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$GABA_C$ receptor antagonists differentiate between human ρ1 and ρ2 receptors expressed in *Xenopus* oocytes

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Abstract

The selective $GABA_C$ receptor antagonist, (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA), is eight times more potent against human recombinant ρ1 receptors than ρ2 receptors expressed in *Xenopus* oocytes. (3-Aminopropyl)methylphosphinic acid (CGP35024), the methylphosphinic acid analogue of GABA, and [(*E*)-3-aminopropen-1-yl]methylphosphinic acid (CGP44530), an open chain analogue of TPMPA, were five and four times, respectively, more potent as antagonists of ρ1 receptors than as antagonists of ρ2 receptors. Isoguvacine was a weak partial agonist at both ρ1 and ρ2 receptors with intrinsic activities (calculated as a percentage of the maximum whole cell current produced by a maximum dose of GABA) of 45 and 68%, respectively, of the maximum response produced by GABA. In agreement with other workers, it was found that imidazole-4-acetic acid was a partial agonist at both ρ1 and ρ2 receptors, showing higher intrinsic activity at ρ2 than at ρ1 receptors. The ρ1 receptor antagonist, *trans*-4-amino-2-methylbut-2-enoic acid (2-MeTACA), was a partial agonist at ρ2 receptors with an intrinsic activity of 34%. 2-MeTACA may be useful in differentiating between homo-oligomeric ρ1 and ρ2 receptors in native systems. These studies reveal significant differences in the antagonist profile of human recombinant ρ1 and ρ2 $GABA_C$ receptors.

Keywords: $GABA_C$ receptor; $GABA_C$ receptor antagonist; 2-MeTACA (*trans*-4-amino-2-methylbut-2-enoic acid); TPMPA ((1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid); *Xenopus* oocyte

1. Introduction

Mammalian $GABA_C$ receptors are transmitter-gated Cl^- channels for the inhibitory neurotransmitter, γ-aminobutyric acid (GABA). These receptors are physiologically and pharmacologically different to $GABA_A$ and $GABA_B$ receptors. In contrast to $GABA_A$ receptors, $GABA_C$ receptors are more sensitive to GABA, the channels remain open longer with maintained agonist application (Feigenspan and Bormann, 1994), have slower onset and offset times and are less prone to desensitisation (Amin and Weiss, 1994). Pharmacologically, $GABA_C$ receptors are insensitive to the $GABA_A$ receptor antagonist, bicuculline, and to the $GABA_B$ receptor antagonist, phaclofen (Johnston, 1996). (1,2,5,6-Tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA) has been described as 'the first selective antagonist for $GABA_C$ receptors' (Murata et al., 1996). This compound is at least 100 times more potent in antagonising human homo-oligomeric ρ1 $GABA_C$ receptors ($K_B = 2$ μM) than in antagonising rat brain $GABA_A$ receptors ($K_B = 320$ μM) expressed in *Xenopus* oocytes. Furthermore, TPMPA is at least 500 times less potent as an agonist at $GABA_B$ receptors ($EC_{50} = 500$ μM) in rat hippocampal slices compared to its activity at human recombinant ρ1 $GABA_C$ receptors (Ragozzino et al., 1996).

Evidence for $GABA_C$ receptors have been described in mammalian retina (Feigenspan et al., 1993) and other parts of the central nervous system (CNS), including the cerebellum (Drew et al., 1984) and spinal cord (Johnston et al., 1975). In vivo $GABA_C$ receptors are most likely to consist of ρ subunits. In situ hybridisation and reverse transcriptase-polemerase chain reactions (RT-PCR) have been used to examine the distribution of three types of ρ subunits (ρ1–3) in different rat brain regions (Enz et al., 1995; Boue-Grabot et al., 1998). The distribution for each subunit type differs slightly; most have been found in the retina, cerebellum, hippocampus, superior colliculus and neocortex. Up to five different ρ-subunits have been cloned,

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including two from human retinal cDNA library (ρ1 and ρ2; Cutting et al., 1991, 1992), three from rat (ρ1–3; Zhang et al., 1995; Ogurusu et al., 1995; Ogurusu and Shingai, 1996; Wegelius et al., 1996), two from chick (ρ1 and ρ2; Albrecht and Darlison, 1995), five from white perch (ρ1–5; Qian et al., 1997) and two from mouse (ρ1 and ρ2; Greka et al., 1998) retinas. These have a high percentage sequence homology between the different subunits and species.

ρ-Subunits do not combine with any of the α, β or γ subunits that form $GABA_A$ receptors. Furthermore, double immunolabelling experiments have shown that there are no synaptic GABA receptors in the rat retina in which $GABA_C$ and $GABA_A$ receptor subunits are co-assembled (Koulen et al., 1998). Instead, $GABA_C$ receptor subunits combine to form either homo-oligomeric receptors (made up of either ρ1, ρ2 or ρ3 subunits; Cutting et al., 1991; Shimada et al., 1992; Kusama et al., 1993a,b; Shingai et al., 1996), or hetero-oligomeric receptors (made up of ρ1 and ρ2 subunits; Zhang et al., 1995).

