

(3-Aminocyclopentyl)methylphosphinic acids: Novel GABA_C receptor antagonists[☆]

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Abstract

Our understanding of the role GABA_C receptors play in the central nervous system is limited due to a lack of specific ligands. Here we describe the pharmacological effects of (±)-*cis*-3- and (±)-*trans*-3-(aminocyclopentyl)methylphosphinic acids ((±)-*cis*- and (±)-*trans*-3-ACPMPA) as novel ligands for the GABA_C receptor showing little activity at GABA_A or GABA_B receptors. (±)-*cis*-3-ACPMPA has similar potency to (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA) at human recombinant ρ1 ($K_B = 1.0 \pm 0.2 \mu\text{M}$) and rat ρ3 ($K_B = 5.4 \pm 0.8 \mu\text{M}$) but is 15 times more potent than TPMPA on human recombinant ρ2 ($K_B = 1.0 \pm 0.3 \mu\text{M}$) GABA_C receptors expressed in *Xenopus* oocytes. (±)-*cis*- and (±)-*trans*-3-ACPMPA are novel lead compounds for developing into more potent and selective GABA_C receptor antagonists with increased lipophilicity for in vivo studies.

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Keywords: GABA; GABA_C receptors; GABA antagonists; (1,2,5,6-Tetrahydropyridin-4-yl)methylphosphinic acid; (±)-*cis*-3- and (±)-*trans*-3-(Aminocyclopentyl)methylphosphinic acids

1. Introduction

The GABAergic system consists of three major receptor classes termed GABA_A, GABA_B and GABA_C (Bormann, 2000; Chebib and Johnston, 2000). The GABA_A and GABA_C receptors are members of the ligand-gated ion channel superfamily, which include the nicotinic acetylcholine (nACh), GABA_A, strychnine-sensitive glycine and serotonin type 3 (5-HT₃) receptors (Bormann, 2000; Chebib and Johnston, 2000). At least 16 human GABA_A receptor subunits have been described and classified under seven subfamilies of

protein subunits: α, β, γ, δ, ε, θ, and π. This provides great diversity among the GABA_A receptors with the most common being the α1β2γ2 subtype constituting approximately 18% of all GABA_A receptors in the human brain (Whiting, 2003).

In contrast, the GABA_C receptor is generally made up solely of ρ-subunits indicating a more simple class of receptor. The ρ-subunits have been cloned from human, rat, mouse, perch and chick retinas. In total, five ρ-subunit types have been identified, termed ρ1–5 (Bormann, 2000; Chebib and Johnston, 2000). Two subunits (ρ1 and ρ2) have been cloned from human, while in rat, three subunits (ρ1–3) have been cloned. There is a high degree of sequence homology (>92%) shared between human and rat ρ-subunits, while 60–74% sequence homology is exhibited between the various ρ-subunits. The subunits form functional homomeric receptors (formed from ρ1, ρ2 or ρ3 subunits; Kusama et al., 1993a,b; Enz and Cutting, 1998; Ogurusu et al., 1999) or pseudo-heteromeric receptors

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(formed from a combination of $\rho 1$ and $\rho 2$ subunits, or $\rho 2$ and $\rho 3$ subunits; Enz and Cutting, 1998; Ogurusu et al., 1999).

The GABA_B receptor is a member of the Family 3 class of G-protein coupled receptors. These receptors exist as heterodimers consisting of GABA_{B1} and GABA_{B2} subunits, which are required to form functional receptors both in vivo and in vitro (reviewed in Cryan and Kaupmann, 2005; Ong and Kerr, 2000; White et al., 1998). These receptors couple to Gi/o and activate second messenger systems and ion channels including inwardly rectifying potassium channels (GIRKs) such as GIRK1 and GIRK4. A number of isoforms of the GABA_{B1} receptor exist (GABA_{B(1a-g)}) and to date, the pharmacology of the major isoforms GABA_{B(1a)} and GABA_{B(1b)} has not been shown to be different (reviewed in Cryan and Kaupmann, 2005; Green et al., 2000).

While there has been a plethora of studies regarding the role of GABA_A and GABA_B receptors in the central nervous system (CNS), GABA_C receptors have been less well studied. Found in the retina (Enz et al., 1995), hippocampus (Enz et al., 1995; Boue-Grabot et al., 1998; Alakuijala et al., 2006), pituitary (Boue-Grabot et al., 2000) and gut (Jansen et al., 2000), GABA_C receptors may play a role in visual processing, memory and learning, regulation of hormones and neuroendocrine gastrointestinal secretion.

The pharmacology of GABA_C receptors is quite distinct from that of either the GABA_A or GABA_B receptors. GABA_C receptors are not inhibited by the alkaloid, bicuculline, which affects GABA_A receptors, or activated by the GABA_B receptor agonist (–)-baclofen (Chebib and Johnston, 2000). Instead, GABA_C receptors are activated by (+)-*cis*-2-(aminomethyl)cyclopropanecarboxylic acid ((+)-CAMP; Fig. 1) (Duke et al., 2000) and *cis*-4-aminocrotonic acid (CACA).

The first selective GABA_C receptor antagonist that differentiated GABA_C receptors from both GABA_A and GABA_B receptors was (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA; Fig. 1; Ragazzino et al., 1996). TPMPA has been shown to (1) enhance reinforced memory (Gibbs and Johnston, 2005); (2) inhibit the neuroprotective effects of dihydrohonokiol-B (Liu et al., 2005); (3) affect the sleep-waking behaviour of rats (Arnaud et al., 2001); (4) inhibit ammonia-induced apoptosis in hippocampal neurons (Yang et al., 2003); (5) regulate hormone release in the pituitary (Boue-Grabot et al., 2000); and (6) inhibit synaptic transmission in the neonatal rat spinal cord in vitro (Rozzo et al., 1999). These studies provide some clues to the role GABA_C receptors play in the CNS but a lack of specific ligands for this receptor limits

study in this area (Johnston, 2002; Johnston et al., 2003). There have been no reports describing the central effects of TPMPA upon systemic administration suggesting it does not cross the blood brain barrier. Thus selective, more lipophilic agents are required as pharmacological tools to evaluate the role GABA_C receptors play in the CNS (Johnston, 2002; Johnston et al., 2003). In this study, we identify (±)-*cis*-(3-aminocyclopentyl)methylphosphinic acid ((±)-*cis*-3-ACMPA) and (±)-*trans*-(3-aminocyclopentyl)methylphosphinic acid ((±)-*trans*-3-ACMPA) as potent and selective GABA_C receptor antagonists.

