British Journal of Pharmacology (2002) 135, 883–890

www.nature.com/bjp

trans-4-Amino-2-methylbut-2-enoic acid (2-MeTACA) and (±)-*trans*-2-aminomethylcyclopropanecarboxylic acid ((±)-TAMP) can differentiate rat ρ3 from human ρ1 and ρ2 recombinant $GABA_C$ receptors

[1]Jimmy Vien, [1]Rujee K. Duke, [1]Kenneth N. Mewett, [1]Graham A.R. Johnston, [3]Ryuzo Shingai & *,[2]Mary Chebib

[1]Department of Pharmacology, University of Sydney, NSW 2006, Australia; [2]Faculty of Pharmacy, University of Sydney, NSW 2006, Australia and [3]Department of Welfare Engineering, Faculty of Engineering, Iwate University, Morioka, Japan

1 This study investigated the effects of a number of GABA analogues on rat ρ3 $GABA_C$ receptors expressed in *Xenopus* oocytes using 2-electrode voltage clamp methods.

2 The potency order of agonists was muscimol ($EC_{50} = 1.9 \pm 0.1$ μM) (+)-*trans*-3-aminocyclopentanecarboxylic acids ((+)-TACP; $EC_{50} = 2.7 \pm 0.9$ μM) trans-4-aminocrotonic acid (TACA; $EC_{50} = 3.8 \pm 0.3$ μM) GABA ($EC_{50} = 4.0 \pm 0.3$ μM) > thiomuscimol ($EC_{50} = 24.8 \pm 2.6$ μM) > (±)-*cis*-2-aminomethylcyclopropane-carboxylic acid ((±)-CAMP; $EC_{50} = 52.6 \pm 8.7$ μM) > *cis*-4-aminocrotonic acid (CACA; $EC_{50} = 139.4 \pm 5.2$ μM).

3 The potency order of antagonists was (±)-*trans*-2-aminomethylcyclopropanecarboxylic acid ((±)-TAMP; $K_B = 4.8 \pm 1.8$ μM) (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA; $K_B = 4.8 \pm 0.8$ μM) > (piperidin-4-yl)methylphosphinic acid (P4MPA; $K_B = 10.2 \pm 2.3$ μM) 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol (THIP; $K_B = 10.2 \pm 0.3$ μM) imidazole-4-acetic acid (I4AA; $K_B = 12.6 \pm 2.7$ μM) > 3-aminopropylphosphonic acid (3-APA; $K_B = 35.8 \pm 13.5$ μM).

4 *trans*-4-Amino-2-methylbut-2-enoic acid (2-MeTACA; 300 μM) had no effect as an agonist or an antagonist indicating that the C2 methyl substituent is sterically interacting with the ligand-binding site of rat ρ3 $GABA_C$ receptors.

5 2-MeTACA affects ρ1 and ρ2 but not ρ3 $GABA_C$ receptors. In contrast, (±)-TAMP is a partial agonist at ρ1 and ρ2 $GABA_C$ receptors, while at rat ρ3 $GABA_C$ receptors it is an antagonist. Thus, 2-MeTACA and (±)-TAMP could be important pharmacological tools because they may functionally differentiate between ρ1, ρ2 and ρ3 $GABA_C$ receptors *in vitro*.
British Journal of Pharmacology (2002) **135,** 883–890

Keywords: γ-Aminobutyric acid (GABA); $GABA_C$ receptors; ρ3 subunits; structure-activity relationship profiles; two-electrode voltage clamp; *Xenopus* oocytes

Abbreviations: (+)-TACP, (+)-*trans*-3-aminocyclopentanecarboxylic acid; (±)-CAMP, (±)-*cis*-2-aminomethylcyclopropanecarboxylic acid; (±)-TAMP, (±)-*trans*-2-aminomethylcyclopropanecarboxylic acid; 2-MeTACA, *trans*-4-amino-2-methylbut-2-enoic acid; 3-APA, 3-aminopropylphosphonic acid; CACA, *cis*-4-aminocrotonic acid; GABA, γ-aminobutyric acid; I4AA, imidazole-4-acetic acid; P4MPA, (piperidin-4-yl)methylphosphinic acid; SAR, structure-activity relationship; TACA, *trans*-4-aminocrotonic acid; THIP, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol; TPMPA, (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid

Introduction

The inhibitory neurotransmitter γ-aminobutyric acid (GABA) activates three major classes of receptors termed $GABA_A$, $GABA_B$ and $GABA_C$ receptors. $GABA_A$ and $GABA_C$ receptors are members of the ligand-gated ion channels superfamily that includes nicotinic acetylcholine, strychnine-sensitive glycine, serotonin type 3 and some invertebrate anionic glutamate receptors. Both $GABA_A$ and $GABA_C$ receptors are Cl^- channels producing fast synaptic inhibition when activated by GABA (Figure 1; see review by Chebib & Johnston, 2000). In contrast, $GABA_B$ receptors are members of the G-protein coupled receptor superfamily. These receptors are heterodimeric G-protein coupled receptors, which produce slow, longer lasting inhibition, and function to inhibit neurotransmitter release (see reviews by Bowery & Enna, 2000; Blein *et al*., 2000; Ong & Kerr, 2000). All three classes of GABA receptors are pharmacologically, physiologically and biochemically distinct (see reviews by Bormann, 2000; Chebib & Johnston, 2000; Bowery & Enna, 2000; Blein *et al*., 2000; Ong & Kerr, 2000).

$GABA_C$ receptors have been identified by their distinct pharmacology. These receptors are not blocked by the alkaloid bicuculline nor modulated by benzodiazepines and barbiturates, which typically affect $GABA_A$ receptors. Furthermore, $GABA_C$ receptors are not activated by (−)-baclofen or inhibited by (−)-phaclofen, which typically affect $GABA_B$ receptors. Instead, $GABA_C$ receptors are selectively activated

*Author for correspondence at: Faculty of Pharmacy, A15, The University of Sydney, NSW 2006, Australia
E-mail: maryc@pharm.usyd.edu.au

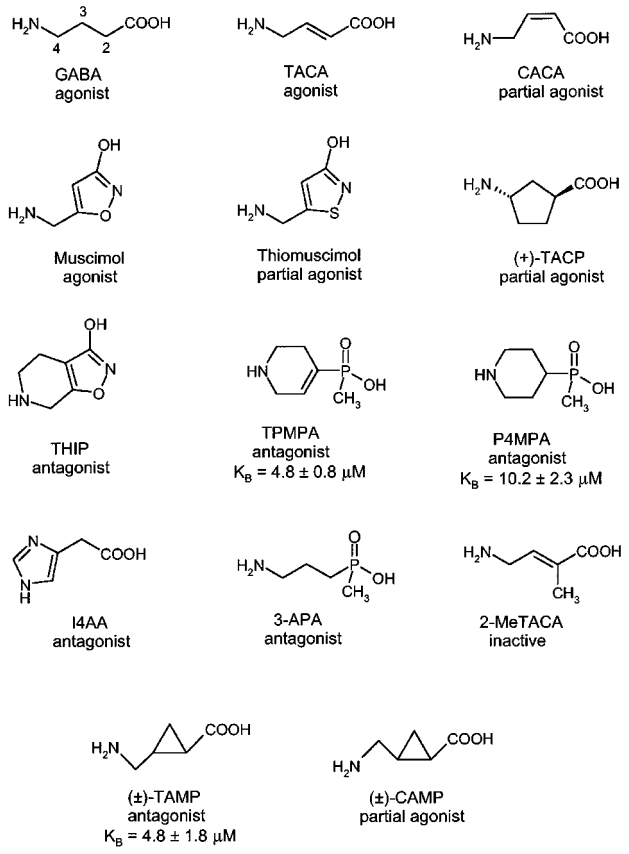

Figure 1 Structures of GABA analogues that have agonist, partial agonist and antagonist effects at ρ3 GABA$_C$ receptors.

by (+)-*cis*-2-aminomethylcyclopropane-carboxylic acid ((+)-CAMP) (Figure 1; Duke *et al*., 2000) and blocked by (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA) (Figure 1; Murata *et al*., 1996; Ragozzino *et al*., 1996).