Most pharmacological studies on recombinant $GABA_C$ receptors have been carried out by expressing human ρ1 cDNA in *Xenopus* oocytes. However, Cutting et al. (1992) identified a second subunit, ρ2, in a human retinal cDNA library, with 74% similarity to the ρ1 subunit, indicating that $GABA_C$ receptor subtypes exist in vivo. Like the human ρ1 subunit, the ρ2 subunit forms functional homo-oligomeric receptors when expressed in *Xenopus* oocytes (Cutting et al., 1992; Kusama et al., 1993b; Wang et al., 1994). These receptors were found to have a pharmacological profile similar to that of expressed ρ1 receptors. This led to the suggestion that the amino acid residues important for most aspects of GABA receptor agonist action may not be among those that differ between ρ1 and ρ2 subunits, including the 20% amino acid difference in the N-terminal region (Kusama et al., 1993b). Some pharmacological differences between ρ1 and ρ2 receptors have been noted, however, including the intrinsic activity (calculated as a percentage of the maximum whole cell current produced by a maximum dose of GABA) of the partial agonist, imidazole-4-acetic acid, being seven-fold higher on ρ2 than on ρ1 receptors (Kusama et al., 1993b).

We have previously reported that a number of unsaturated analogues of GABA and 3-aminopropylphosphinic acid are antagonists at ρ1 receptors (Chebib et al., 1997a,b). The present study is directed towards the possibility of detecting pharmacological differences in antagonist selectivity between human homo-oligomeric ρ1 and ρ2 receptors expressed in *Xenopus* oocytes.

2. Materials and methods

2.1. Materials

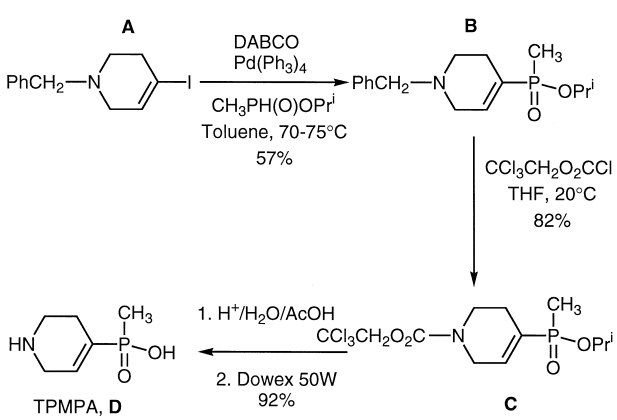
trans-4-Amino-2-methylbut-2-enoic acid (2-MeTACA) was prepared as described previously by Dr. Robin D. Allan (Department of Pharmacology, The University of Sydney, Sydney, Australia) (Allan and Twitchin, 1978). Isoguvacine was purchased from Tocris Cookson (Bristol, UK). GABA and imidazole-4-acetic acid were purchased from Sigma (St. Louis, MO, USA). (3-Aminopropyl)methylphosphinic acid (CGP35024) and [(*E*)-3-aminopropen-1-yl]methylphosphinic acid (CGP44530) were gifts from Dr. W. Froestl (Novartis, Basle, Switzerland).

2.2. Synthesis of TPMPA

TPMPA (D) was prepared (Scheme 1) by modification of the procedure described by Murata et al. (1996). Our procedure utilised alternative reagents that were more readily synthesised or commercially available and gave a comparable overall yield. It avoided the use of phosgene gas which is a prohibited import into Australia.

2.2.1. Isopropyl (1-benzyl-1,2,5,6-tetrahydropyridin-4-yl)methylphosphinate (B)

A solution of 1-benzyl-4-iodo-1,2,5,6-tetrahydropyridine (A) (4.50 g, 15 mmol), isopropyl methylphosphinate (Gallagher et al., 1996) (2.70 g, 22.1 mmol), 1,4-diazabicyclo[2.2.2]octane (DABCO) (5.1 g, 46 mmol), and tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) (500 mg, 0.5 mmol) in toluene (150 ml) under N_2 was stirred and slowly heated to 70°C over 1 h, then maintained at 70–75°C for a further 3 h. The reaction mixture was filtered, the filtrate concentrated under reduced pressure (ca. 20 mm) and the viscous residue purified by short column vacuum chromatography on silica gel (ethyl acetate containing 10% isopropanol) to afford the title compound as a light brown viscous oil (2.51 g, 57%): ^1H Nuclear Magnetic Resonance Spectroscopy (NMR) (300 MHz, $CDCl_3$) δ 7.20–7.35 (5H, m, Ph), 6.70 (1H, dm, $J = 18.9$ Hz, C*H*=C), 4.55 (1H, d of septet, $J = 8.9$, 6.2 Hz, OC*H*), 3.61 (2H, s, CH_2Ph), 3.03–3.26 (2H, m, NCH_2C=C), 2.64–2.73 and 2.48–2.57 (each 1H, m, NCH_2CH_2), 2.24–2.33 (2H, m, NCH$_2$CH_2), 1.45 (3H, d,



Scheme 1. Synthetic pathway of TPMPA.

$J = 14.1$ Hz, PCH_3), 1.33 and 1.25 (each 3H, d, $J = 6.2$ Hz, CH_3CH); ^{13}C NMR (75.46 MHz, $CDCl_3$) δ 139.6 (d, $J = 8.1$ Hz), 137.8, 129.9 (d, $J = 128$ Hz), 129.1, 128.3, 68.9 (d, $J = 6.1$ Hz), 62.5 (d, $J = 1.5$ Hz), 53.1 (d, $J = 15.2$ Hz), 49.0 (d, $J = 9.3$ Hz), 25.6 (d, $J = 9.9$ Hz), 24.6 (d, $J = 3.3$ Hz), 24.1 (d, $J = 4.7$ Hz), 14.0 (d, $J = 100.5$ Hz); Low Resolution Mass Spectrometry (LRMS) (Chemical Ionization (CI), CH_4) m/z 294 (MH^+).