2. Methods

2.1. Materials

(1,2,5,6-Tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA), (piperidin-4-yl)methylphosphinic acid (P4MPA), (±)-*cis*-(3-aminocyclopentyl)methylphosphinic acid ((±)-*cis*-3-ACMPA) and (±)-*trans*-(3-aminocyclopentyl)methylphosphinic acid ((±)-*trans*-3-ACMPA) were synthesised according to our previously published methods (Hanrahan et al., 2001, 2006). GABA was obtained from Sigma Chemical Co. (St Louis, MO, USA).

Human $\rho 1$ cDNA encapsulated in the pcDNA1.1 vector (Invitrogen, San Diego, CA, USA) was donated by Dr George Uhl (National Institute for Drug Abuse, Baltimore, MD, USA). Human $\rho 2$ cDNA encapsulated in the pKS vector was kindly donated by Dr Garry Cutting (Center for Medical Genetics, Johns Hopkins University, School of Medicine, Baltimore, MD, USA). Rat $\rho 3$ cDNA encapsulated in pBluescript KS(–) vector was a kind gift from Dr Ryuzo Shingai (Department of Welfare Engineering, Iwate University, Morioka, Japan). Human $\alpha 1$, $\beta 2$ and $\gamma 2$ GABA_A cDNAs encapsulated in pcDM8 were gifts from Dr Paul Whiting (Merck Sharpe and Dohme, Harlow, UK). Human GABA_{B(1b)}, GABA_{B2} cDNA and rat G-protein coupled inwardly rectifying potassium channels (GIRK) 1 and 4 were provided by Dr Fiona Marshall (GlaxoWellcome, UK). Human GABA_{B(1b)} was encapsulated in the pcDNA3.1(–) (Invitrogen USA), GABA_{B2} and rat GIRK1 were encapsulated in the pcDNA3 (Invitrogen USA) while the rat GIRK4 was encapsulated in pBluescript KS(–) (Stratagene USA).

Xenopus laevis were obtained from an African *Xenopus* colony and housed in the Department of Veterinary Science at the University of Sydney.

2.2. Isolation and purification of cRNA

Escherichia coli containing $\rho 1$, $\rho 2$, $\rho 3$, GABA_A $\alpha 1$, $\beta 2$ and $\gamma 2$, GABA_{B(1b)}, GABA_{B2}, GIRK1 and GIRK4 cDNA encapsulated in the relevant plasmid vector were cultured and released using the Wizard[®] Plus Minipreps kit (Promega Corporation, Madison, WI, USA). cDNAs were linearised with restriction endonuclease (Table 1) for 2 h at 37 °C.

Linearised cDNA was then purified and precipitated with ethanol and 10% sodium acetate (pH 5.2). Capped RNA was synthesised from linearised plasmid containing cDNAs using the T7 “mMESSAGE mMACHINE” kit from Ambion Inc. (Austin, Texas, USA) with the exception of $\rho 3$ cDNA where T3 mMESSAGE mMACHINE kit (Ambion Inc., Austin, TX, USA) was used instead.

2.3. Electrophysiological recording

Female *X. laevis* were anaesthetised with 0.17% 3-aminobenzoic acid ethyl ester and a lobe of the ovaries was removed. Lobes were thoroughly rinsed with Ca²⁺-free OR-2 buffer (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES, pH 7.5) and treated with Collagenase A (2 mg/mL in OR-2) for 2 h to separate oocytes from connective tissue and follicular cells. Released oocytes were then rinsed in ND96 storage solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, pH 7.5, supplemented with 2.5 mM pyruvate, 0.5 mM theophylline and 50 μ M/mL gentamycin). Stage

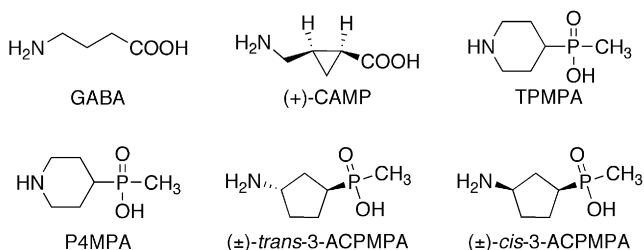


Fig. 1. Structures of GABA_C ligands.

Table 1
List of cDNAs with corresponding endonuclease used in this study

cDNA	Enzyme	cDNA	Enzyme	cDNA	Enzyme	cDNA	Enzyme
$\rho 1$	<i>Xba1</i>	$\rho 3$	<i>ECORI</i>	$\rho 2$	<i>ECORI</i>	$\alpha 1$	<i>NOT1</i>
$\beta 2$	<i>NOT1</i>	$\gamma 2$	<i>NOT1</i>	GABA _{B(1b)}	<i>BAMH1</i>	GABA _{B2}	<i>Xba1</i>
GIRK1	<i>NOT1</i>	GIRK4	<i>Xba1</i>				

V–VI oocytes were collected and stored at 16 °C in ND96 storage solution with constant mixing in an orbital shaker.

$\rho 1$ cRNA (10 ng/50 nL), $\rho 2$ cRNA (10–50 ng/50 nL), $\rho 3$ cRNA (10 ng/50 nL), $\alpha 1\beta 2\gamma 2$ cRNAs (20 ng/50 nL) in a 1:1:2 ratio, or GABA_{B(1b)}, GABA_{B2}, GIRK1 and GIRK4 cRNAs (20 ng/50 nL) in a 1:2:1:1 ratio were injected into the cytoplasm of defolliculated Stage V *Xenopus* oocytes.