GABA$_C$ receptors are believed to comprise of only one subunit type, the rho (ρ) subunit. To date, several ρ-subunits have been cloned including two from human (ρ1 and ρ2) (Cutting *et al*., 1991; 1992) and three from rat (ρ1–3) (Wang *et al*., 1994; Zhang *et al*., 1995; Ogurusu *et al*., 1995; Ogurusu & Shingai, 1996). These subunits exhibit high sequence homology between species and with each other. The human ρ subunits have approximately 95% sequence homology with rat ρ subunits while the sequence homology between ρ1 and ρ2 subunits is approximately 75%. Most of the diversity between the ρ1 and ρ2 subunits is in the N-terminal domain where there is a 20% sequence divergence (Cutting *et al*., 1992). In contrast, rat ρ3 subunits exhibits lower homology to rat ρ1 (65%) and ρ2 (61%) subunits (Ogurusu & Shingai, 1996).

The human ρ3 subunit gene has been found on chromosome 3q11-q13.3 but, as yet, has not been cloned (Bailey *et al*., 1999). However, expression pattern of rat ρ3 mRNA was studied along with ρ1 and ρ2 mRNA using immunohistochemistry (Boue-Grabot *et al*., 1998), *in situ* hybridization and RT–PCR (Wegelius *et al*., 1998; Boue-Grabot *et al*., 1998). These studies showed the expression pattern of the ρ3 was somewhat different from that of ρ1 and ρ2, being strongest in the hippocampus and significantly lower in the retina, dorsal root ganglia and cortex. Interestingly, no ρ3 expression was observed in the superior colliculus (Wegelius *et al*., 1998; Boue-Grabot *et al*., 1998).

The human ρ1 and ρ2, and rat ρ3 subunits form functional receptors when expressed either as homomeric receptors or as combinations to form pseudoheteromeric receptors when expressed in *Xenopus laevis* oocytes (Cutting *et al*., 1991; 1992; Kusama *et al*., 1993a, b; Zhang *et al*., 1995; Shingai *et al*., 1996; Chebib *et al*., 1997; 1998; Duke *et al*., 2000) or mammalian cell expression systems (Enz & Bormann, 1995; Enz & Cutting, 1998). These recombinant receptors have similar physiological and pharmacological properties to GABA$_C$ receptors found on native cells such as rat rod bipolar cells (Feigenspan *et al*., 1993), indicating that these combinations may exist *in vivo*. Some evidence exists for heteromeric assembly of ρ-subunits with the γ2-subunit of the GABA$_A$ receptor, particularly with perch ρ-subunits (Qian & Ripps, 1999). However, human ρ1 and ρ2-subunits do not assemble with the classical α, β and γ-subunits of the GABA$_A$ receptor (Hackam *et al*., 1998), indicating that these ρ-subunits do not form part of the GABA$_A$ receptor subunit family.

Structure-activity relationship (SAR) studies on GABA$_C$ receptors have been carried out using bovine retinal poly(A)$^+$ RNA expressed in *Xenopus* oocytes (Woodward *et al*., 1993) and human homooligomeric ρ1 and ρ2 cRNAs expressed in *Xenopus* oocytes (Kusama *et al*., 1993a, b; Ragozzino *et al*., 1996; Chebib *et al*., 1997; 1998; Duke *et al*., 2000). These studies have led to the discovery of a variety of compounds, including TPMPA (Murata *et al*., 1996; Ragozzino *et al*., 1996), (+)-CAMP (Duke *et al*., 2000) and *trans*-4-amino-2-methylbut-2-enoic acid (2-MeTACA) (Figure 1; Chebib *et al*., 1997; 1998), which are useful pharmacological tools to study ρ1 and ρ2 GABA$_C$ receptors. TPMPA was the first selective GABA$_C$ receptor antagonist that differentiated GABA$_C$ receptors from GABA$_A$ and GABA$_B$ receptors. (+)-CAMP was shown to be the most selective agonist at human ρ1 and ρ2 GABA$_C$ receptors and 2-MeTACA was shown to functionally distinguish between homomeric ρ1 and ρ2 GABA$_C$ receptors expressed in *Xenopus* oocytes.