2.2.2. Isopropyl [1-(2,2,2-trichloroethoxycarbonyl)-1,2,5,6-tetrahydropyridin-4-yl]methylphosphinate (C)

2,2,2-Trichloroethyl chloroformate (1.90 g, 8.97 mmol) was added in one portion to a stirred solution of (B) (2.56 g, 8.74 mmol) in anhydrous tetrahydrofuran (10 ml) under a N_2 atmosphere and the reaction mixture was stirred at room temperature (20°C) for 20 h. The small amount of solid which precipitated was filtered off and the filtrate concentrated under reduced pressure. The residue was purified by short column vacuum chromatography on silica gel (ethyl acetate containing 10% isopropanol) to afford (C) as an off-white solid. Recrystallisation from hexane gave pure (C) as small white needles (2.70 g, 82%): melting point (m.p.) 98–99°C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.72 (1H, dm, $J = 19.5$ Hz, CH=C), 4.78 (2H, s, OCH_2CCl$_3$), 4.58 (1H, d of septet, $J = 8.8$, 6.2 Hz, OCH), 4.07–4.34 (2H, m, NCH_2C=C), 3.71–3.83 and 3.49–3.66 (each 1H, m, NCH_2CH$_2$), 2.25–2.45 (2H, m, NCH$_2$CH_2), 1.49 (3H, d, $J = 14.2$ Hz, PCH_3), 1.35 and 1.26 (each 3H, d, $J = 6.2$ Hz, CH_3CH); ^{13}C NMR (75.46 MHz, $CDCl_3$) δ 153.6* and 153.3*, 137.2* and 136.8* (each d, $J = 7.5$ Hz), 131.1 (d, $J = 124$ Hz), 95.5, 75.2, 69.3 (d, $J = 6.4$ Hz), 44.4* and 44.1* (each d, $J = 15.2$ Hz), 40.5* and 40.2* (each d, $J = 8.0$ Hz), 24.7* and 24.3* (each d, $J = 9.2$ Hz), 24.5 (d, $J = 3.5$ Hz), 24.1 (d, $J = 4.7$ Hz), 13.9 (d, $J = 101$ Hz); LRMS (CI, CH_4) m/z 378/380/382/384 (in correct isotopic abundances) (MH^+).

Note: * signifies contributions from discrete rotamers in solution.

2.2.3. (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA) (D)

A solution of (C) (2.62 g, 6.91 mmol) in 48% aqueous hydrogen bromide (40 ml) and glacial acetic acid (40 ml) was refluxed gently for 30 h. The mixture was concentrated under reduced pressure and final traces of residual acid removed by the sequential addition of H_2O and concentration under reduced pressure (several cycles). The final residue was purified by ion-exchange chromatography (Dowex 50W). The column was eluted initially with distilled H_2O (200 ml) until the eluent was neutral. Further elution with 1 M aqueous pyridine gave a ninhydrin positive fraction which was concentrated under reduced pressure. The final traces of pyridine were removed by the sequential addition of H_2O and concentration as described above to afford crude TPMPA as an off-white solid (1.11 g, 92%). Recrystallisation from ethanol (EtOH)/H_2O and vacuum drying gave pure TPMPA: m.p. 252–254°C; ^{1}H NMR (300 MHz, CD_3OD) δ 6.32 (1H, dm, $J = 17.4$ Hz, CH=C), 3.71 (2H, m as apparent quintet, $J =$ ca. 2.7 Hz, NCH_2C=C), 3.28 (2H, dt, $J =$ ca. 0.8 Hz, 6.0 Hz, NCH_2CH$_2$), 2.49–2.57 (2H, m, NCH$_2$CH_2, 1.25 (3H, d, $J = 13.9$ Hz, PCH_3); LRMS (CI, CH_4) m/z 162 (MH^+).

2.3. Electrophysiological recording

Xenopus laevis were anaesthetised with 0.17% ethyl 3-aminobenzoate and a lobe of the ovaries was removed. The lobe of the ovary was rinsed with oocyte releasing buffer 2 (OR2) (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 5 mM HEPES, pH 7.5) and treated with Collagenase A (2 mg/ml in OR2, Bohringer Manheim) for 2 h. Released oocytes were then rinsed in frog ringer solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5) supplemented with 2.5 mM pyruvate, 0.5 mM theophylline and 50 μg/ml gentamycin, and stage V–VI oocytes collected.