Two to eight days after injection of the oocyte with mRNA, receptor activity was measured by two-electrode voltage clamp recording using a Geneclamp 500 amplifier (Axon Instruments Inc., Foster City, CA, USA), a MacLab 2e recorder (ADInstruments, Castle Hill, NSW, Australia) and Chart version 3.6.3 software (ADInstruments, Castle Hill, NSW, Australia). Oocytes were clamped at –60 mV using two micropipettes containing 3 M KCl. Oocytes were continually superfused with ND96 recording solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, pH 7.5) at a rate of 5 mL/min with the exception of the GABA_B receptor where high potassium buffer (45 mM KCl, 51 mM NaCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, pH 7.5) was used for recording.

Oocytes were first screened for receptor activity by the addition of maximal dose of GABA (100 μ M or 1 mM) and, where currents were greater than 50 nA, were used for further recording. All compounds were dissolved in distilled water to a stock concentration of 100 mM and stored at –20 °C. All

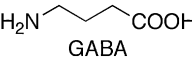
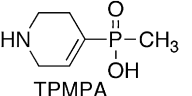
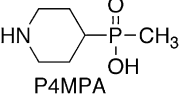
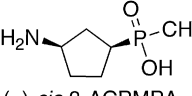
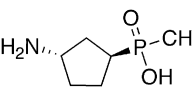
compounds were further diluted using ND96 or high potassium buffer recording solution to the required concentration.

Compounds were screened for agonist activity by applying increasing concentrations of the compound to the cell bath until the maximal current was attained. Where compounds showed agonist effects, a current dose–response relationship was obtained and compared to the maximum effect of GABA (100 μ M or 300 μ M) for that cell. Compounds were also screened for antagonist effects by testing the compound in the presence of a submaximal dose of GABA (1 μ M, 3 μ M or 10 μ M). The effects of antagonists were further evaluated for their competitive actions. For each drug dose–response, a minimum of three cells were used to ensure that an accurate and replicable dose–response relationship was produced.

2.4. Analysis of kinetic data

Current dose–response relationships for agonists and antagonists were measured by recording the peak amplitude of current obtained for each concentration of drug and standardised by calculating the ratio, I/I_{\max} , where I is the peak amplitude of current at a given concentration of agonist, GABA, and I_{\max} is the maximal current generated by GABA at individual oocytes.

Table 2
The effects of GABA, TPMPA, P4MPA, (\pm)-*cis*- and (\pm)-*trans*-3-ACPMA on recombinant GABA receptors expressed in oocytes^a

Compound	GABA _A human $\alpha 1\beta 2\gamma 2$	GABA _B human GABA _{B(1b2)}	Human $\rho 1$	GABA _C human $\rho 2$	Rat $\rho 3$
 GABA	16.5 \pm 0.5 μ M ^b 1.3 \pm 0.1 ^c (<i>n</i> = 6)	2.3 \pm 0.4 μ M ^b 1.0 \pm 0.1 ^c (<i>n</i> = 4)	1.0 \pm 0.1 μ M ^b 2.4 \pm 0.3 ^c (<i>n</i> = 6)	0.8 \pm 0.1 μ M ^b 1.2 \pm 0.2 ^c (<i>n</i> = 7)	4.0 \pm 0.2 μ M ^b 1.8 \pm 0.1 ^c (<i>n</i> = 4)
 TPMPA	67 \pm 3% ^d (<i>n</i> = 3)	>>300 μ M ^b (<i>n</i> = 4)	2.3 \pm 0.4 μ M (<i>n</i> = 3)	14.9 \pm 1.5 μ M (<i>n</i> = 3)	4.5 \pm 0.8 μ M (<i>n</i> = 3)
 P4MPA	>100 μ M ^c	>1000 μ M ^{a,e}	6.0 \pm 1.2 μ M ^c	4.2 \pm 0.2 μ M ^c	10.2 \pm 23 μ M ^f
 (\pm)- <i>cis</i> -3-ACPMPA	8 \pm 1% ^d (<i>n</i> = 3)	50.7 \pm 3.0 μ M ^b 1.4 \pm 0.1 ^c 84 \pm 2% ^g (<i>n</i> = 3)	1.0 \pm 0.2 μ M (<i>n</i> = 6)	1.0 \pm 0.3 μ M (<i>n</i> = 4)	5.4 \pm 0.8 μ M (<i>n</i> = 4)
 (\pm)- <i>trans</i> -3-ACPMPA	11 \pm 1% ^d (<i>n</i> = 3)	131.7 \pm 3.8 μ M ^b 1.3 \pm 0.1 ^c 85 \pm 1% ^g (<i>n</i> = 3)	6.6 \pm 0.7 μ M (<i>n</i> = 3)	5.4 \pm 0.8 μ M (<i>n</i> = 4)	17.7 \pm 2.2 μ M (<i>n</i> = 4)

^a Unless otherwise stated, values in the table are the dissociation constants (K_B) of the antagonist. Data are mean \pm s.e.m.

^b EC₅₀ values of agonists. The EC₅₀ is the effective dose that activates 50% of the I_{\max} and I_{\max} is the maximum current produced by the agonist. Data are mean \pm s.e.m.

^c The Hill coefficient (n_H). Data are mean \pm s.e.m.

^d Percentage inhibition by 300 μ M of compound (for direct comparison to TPMPA) of the current produced by a submaximal dose of GABA (10 μ M; EC₂₀).

^e Data are from Johnston et al. (1998).

^f Data are from Vien et al. (2002).

^g I_{\max} is the intrinsic activity calculated as a percentage of the maximum whole cell current produced by a maximum dose of GABA, which has been assigned as 100%. Data are mean \pm s.e.m.

