaard.

JOHNSTON, G.A.R., CURTIS, D.R., BEART, P.M., GAME, C.J.A., MCCULLOCH, R.M. & TWITCHIN, B. (1975). *cis*- and *trans*-4-Aminocrotonic acid as GABA analogues of restricted conformation. *J. Neurochem.*, **24,** 157 – 160.

KAUPMANN, K., HUGGEL, K., HEID, J., FLOR, P.J., 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*, **386,** 239 – 246.

KERR, D.I.B. & ONG, J. (1995). $GABA_B$ receptors. *Pharmacol. Ther.*, **67,** 187 – 246.

KROGSGAARD-LARSEN, P. & FALCH, E. (1981). GABA agonists. Development and interaction with the GABA receptor complex. *Mol. Cellular Biochem.*, **38,** 129 – 146.

KROGSGAARD-LARSEN, P., FALCH, E., PEET, M.J., LEAH, J.D. & CURTIS, D.R. (1983). Molecular pharmacology of the GABA receptor and GABA agonists. *Adv. Biochem. Psychopharmacol.*, **37,** 1 – 13.

KROGSGAARD-LARSEN, P., FROLUND, B., JORGENSEN, F.S. & SCHOUSBOE, A. (1994). $GABA_A$ receptor agonists, partial agonists and antagonists. Design and therapeutic prospects. *J. Med. Chem.*, **37,** 2489 – 2505.

KROGSGAARD-LARSEN, P., HJEDS, H., CURTIS, D.R., LODGE, D. & JOHNSTON, G.A.R. (1979). Dihydromuscimol, thiomuscimol and related heterocyclic compounds as GABA analogues. *J. Neurochem.*, **32,** 1717 – 1724.

KROGSGAARD-LARSEN, P., JOHNSTON, G.A.R., LODGE, D. & CURTIS, D.R. (1977). A new class of GABA agonists. *Nature*, **268,** 53 – 58.

KUSAMA, T., SPIVAK, C.E., WHITING, P., DAWSON, V.L., SCHAEFFER, J.C. & UHL, G.R. (1993a). Pharmacology of GABA ρ1 and GABA α/β receptors expressed in *Xenopus* oocytes and COS cells. *Br. J. Pharmacol.*, **109,** 200 – 206.

KUSAMA, T., WANG, T.-L., GUGGINO, W.B., CUTTING, G.R. & UHL, G.R. (1993b). GABA ρ2 receptor pharmacology profile: GABA recognition site similarities to ρ1. *Eur. J. Pharmacol.-Mol. Pharmacol. Sect.*, **245,** 83 – 84.

LUKASIEWICZ, P.D., MAPLE, B.R. & WEBLIN, F.S. (1994). A novel GABA receptor on bipolar cell terminals in the tiger salamander retina. *J. Neurosci.*, **14,** 1202 – 1212.

MURATA, Y., WOODWARD, R.M., MILEDI, R. & OVERMAN, L.E. (1996). The first selective antagonist for a $GABA_C$ receptor. *Bioorg. Med. Chem. Lett.*, **6,** 2071 – 2076.

OGURUSU, T. & SHINGAI, R. (1996). Cloning of a putative γ-aminobutyric acid GABA receptor subunit ρ_3 cDNA. *Biochim. Biophys. Acta*, **1305,** 15 – 18.

POLENZANI, L., WOODWARD, R.M. & MILEDI, R. (1991). Expression of mammalian γ-aminobutyric acid receptors with distinct pharmacology in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 4318 – 4322.

QIAN, H. & DOWLING, J.E. (1993). Novel GABA responses from rod-driven retinal horizontal cells. *Nature*, **361,** 162 – 164.

RAGOZZINO, D., WOODWARD, R.M., MURATA, F., EUSEBI, 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.

SHIMADA, S., CUTTING, G. & UHL, G.R. (1992). γ-Aminobutyric acid A or C receptor? γ-Aminobutyric acid ρ1 receptor RNA induces bicuculline-, barbiturate-, and benzodiazepine-insensitive γ-aminobutyric acid responses in *Xenopus* oocytes. *Mol. Pharmacol.*, **41,** 683 – 687.

WANG, T.-L., GUGGINO, W.B. & CUTTING, G.R. (1994). A novel γ-aminobutyric acid receptor subunit (ρ2) cloned from human retina forms bicuculline-insensitive homooligomeric receptors in *Xenopus* oocytes. *J. Neurosci.*, **14,** 6524–6531.

WOODWARD, R.M., POLENZANI, L. & MILEDI, R. (1993). Characterization of bicuculline/baclofen-insensitive (ρ-like) γ-aminobutyric acid receptors expressed in *Xenopus* oocytes. II. Pharmacology of γ-aminobutyric acid$_A$ and γ-aminobutyric acid$_B$ receptor agonists and antagonists. *Mol. Pharmacol.*, **43,** 609–625.

ZHANG, D.X., PAN, Z.H., ZHANG, X.H., BRIEAU, A.D. & LIPTON, S.A. (1995). Cloning of gamma-aminobutyric acid type C receptor subunit in rat retina with a methionine residue critical for picrotoxinin channel block. *Proc. Natl. Acad. Sci. U.S.A.*, **92,** 11756–11760.

(Received June 18, 1997
Revised August 22, 1997
Accepted September 10, 1997)