Human ρ1 cDNA in pcDNA (Invitrogen, San Diego, CA, USA) was provided by Dr. George Uhl (National Institute for Drug Abuse, Baltimore, MD, USA). Human ρ2 cDNA in pKS (Invitrogen, San Diego, CA, USA) was a gift from Dr. Garry Cutting (Center for Medical Genetics, Johns Hopkins University School of Medicine, Baltimore, MD, USA). Capped RNA was synthesised from linearized plasmid containing the ρ1 cDNA and ρ2 cDNA using Xba 1 and ECO RV enzymes, respectively. cRNA was synthesised using the 'Mmessage Mmachine' kit from Ambion (Austin, TX, USA). ρ1 cRNA (10 ng/50 nl) and 50 ng/50 nl of ρ2 cRNA were injected into defolliculated Stage V–VI *Xenopus* oocytes. Oocytes containing ρ1 cRNA were stored at 9°C while oocytes containing ρ2 cRNA were stored at 21–22°C. Two to eight days later, receptor activity was measured by two electrode voltage clamp recording using a Geneclamp 500 amplifier (Axon Instruments, Foster City, CA, USA), a MacLab 2e recorder (AD Instruments, Sydney, NSW, Australia) and Chart version 3.5 program. Oocytes were voltage clamped at either −60 mV or −100 mV and continuously superfused with frog Ringer solution. For receptor activation measurements, the indicated concentrations of drug were added to the buffer solution.

2.4. 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 maximum current, K_d is the effective dose that activates 50% of the maximum current and n_H is the Hill coefficient. K_d values are expressed as mean ± S.E.M. ($n = 3$–6 oocytes) and are determined by fitting

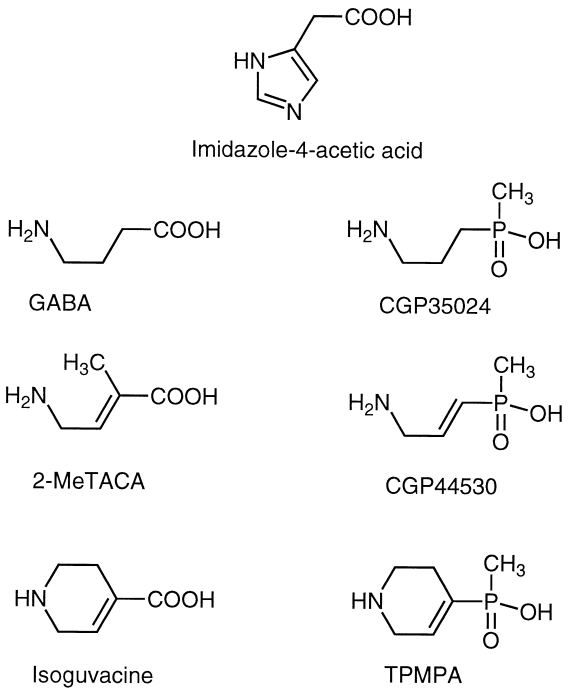


Fig. 1. Structures of agonists and antagonists tested at human homo-oligomeric ρ1 and ρ2 receptors expressed in *Xenopus* oocytes.

data from individual oocytes using Kaleidagraph 3.0 (1993). The intrinsic activity of partial agonists, I_m, is calculated as a percentage of the maximum whole cell current produced by a maximum dose of GABA. K_B values are the apparent binding constants for the antagonists and were determined using Schild plot analysis (Arunlakshana and Schild, 1959). $-\log K_B$ values were determined using the following equation $\log\{(A)/(A*)-1\} = m\log[\text{Ant}] - \log K_B$, 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 using Kaleidagraph 3.0 (1993).

3. Results

As described by other workers (Kusama et al., 1993b; Wang et al., 1994), the amplitudes of the whole-cell currents recorded from the human ρ2 receptors when expressed in oocytes were smaller than those of ρ1 receptors. We recorded currents of 30–600 nA with ρ2 receptors held at −100 mV compared to 400–2000 nA with ρ1 receptors at −60 mV. Furthermore, in order to facilitate expression of ρ2 receptors, oocytes containing ρ2 cRNA were stored at 21–22°C compared to 9°C for ρ1. The results we obtain under these conditions are similar to those reported by Kusama et al. (1993a,b). Storing oocytes injected with ρ2 cRNA at 9°C only produced current ranges between 0–10 nA which are inappropriate for determining dose–response curves. Therefore, it is unlikely that the different experimental conditions by which ρ1 and ρ2 receptors are expressed and recorded contribute to the pharmacological differences observed by the antagonists used in this study.

The structures (drawn to emphasise structural similarities) of the GABA analogues tested in this study are shown in Fig. 1 and their activities at ρ1 and ρ2 receptors are summarised in Table 1.

GABA was marginally less potent and showed higher positive cooperativity (although not statistically significant; unpaired *t*-test, $p > 0.05$) as an agonist at ρ1 than at

Table 1
Effects of agonists and antagonists at human homo-oligomeric $GABA_C$ receptor subtypes

Compound	Human ρ1 $GABA_C$ receptors				Human ρ2 $GABA_C$ receptors			
	K_d (μM)[a]	K_B (μM)[b]	n_H[c]	I_m (%)[d]	K_d (μM)[a]	K_B (μM)[b]	n_H[c]	I_m (%)[d]
GABA	1.01 ± 0.06[e,f]		2.2 ± 0.3	100	0.52 ± 0.10		1.7 ± 0.2	100
Imidazole-4-acetic acid	7.80 ± 028	1.45 ± 0.12	1.1 ± 0.1	9 ± 1	3.0 ± 0.2	ND[h]	1.2 ± 0.1	38 ± 1
TPMPA	NC[g]	2.0 ± 0.4			NC	15.6 ± 1.6		
CGP35024	NC	0.75 ± 0.07[e]			NC	3.5 ± 0.3		
CGP44530	NC	8.6 ± 1.6[e]			NC	37.0 ± 4.5		
2-MeTACA	NC at 100 μM	45.5 ± 5.4[f]			101 ± 11	ND	1.4 ± 0.2	34 ± 0.1
Isoguvacine	137 ± 6	ND	1.6 ± 0.1	45 ± 1	110 ± 12	ND	1.1 ± 0.1	68 ± 3

[a] K_d is the dissociation constant for the agonist.
[b] K_B is the binding constant for the antagonist. These were determined using Schild plot analysis assuming competitive antagonism over the tested concentrations.
[c] n_H is the Hill coefficient.
[d] I_m is the intrinsic activity calculated as a percentage of the maximum whole cell current produced by a maximum dose of GABA.
[e] Data obtained from Chebib et al. (1997a).
[f] Data obtained from Chebib et al. (1997b).
[g] NC for no current response.
[h] ND for not determined.