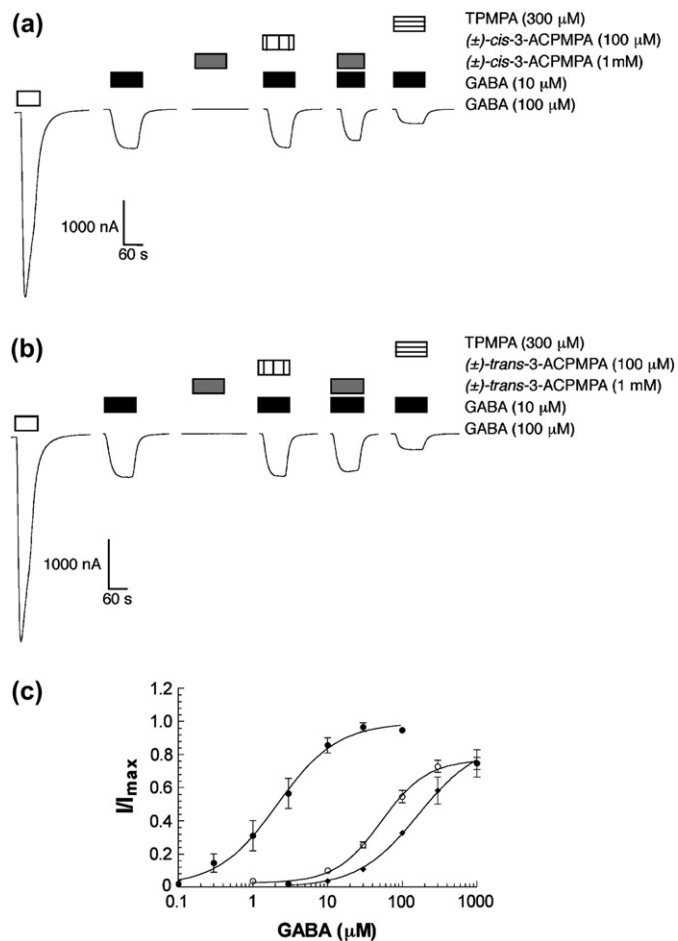


Fig. 2. (a) GABA (100 μ M) (duration indicated by open bar) activated a maximal inward current in oocytes expressing $\alpha 1\beta 2\gamma 2$ GABA_A receptors and clamped at -60 mV. GABA (10 μ M) (duration indicated by black bar) activated an inward current 20% of the maximal current produced by GABA (100 μ M). (\pm)-*cis*-3-ACPMPA (1 mM, duration indicated by the grey bar) did not activate a current. When co-applied with GABA (10 μ M) (\pm)-*cis*-3-ACPMPA (100 μ M, duration indicated by vertically striped bar) did not significantly reduce the GABA response. When co-applied with GABA (10 μ M) (\pm)-*cis*-3-ACPMPA (1 mM) and TPMPA (300 μ M, duration indicated by horizontally striped bar) reduced the GABA response by 21% and 63%, respectively; (b) GABA (100 μ M) (duration indicated by open bar) activated a maximal inward current in oocytes expressing $\alpha 1\beta 2\gamma 2$ GABA_A receptors and clamped at -60 mV. GABA (10 μ M) (duration indicated by black bar) activated an inward current 20% of the maximal current produced by GABA (100 μ M). (\pm)-*trans*-3-ACPMPA (1 mM, duration indicated by the grey bar) did not activate a current. When co-applied with GABA (10 μ M) (\pm)-*trans*-3-ACPMPA (100 μ M, duration indicated by vertically striped bar) did not significantly reduce the GABA response. When co-applied with GABA (10 μ M) (\pm)-*trans*-3-ACPMPA (1 mM) and TPMPA (300 μ M, duration indicated by horizontally striped bar) reduced the GABA response by 13% and 63%, respectively (c) Dose–response curves for GABA (\bullet , $n = 4$), (\blacksquare , $n = 3$) and (\blacklozenge , $n = 3$) at human GABA_{B(1b,2)} receptors coupled to GIRK1 and GIRK4 expressed in *Xenopus* oocytes. Data are the mean \pm s.e.m.

Data are expressed as the mean current (nA) or ratio of the maximal GABA response (I/I_{max}) \pm standard error of the mean (s.e.m.).

EC_{50} values were calculated from dose–response data by fitting ratios of maximal GABA current as a function of agonist concentration by least squares method to the Hill equation $I = I_{max}[A]^{n_H}/(EC_{50}^{n_H} + [A]^{n_H})$ using Kaleidagraph 4.01, where I is the peak current at a given concentration of agonist, I_{max} is the

maximal current generated by the concentration of agonist, $[A]$ is the concentration of the agonist, n_H is the Hill coefficient, and EC_{50} is the effective dose that activates 50% of the maximal current for individual cells. EC_{50} values are expressed as mean \pm s.e.m.

The dissociation constant of an antagonist (K_B) is the effective concentration at which an antagonist binds to half the receptor population. Where the EC_{50} of GABA was determined in multiple concentrations of the antagonist, competitive antagonism was tested for via the method of Lew and Angus (1995). This involved using a non-linear regression and an f -test to determine which of the following equations fit the data more accurately:

$$\log([GABA^*]) = -\log([Ant] + 10^{\log(K_B)}) - P \quad (1)$$

$$\log([GABA^*]) = -\log([Ant]^{slope} + 10^{\log K}) - P \quad (2)$$

where $[GABA^*]$ is the EC_{50} of GABA, $[Ant]$ is the concentration of antagonist and P is a constant. In the case of the simpler model (Eq. (1)) being more suitable, the interaction is defined as competitive antagonism (Lew and Angus, 1995) and pA_2 ($-\log K_B$) values determined from the fitting result.

Where the EC_{50} of GABA in the presence of only a single concentration of antagonist was determined, competitive antagonism was assumed based on the lack of intrinsic efficacy and a linear shift of the GABA dose response curve to the right. The apparent pA_2 values were determined via Eq. (1) above.

The K_B for each antagonist were subsequently determined from the calculated pA_2 values, and expressed as mean \pm s.e.m.

Statistical significance of results was tested for via a t -test and p values stated where needed.

3. Results

Expression of GABA_A, GABA_B and GABA_C receptors in *Xenopus* oocytes generated GABA gated responses similar to those described in the literature (Kusama et al., 1993a,b; White et al., 1998; Ogurusu et al., 1999). The amplitude of the whole cell currents recorded ranged between 50 and 5000 nA when the cell was clamped at -60 mV. Increasing concentrations of GABA produced a dose dependent effect at the receptors and the EC_{50} values for GABA at human $\alpha 1\beta 2\gamma 2$ GABA_A, GABA_{B(1b,2)}}, $\rho 1$ and $\rho 2$ GABA_C receptors and rat $\rho 3$ GABA_C receptors are summarised in Table 2.

The effects of TPMPA, (\pm)-*cis*- and (\pm)-*trans*-3-ACPMPA were studied on all three classes of GABA receptors. TPMPA had similar effects on recombinant GABA receptors as previously reported (summarised in Table 2; Ragazzino et al., 1996; Chebib et al., 1998; Vien et al., 2002).

Fig. 2 ((a) and (b)) shows the effects of TPMPA, (\pm)-*cis*- and (\pm)-*trans*-3-ACPMPA on GABA_A and GABA_B receptors. (\pm)-*cis*- (1 mM) and (\pm)-*trans*-3-ACPMPA (1 mM) had weak antagonist effects on human $\alpha 1\beta 2\gamma 2$ GABA_A receptors inhibiting the EC_{20} of GABA (10 μ M) by $21 \pm 1\%$ and $13 \pm 1\%$, respectively, whereas no significant effect was observed at 100 μ M. Thus the effects of these compounds were not significantly different ($p < 0.05$) at GABA_A receptors. TPMPA (300 μ M, $K_B = 320$ μ M, Ragazzino et al., 1996) inhibited the response of 10 μ M GABA by $62 \pm 3\%$, which was significantly more active than (\pm)-*cis*- ($p < 0.0001$) and (\pm)-*trans*-3-ACPMPA ($p < 0.0001$) at the same concentration. Complete K_B determination was not performed due to the weak observed activity and the large amounts of compound required.

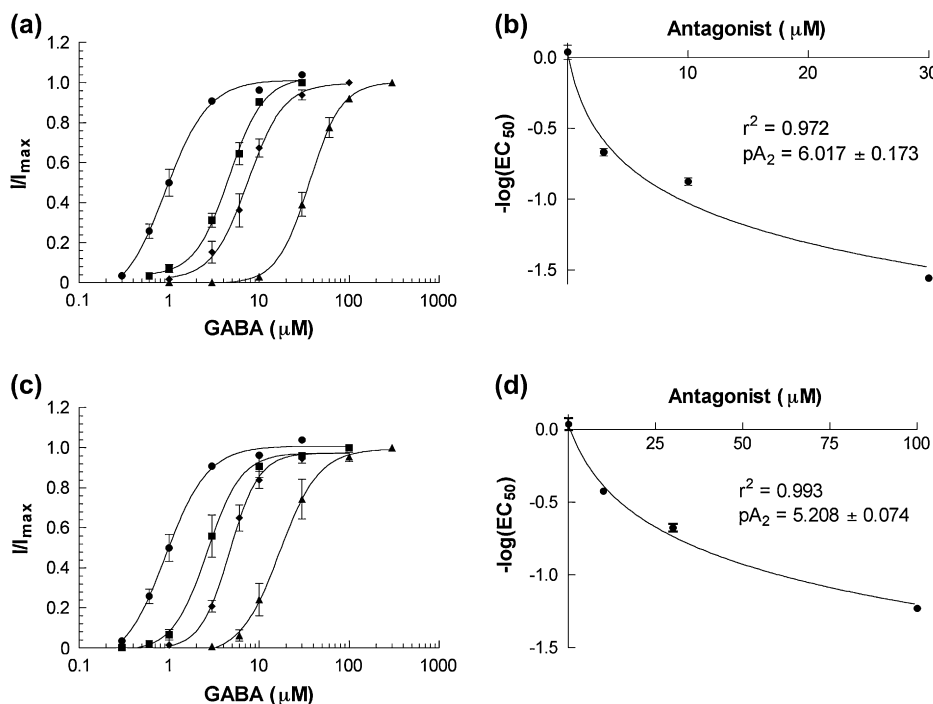


Fig. 3. (a) Dose–response curves for GABA alone (\bullet , $n = 6$) and GABA in the presence of 3 μM (\blacksquare , $n = 3$), 10 μM (\blacklozenge , $n = 6$) and 30 μM (\blacktriangle , $n = 3$) (\pm)-*cis*-3-ACPMPA at human $\rho 1$ GABA_C receptors expressed in *Xenopus* oocytes; (b) dependence of GABA $\log(\text{EC}_{50})$ on (\pm)-*cis*-3-ACPMPA concentration and results of non-linear regression; (c) dose–response curves for GABA alone (\bullet , $n = 6$) and GABA in the presence of 10 μM (\blacksquare , $n = 3$), 30 μM (\blacklozenge , $n = 3$) and 100 μM (\blacktriangle , $n = 3$) (\pm)-*trans*-3-ACPMPA at human $\rho 1$ GABA_C receptors expressed in *Xenopus* oocytes; (d) dependence of GABA $\log(\text{EC}_{50})$ on (\pm)-*trans*-3-ACPMPA concentration and results of non-linear regression. The pA_2 values determined are significantly different for the results obtained in (b) and (d) ($p < 0.0001$). Data are the mean \pm s.e.m.