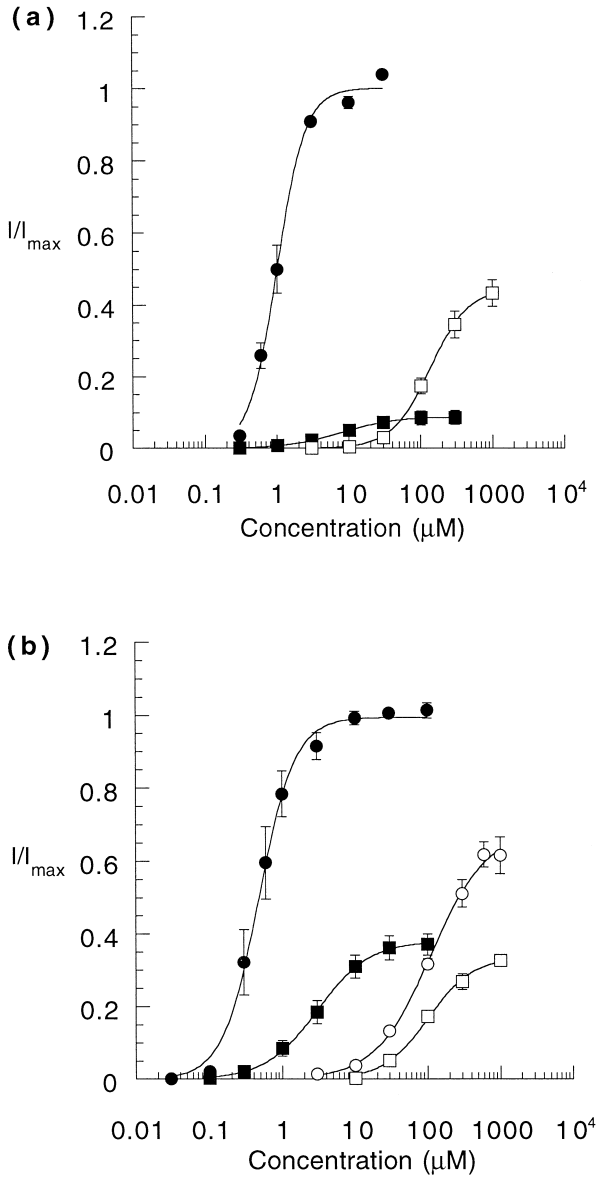


Fig. 2. (a) Agonist dose–response curves for (●) GABA, (■) imidazole-4-acetic acid and (□) isoguvacine obtained from *Xenopus* oocytes expressing ρ1 cRNA. Data are the mean ± S.E.M. ($n = 3–6$ oocytes). (b) Agonist dose–response curves for (●) GABA, (■) imidazole-4-acetic acid, (□) isoguvacine and (○) 2-MeTACA obtained from *Xenopus* oocytes expressing ρ2 cRNA. Data are the mean ± S.E.M. ($n = 3–6$ oocytes).

ρ2 receptors as indicated by the K_d values and Hill coefficients in Table 1. These have been calculated from the dose–response relationships shown in Fig. 2a and b.

2-MeTACA, a carboxylic acid analogue of TPMPA, and of CGP44530 which we have shown previously to be a competitive antagonist at ρ1 receptors (Chebib et al., 1997b) was a moderately potent partial agonist at ρ2 receptors (Fig. 3a and b; $K_d = 101$ μM; $I_m = 34\%$; Fig. 2b). 2-MeTACA showed no agonist activity at ρ1 receptors at concentrations up to 100 μM (Chebib et al., 1997b).

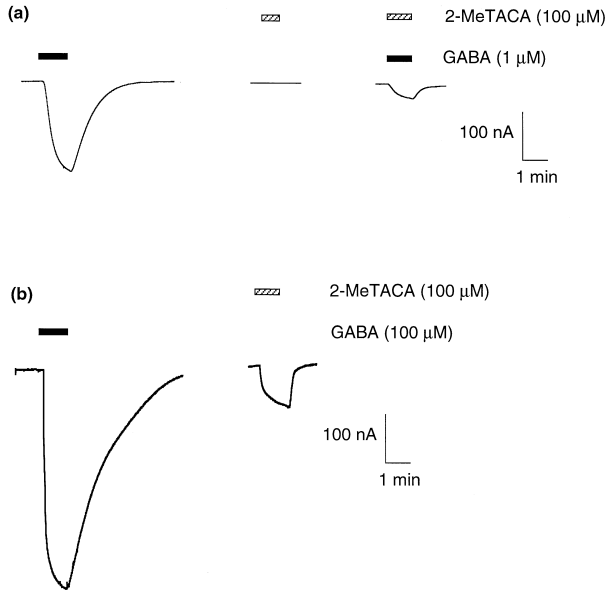


Fig. 3. (a) At homo-oligomeric ρ1 receptors expressed in *Xenopus* oocytes, GABA (1 μM) (duration indicated by filled bar) activates the receptor while 2-MeTACA (100 μM) does not activate the receptor (duration indicated by hatched bar). However, when 2-MeTACA (100 μM) is co-applied with GABA (1 μM) (duration indicated by filled bar), the GABA response is reduced. (b) At homo-oligomeric ρ2 receptors expressed in *Xenopus* oocytes, a maximum dose of GABA (100 μM) (duration indicated by filled bar) and 2-MeTACA (100 μM) (duration indicated by hatched bar) activate the receptor.

Isoguvacine, the carboxylic acid analogue of TPMPA, was shown to be a moderately potent partial agonist at both ρ1 (Fig. 2a; $K_d = 137$ μM; $I_m = 45\%$) and ρ2 receptors (Fig. 2b; $K_d = 110$ μM; $I_m = 68\%$).

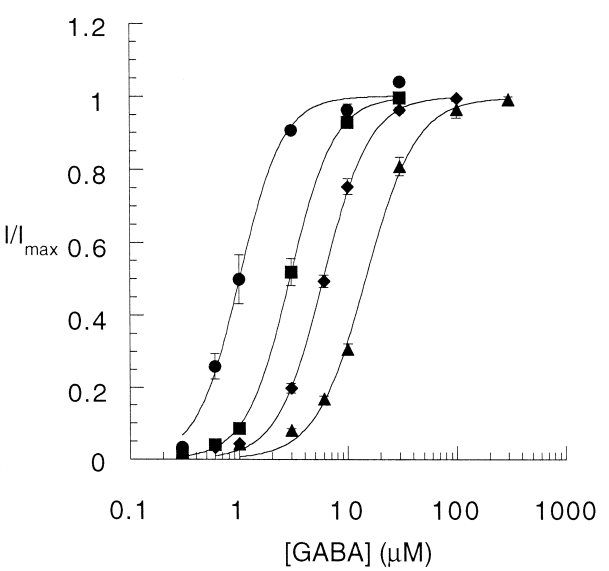


Fig. 4. Schild analysis of GABA (●) and GABA in the presence of 3 μM (■), 10 μM (♦) and 30 μM (▲) Imidazole-4-acetic acid obtained from *Xenopus* oocytes expressing ρ1 cRNA. Data are the mean ± S.E.M. ($n = 3–6$ oocytes).

In agreement with Kusama et al. (1993b), we found that the partial agonist action of imidazole-4-acetic acid was of higher affinity and higher intrinsic activity at ρ2 (Table 1; Fig. 2b; $K_d = 3.0$ μM; $I_m = 38\%$) than at ρ1 receptors (Table 1; Fig. 2a; $K_d = 7.8$ μM; $I_m = 9\%$). In addition, imidazole-4-acetic acid was a potent antagonist of ρ1 receptors (Fig. 4; $K_B = 1.45$ μM) as described previously by Kusama et al. (1993a).

In agreement with the studies of Murata et al. (1996) and Ragozzino et al. (1996), TPMPA was found to be a potent antagonist at ρ1 receptors (Fig. 5a; apparent K_B 2 μM, Schild slope 1.3 ± 0.1). TPMPA was eight times less potent as an antagonist at ρ2 receptors compared to ρ1 receptors (Fig. 5b; apparent K_B of 15.6 μM; Schild slope 0.7 ± 0.1).