(\pm)-*cis*- and (\pm)-*trans*-3-ACPMPA had moderate agonist effects on GABA_B receptors (Fig. 2 (c)). (\pm)-*cis*-3-ACPMPA ($I_{\text{max}} = 84 \pm 2\%$) and (\pm)-*trans*-3-ACPMPA ($I_{\text{max}} = 85 \pm 1\%$) were partial agonists with an EC_{50} of $50.7 \pm 3.0 \mu\text{M}$ and $131.7 \pm 3.8 \mu\text{M}$, respectively. The EC_{50} values for these compounds were found to be significantly different ($p < 0.0001$). Both compounds had significantly greater activity as agonists than TPMPA ($\text{EC}_{50} = 500 \mu\text{M}$, Ragazzino et al., 1996, $p < 0.0001$ in both cases).

In contrast, (\pm)-*cis*-3-ACPMPA and (\pm)-*trans*-3-ACPMPA were potent antagonists at GABA_C receptors. Fig. 3 shows the effects of (\pm)-*cis*- and (\pm)-*trans*-3-ACPMPA on human $\rho 1$ GABA_C receptors. Both (\pm)-*cis*-3-ACPMPA and (\pm)-*trans*-3-ACPMPA were competitive antagonists over the concentrations tested. The K_B values for (\pm)-*cis*-3-ACPMPA and (\pm)-*trans*-3-ACPMPA were found to be $1.0 \pm 0.2 \mu\text{M}$ and $6.6 \pm 0.7 \mu\text{M}$, respectively, and these are significantly different ($p = 0.0015$). The K_B values obtained show that (\pm)-*cis*-3-ACPMPA is significantly more active than TPMPA ($K_B = 2.3 \pm 0.3 \mu\text{M}$, $p = 0.0438$), whereas (\pm)-*trans*-3-ACPMPA is significantly less active than TPMPA ($p = 0.0015$) at human $\rho 1$ GABA_C receptors.

The compounds were further tested at human $\rho 2$ and rat $\rho 3$ receptors. (\pm)-*cis*-3- and (\pm)-*trans*-3-ACPMPA appear to be competitive antagonists at $\rho 2$ GABA_C receptors (Fig. 4). The apparent K_B values are $1.0 \pm 0.3 \mu\text{M}$ and $5.4 \pm 0.8 \mu\text{M}$, respectively, and these are significantly different ($p = 0.0067$). The apparent K_B values obtained show that both (\pm)-*cis*-3- and

(\pm)-*trans*-ACPMPA are significantly more active than TPMPA ($K_B = 14.9 \pm 1.5 \mu\text{M}$, $p = 0.0008$ and 0.0050 , respectively) at human $\rho 2$ GABA_C receptors.

Furthermore, both (\pm)-*cis*-3-ACPMPA and (\pm)-*trans*-3-ACPMPA appear to be competitive antagonists at rat $\rho 3$ receptors (Fig. 5), with apparent K_B values of 5.4 ± 0.8 and $17.7 \pm 2.2 \mu\text{M}$, respectively, and these are significantly different ($p = 0.0063$). No significant difference was observed in the activities of TPMPA ($K_B = 4.5 \pm 0.8 \mu\text{M}$) and (\pm)-*cis*-3-ACPMPA ($p = 0.4709$) at rat $\rho 3$ GABA_C receptors, whereas TPMPA was significantly more active than (\pm)-*trans*-3-ACPMPA at these receptors ($p = 0.0049$).

Table 2 summarises the effect of the compounds, TPMPA and reduced analogue of TPMPA, P4MPA, on all three GABA receptors.

4. Discussion

The early pharmacological characterization of GABA_C receptors (Feiganspan et al., 1993; Woodward et al., 1993) showed that the methylphosphinic acid moiety is able to differentiate GABA_A from GABA_C receptors, but not from GABA_B receptors while the tetrahydropyridine ring of isoguvacine is able to differentiate GABA_B from GABA_C receptors. Thus TPMPA was developed as a chimera of isoguvacine and (3-aminopropyl)methylphosphinic acid (3-APMPA), leading to

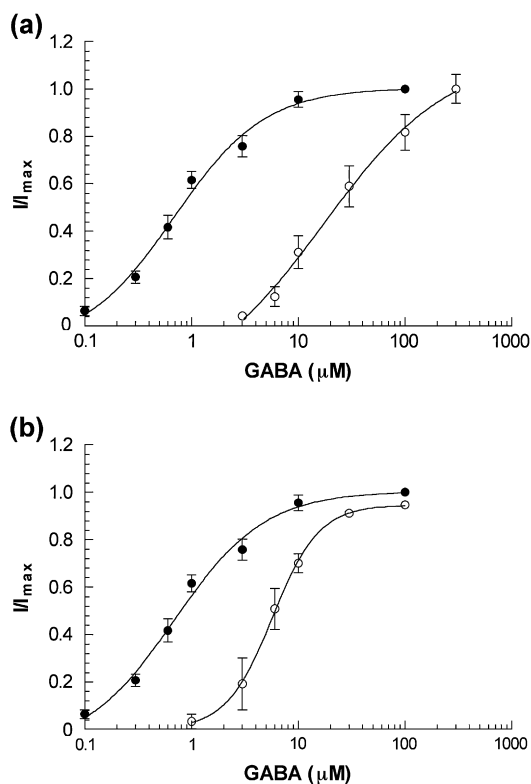


Fig. 4. (a) Dose–response curves for GABA alone (\bullet , $n = 7$) and GABA in the presence of 30 μM (\pm)-*cis*-3-ACPMPA (\circ , $n = 4$) at human $\rho 2$ GABA_C receptors expressed in *Xenopus* oocytes; (b) dose–response curves for GABA alone (\bullet , $n = 7$) and GABA in the presence of 30 μM (\pm)-*trans*-3-ACPMPA (\circ , $n = 4$) at human $\rho 2$ GABA_C receptors expressed in *Xenopus* oocytes. Data are the mean \pm s.e.m.

the first selective competitive antagonist of GABA_C receptors, being at least 100 times more potent as an antagonist at human $\rho 1$ GABA_C than at GABA_A receptors and 250 times more potent at $\rho 1$ GABA_C than at GABA_B receptors. However, TPMPA was approximately eight and two times weaker at human $\rho 2$ GABA_C and rat $\rho 3$ GABA_C receptors, respectively (Vien et al., 2002; Chebib et al., 1998).