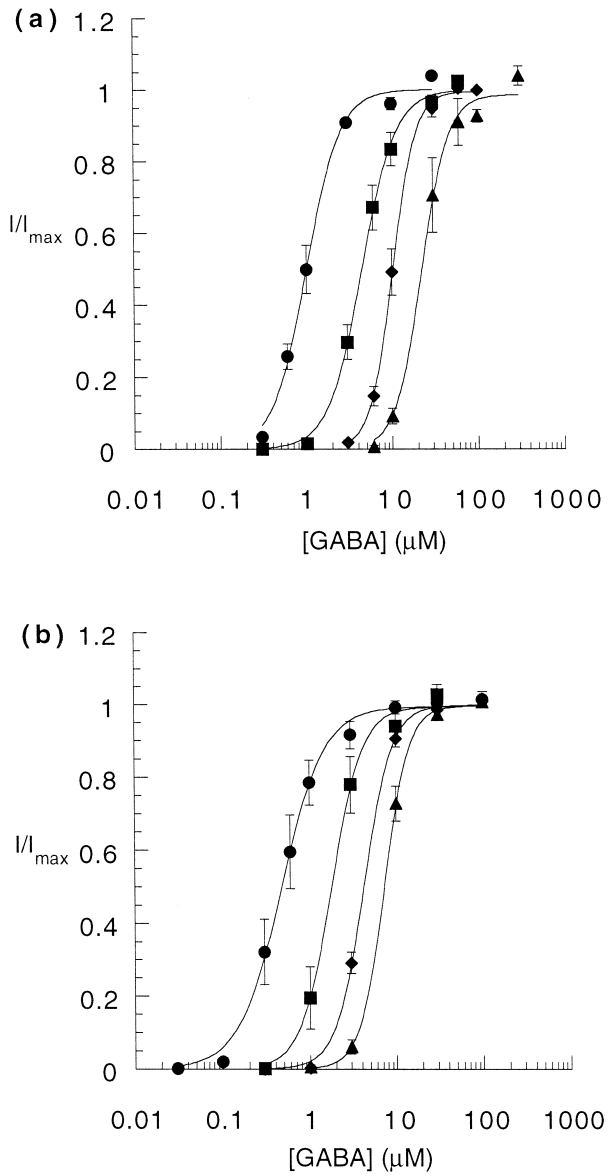


Fig. 5. (a) Schild analysis of GABA (●) and GABA in the presence of 10 μM (■), 30 μM (♦) and 100 μM (▲) TPMPA obtained from *Xenopus* oocytes expressing ρ1 cRNA. Data are the mean ± S.E.M. ($n = 3$–6 oocytes). (b) Schild analysis of GABA (●) and GABA in the presence of 30 μM (■), 60 μM (♦) and 100 μM (▲) TPMPA obtained from *Xenopus* oocytes expressing ρ2 cRNA. Data are the mean ± S.E.M. ($n = 3$–6 oocytes).

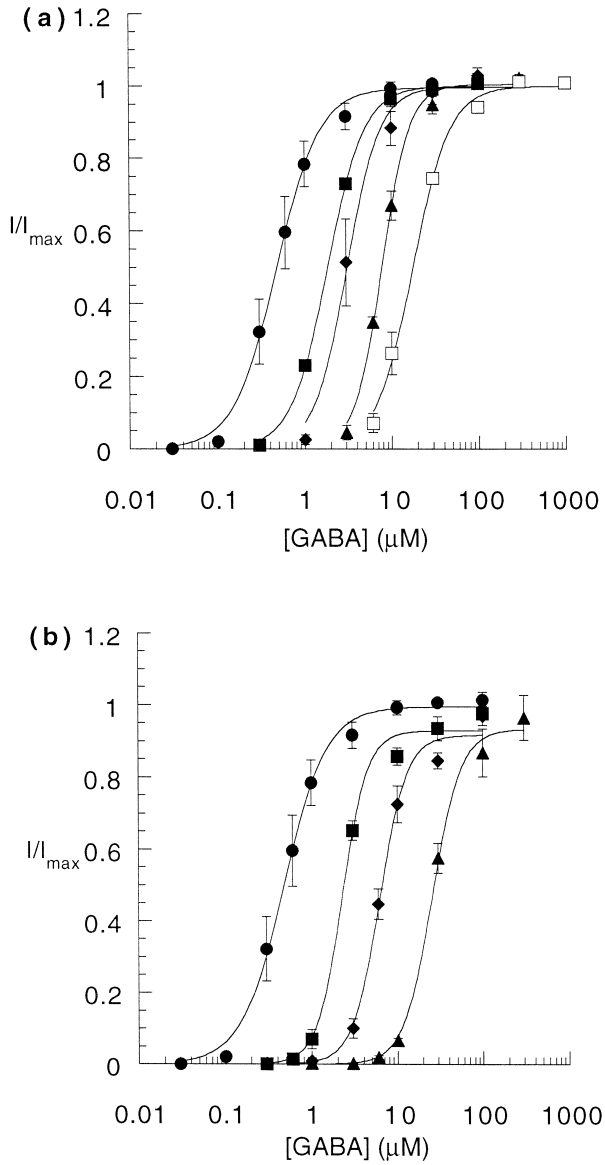


Fig. 6. (a) Schild analysis of GABA (●) and GABA in the presence of 60 μM (■), 100 μM (♦), 300 μM (▲) and 600 μ M (□) CGP44530 obtained from *Xenopus* oocytes expressing ρ2 cRNA. Data are the mean ± S.E.M. ($n = 3$–6 oocytes). (b) Schild analysis of GABA (●) and GABA in the presence of 10 μM (■), 30 μM (♦) and 100 μM (▲) CGP35024 obtained from *Xenopus* oocytes expressing ρ2 cRNA. Data are the mean ± S.E.M. ($n = 3$–6 oocytes).

CGP44530, an open chain analogue of TPMPA, shown previously to be a competitive antagonist at ρ1 receptors (Chebib et al., 1997a), proved to be 4 times less potent as a competitive antagonist of ρ2 receptors compared to ρ1 receptors (Fig. 6a; $K_B = 24.2$ μM; Schild slope 0.9 ± 0.1)

CGP35024, the methylphosphinic acid analogue of GABA, was five times less potent as a competitive antago-

nist of ρ2 receptors compared to ρ1 receptors (Fig. 6b; $K_B = 3.5$ μM; Schild slope 0.9 ± 0.1). To date, this compound is the most potent antagonist of $GABA_C$ receptors. However, it is not selective, as it is a very potent agonist of $GABA_B$ receptors.

4. Discussion

$GABA_C$ ρ1 and ρ2 receptor subunits show an overall 74% sequence homology with a 20% sequence divergence in the N-terminal domain (Cutting et al., 1992). Sequences within a 100 amino acid domain of the N-terminus of ρ1 subunits contain subunit assembly signals that confer robust homo-oligomeric expression (Hackam et al., 1997). The N-terminal domain differences between ρ1 and ρ2 may also explain the less robust expression of ρ2 receptors and their lower current amplitudes in response to GABA (Hackam et al., 1997). Thus, with ρ1 receptors the amino acid residues that are the N-terminal domain are more efficient in forming homo-oligomeric receptors. Further studies comparing intracellular protein levels and cell surface expression may determine the mechanism by which ρ1 receptor formation is more efficient. Since the studies by Hackam et al. (1997) have determined areas of the protein that may explain the physiological differences between ρ1 and ρ2 receptors, that is, signals that confer robust homo-oligomeric expression and current amplitudes between the receptor subtypes, studies on the pharmacological differences may also aid in differentiating between the subtypes for future studies in vivo.

Kusama et al. (1993b) studied a series of agonists on recombinant ρ1 and ρ2 receptors expressed in *Xenopus* oocytes and showed that the pharmacological profiles of the agonists on ρ2 receptors displayed many similarities to those displayed by ρ1 receptors. One of the compounds studied was imidazole-4-acetic acid which, at lower concentration levels, displayed antagonist properties at ρ1 receptors ($K_B = 1.45$ μM) while at concentrations 5 times higher, was a partial agonist ($K_d = 7.8$ μM; $I_m = 9\%$). At ρ2 receptors, imidazole-4-acetic acid was a potent partial agonist ($K_d = 3$ μM; $I_m = 38\%$) and, hence, was the first compound to show some pharmacological difference between ρ1 and ρ2 receptors. However, the other compounds, *cis*- and *trans*-4-aminobut-2-enoic acid (CACA and TACA), GABA, muscimol, *cis*- and *trans*-2-(aminomethyl)-1-carboxycyclopropanes (CAMP and TAMP) did not significantly differentiate between the ρ1 and ρ2 receptor subtypes. Therefore, these authors concluded that many of the amino acid residues involved in the recognition of $GABA_C$ receptor agonists and antagonists were similar for ρ1 and ρ2 receptors (Kusama et al., 1993b).

Murata et al. (1996) showed that TPMPA is a selective antagonist of human recombinant ρ1 $GABA_C$ receptors expressed in oocytes, being 500 times less potent against $GABA_B$ receptors in rat hippocampal slices and 100 times less potent against rat brain $GABA_A$ receptors expressed in oocytes. The present study confirms the high potency of TPMPA as an antagonist of human recombinant ρ1 $GABA_C$ receptors expressed in oocytes and shows that TPMPA is eight times less potent as an antagonist at human recombinant ρ2 than at ρ1 $GABA_C$ receptors. Therefore, TPMPA is not as selective on ρ2 as it is on ρ1 receptors when compared to $GABA_A$ receptors.

Increased conformational flexibility of CGP44530 resulted in a compound that is less active as an antagonist of ρ1 receptors, and less able to discriminate between ρ1 and ρ2 receptors. TPMPA is a conformationally restricted analogue of CGP44530 which suggests that TPMPA represents conformations of CGP44530 that preferentially interact with ρ1 receptors compared to ρ2 receptors.

Like GABA, CGP35024 is a highly flexible compound that can attain many low energy conformations, which are conducive for binding to the GABA receptor agonist/antagonist site. As CGP35024 is weaker at ρ2 than ρ1 receptors, then either the conformations which CGP35024 can adopt do not ideally fit the binding site of the ρ2 receptor or the methylphosphinic acid group is not an ideal moiety to achieve high activity for ρ2 receptors. A similar rationale may explain the effects of TPMPA and CGP44530 at this receptor subtype.

The carboxylic analogue of TPMPA, isoguvacine, displayed similar potencies at the ρ1 and ρ2 receptor subtypes and, hence, did not distinguish between these receptors by receptor affinity. Thus, conformations which isoguvacine can adopt are similar for ρ1 and ρ2 receptor subtypes. Isoguvacine only distinguishes between these receptors by displaying a higher intrinsic activity at ρ2 than at ρ1 receptors. This result was similar to that observed by most of the agonists/partial agonists studied by Kusama et al. (1993b). Therefore, the reasons why TPMPA differentiates between these receptors, and isoguvacine does not, may lie in the chemical nature of and the interactions between the acidic moiety of the ligand and the corresponding amino acid residue(s) of the protein binding site.

2-MeTACA was a competitive antagonist at ρ1 receptors but was a partial agonist at ρ2 receptors, with an intrinsic activity 34% that of the maximum response of GABA. This is an important finding because 2-MeTACA is the first compound to bind to the agonist/antagonist site of $GABA_C$ receptors that shows different pharmacological properties for the ρ1 and the ρ2 receptors. Thus, 2-MeTACA may differentiate between ρ receptor subtypes in native systems. The differences observed with TPMPA, imidazole-4-acetic acid, isoguvacine, CGP44530 and CGP35024 are less dramatic as binding or dissociation constants differ only by 2–8-times when comparing homo-oligomeric ρ1 and ρ2 receptors.

The present study shows that the $GABA_C$ selective antagonist, TPMPA, and structurally related antagonists/partial agonists differentiate between ρ1 and ρ2 receptors.

Given the different regional localisation of ρ1 and ρ2 subunits found in rat retina and brain (Enz et al., 1995), it is important to further develop our understanding of the different pharmacological profiles of $GABA_C$ receptor subtypes. These results may aid in characterising further the agonist/antagonist binding site(s) of the $GABA_C$ receptor subtypes.

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