Analogues of TPMPA and other ligands have been reported. These agents contain (1) an ethyl substituent replacing the methyl on the phosphinic acid moiety with an ethyl group producing (1,2,5,6-tetrahydropyridin-4-yl)ethylphosphinic acid (TPEPA) (Ragazzino et al., 1996); (2) a reduced double bond producing the saturated analogue P4MPA (Johnston et al., 1998; Hanrahan et al., 2001; Vien et al., 2002); (3) various phosphonic and selenic acid bioisosteric analogues of P4MPA (Krehan et al., 2003); or (4) a difluorophenol moiety, which increases the lipophilicity of the ligand (eg. 4-(aminomethyl)-2,6-difluorophenol; Chebib et al., 1999). These compounds were all antagonists at the GABA_C receptor. TPEPA and P4MPA were two and three times weaker than TPMPA at human $\rho 1$ GABA_C receptors, respectively. Interestingly, P4MPA was approximately four times more potent than TPMPA at human $\rho 2$ but two times weaker at rat $\rho 3$ GABA_C receptors (Table 2). (Piperidin-4-yl)selenic acid (SEPI; 0.95 μM), was the most active, being 200 times more active

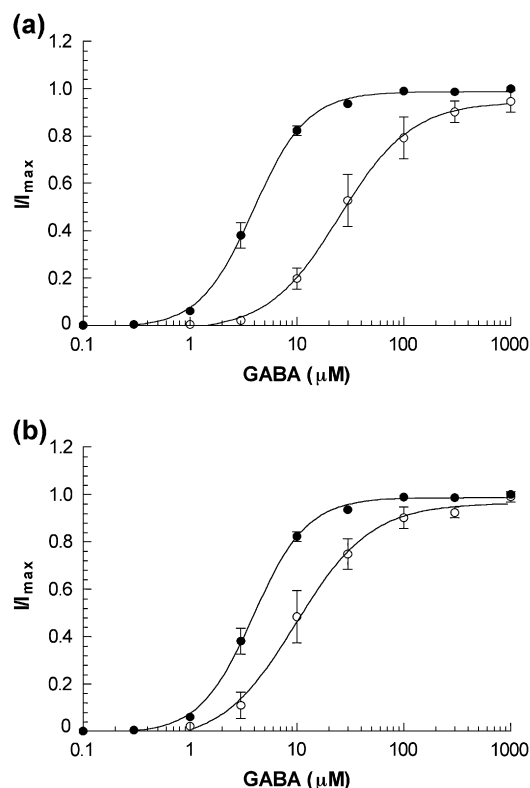


Fig. 5. (a) Dose–response curves for GABA alone (\bullet , $n = 4$) and GABA in the presence of 30 μM (\pm)-*cis*-3-ACPMPA (\circ , $n = 4$) at rat $\rho 3$ GABA_C receptors expressed in *Xenopus* oocytes; (b) dose–response curves for GABA alone (\bullet , $n = 4$) and GABA in the presence of 30 μM (\pm)-*trans*-3-ACPMPA (\circ , $n = 4$) at rat $\rho 3$ GABA_C receptors expressed in *Xenopus* oocytes. Data are the mean \pm s.e.m.

at human $\rho 1$ GABA_C receptors than at human $\alpha 1\beta 3\gamma 2$ GABA_A receptors (Krehan et al., 2003). The effect of SEPI on other GABA_C receptor subtypes or the GABA_B receptor has not been reported. Although 4-(aminomethyl)-2,6-difluorophenol had increased lipophilicity, it was only a weak antagonist at the $\rho 1$ GABA_C receptor. Importantly, apart from P4MPA, which was also shown to enhance reinforced memory (Gibbs and Johnston, 2005) via intracranial injections, no in vivo studies have been reported on these compounds indicating that they may not be able to cross the blood brain barrier following systemic injection.

The effects of the cyclopentane analogues of GABA, (+)- and (–)-*cis*-(3-aminocyclopentyl)carboxylic acids ((+)- and (–)-CACP), and (+)- and (–)-*trans*-(3-aminocyclopentyl)carboxylic acids ((+)- and (–)-TACP), were studied at recombinant GABA_C receptors (Chebib et al., 2001). These compounds were shown to be moderately potent partial agonists at GABA_C receptors. However, they are not selective, affecting both GABA_A receptors and GABA transporters but not GABA_B receptors. Thus, in order to improve selectivity for GABA_C receptors, we developed chimeras of either (\pm)-CACP or (\pm)-TACP with 3-APMPA (Fig. 6) whereby the carboxylic acid moiety was replaced with a methylphosphinic acid group – an approach similar to that used in the development of TPMPA.

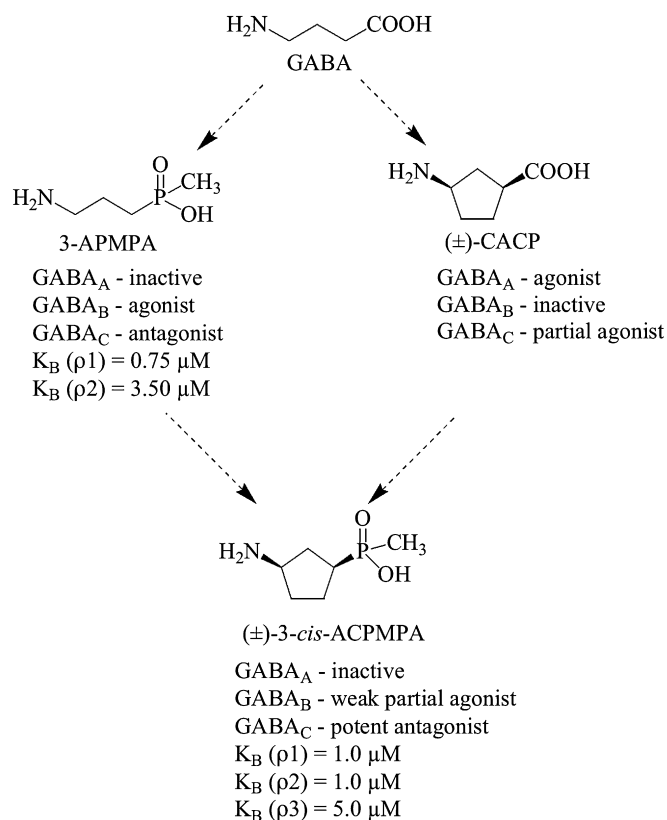


Fig. 6. Strategy for designing GABA_C ligands from (3-aminopropyl)methylphosphinic acid (3-APMPA) and (±)-*cis*-(3-aminocyclopentyl)carboxylic acid ((±)-CACP).

The resulting compounds, (±)-*cis*- and (±)-*trans*-3-ACPMPA, were shown to be very weak inhibitors of GABA_A receptors, moderately potent partial agonists at GABA_B receptors and potent antagonists at GABA_C receptors.

Complete K_B determination was not undertaken at $\alpha 1\beta 2\gamma 2$ receptors, as it was estimated that concentrations of up to 10–30 mM would be needed to produce a statistically significant shift in the GABA dose–response curve and this was not feasible. However, comparative experiments involving the degree of inhibition of the GABA EC₂₀ (10 μ M) produced by (±)-*cis*- and (±)-*trans*-3-ACPMPA and TPMPA show that both compounds produce significantly less inhibition than TPMPA. This indicates that the K_B for (±)-*cis*- and (±)-*trans*-3-ACPMPA is greater than that of TPMPA ($K_B = 320 \mu$ M; Ragazzino et al., 1996) at these receptors. A high affinity, low efficacy effect was ruled out as 100 μ M (±)-*cis*- and (±)-*trans*-3-ACPMPA do not significantly reduce the current produced by 10 μ M GABA at $\alpha 1\beta 2\gamma 2$ GABA_A receptors. Taken together with K_B data determined at GABA_C ρ_1 receptors, it is demonstrated that (±)-*cis*-3-ACPMPA is both significantly more potent at ρ_1 GABA_C receptors than TPMPA and more selective for these receptors with respect to GABA_A receptors. Additionally, (±)-*trans*-3-ACPMPA is less potent than TPMPA at ρ_1 GABA_C receptors with comparable selectivity.

Both (±)-*cis*- and (±)-*trans*-3-ACPMPA have reduced selectivity for GABA_C over GABA_B receptors than TPMPA,

due to an increased agonist effect of these compounds. It has previously been observed that GABA_B receptors do not tolerate substitution in the α or γ positions of the GABA backbone and therefore the observed effect may be due to the greater steric load of TPMPA at the γ position.

However, the overall selectivity of the compounds for GABA_C receptor subtypes is promising. (±)-*cis*-3-ACPMPA differentiated GABA_C from both GABA_A and GABA_B receptors being at least 100 times more potent as an antagonist at ρ_1 , ρ_2 and ρ_3 GABA_C than at GABA_A receptors and 50 times more potent at ρ_1 or ρ_2 GABA_C than at GABA_B receptors. Although the selectivity of this compound is somewhat less than that of TPMPA at GABA_B receptors, (±)-*cis*-3-ACPMPA has similar potency to TPMPA at ρ_1 and rat ρ_3 but is 15 times more potent than TPMPA on ρ_2 GABA_C receptors expressed in *Xenopus* oocytes. This is a significant finding as ρ_2 mRNA is distributed more widely in the CNS areas than ρ_1 mRNA (Boue-Grabot et al., 1998; Ogurusu et al., 1999) indicating a need for subtype selective agents.

Although (±)-*trans*-3-ACPMPA differentiated GABA_A from GABA_C receptors being at least 100 times more potent as an antagonist at ρ_1 , ρ_2 and ρ_3 GABA_C than at GABA_A receptors, it was three times weaker than TPMPA. Furthermore, there was only a 20-fold difference in the activity of (±)-*trans*-3-ACPMPA between GABA_B and either ρ_1 or ρ_2 GABA_C receptors, and only a nine-fold difference in the activity compared to that at the ρ_3 GABA_C receptor. The reduced activity and selectivity of (±)-*trans*-3-ACPMPA may indicate that the binding site of the GABA_C receptor preferentially selects for compounds in the *cis* configuration. A similar observation is noted with the agonist (+)-CAMP (Duke et al., 2000). As the enantiomers of a number of flexible and constrained ligands have been reported to have different effects at GABA_C receptors (Duke et al., 2000; Chebib et al., 2001; Duke et al., 2004; Crittenden et al., 2006), a resolution of the enantiomers of (±)-*cis*-3-ACPMPA may also lead to more active GABA_C agents. Furthermore, it would also be interesting to see if models of the stabilized zwitterionic structures of the cyclopentanephosphinic acid antagonists (Crittenden et al., 2005) fit into the agonist binding pocket described recently for GABA_C ρ_1 agonists by Sedelnikova et al. (2005).

Finally, there is a real need for pharmacological tools that could contribute towards an understanding of the role of GABA_C receptors in the brain. This study identifies (±)-*cis*- and (±)-*trans*-3-ACPMPA as a new class of potent and moderately selective GABA_C receptor antagonists whereby (±)-*cis*-3-ACPMPA is the most potent of the two cyclopentane analogues. Although (±)-*cis*-3-ACPMPA may not cross the blood brain barrier, it is a novel lead compound for developing GABA_C receptor antagonists with increased lipophilicity for in vivo studies. Given the lower abundance, structural simplicity and less widespread distribution of GABA_C receptors in the CNS compared to GABA_A receptors, GABA_C receptors may be a more selective drug target than the GABA_A receptors (Johnston et al., 2003).

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