ρ3 GABA$_C$ receptors, like ρ1 and ρ2 GABA$_C$ receptors, have been shown to be insensitive to bicuculline and the GABA$_A$ receptor modulators, 3-α-hydroxy-5α-pregnan-20-one, pentobarbitone and diazepam (Shingai *et al*., 1996). Few agonists, partial agonists and antagonists were tested on ρ3 GABA$_C$ receptors. From the study using *trans*-4-aminocrotonic acid (TACA; Figure 1), GABA, muscimol (Figure 1), *cis*-4-aminocrotonic acid (CACA; Figure 1) and picrotoxinin, Shingai *et al*. (1996) concluded that ρ3 GABA$_C$ receptors have a similar pharmacological profile as ρ1 and ρ2 GABA$_C$ receptors. In this study, we report the effects of a number of GABA analogues on ρ3 GABA$_C$ receptors in order to (1) further develop the SAR profiles of ρ3 GABA$_C$ receptors and (2) identify compounds that distinguish ρ3 from ρ1 or ρ2 GABA$_C$ receptors.

Methods

Materials

GABA, imidazole-4-acetic acid (I4AA; Figure 1), 3-aminopropylphosphonic acid (3-APA; Figure 1) and muscimol were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.).

4,5,6,7-Tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol (THIP; Figure 1) was purchased from Tocris Cookson (Ballwin, MO, U.S.A.). TACA, CACA, 2-MeTACA, (±)-*trans*-2-aminomethyl-cyclopropanecarboxylic acid ((±)-TAMP; Figure 1), (±)-CAMP (Johnston *et al*., 1975; Allan & Twitchin, 1978; Allan *et al*., 1985; Duke *et al*., 2000), TPMPA (Chebib *et al*., 1997) and (piperidin-4-yl)methylphosphinic acid (P4MPA; Johnston *et al*., 1998) were prepared according to methods described in the literature. (+)-TACP was previously prepared by Associate Professor Robin D. Allan according to methods described in the literature (Allan & Twitchin, 1980). Thiomuscimol was a gift from Professor Povl Krogsgaard-Larsen.

Electrophysiological recording

Xenopus laevis were anaesthetized with 0.17% ethyl 3-aminobenzoate and a lobe of the ovaries was removed. The lobe was rinsed with oocyte releasing buffer 2 (OR2; mM): NaCl 82.5, KCl 2, $MgCl_2.6H_2O$ 1, HEPES 1, 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 (mM): NaCl 96, KCl 2, $MgCl_2.6H_2O$ 1, $CaCl_2$ 1.8, HEPES 5, pH 7.5, supplemented with 2.5 mM pyruvate, 0.5 mM theophylline and 50 g μl^{-1} gentamycin, and stage V–VI oocytes were collected.

Rat ρ3 cRNA was prepared as reported by Shingai *et al*. (1996). In brief, rat ρ3 cDNA subcloned in pBluescript KS(−) vector was linearized using the restriction enzyme *ECOR*-I. Capped RNA was synthesized from linearized plasmid containing ρ3 cDNAs using the 'mMESSAGE mMACHINE' kit from Ambion Inc. (Austin, TX, U.S.A.). ρ3 cRNA (10 ng 50 nl^{-1}) was injected into defolliculated Stage V–VI *Xenopus* oocytes and stored at 18°C. Two to 10 days later, receptor activity was measured by two-electrode voltage clamp recording using a Geneclamp 500 amplifier (Axon Instruments Inc., Foster City, CA, U.S.A.), a MacLab 2e recorder (AD Instruments, Sydney, NSW, Australia) and Chart program version 3.5. Oocytes injected with ρ3 cRNA 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. Antagonist effects were measured at a constant dose in the presence of increasing concentrations of GABA. These solutions were prepared in frog Ringer solution. The receptor recovery time between doses was 15 min.

Analysis of kinetic data

Current (I) as a function of agonist concentration ([A]) was fitted by least squares to $I = I_{max}\ [A]^{nH} / (EC_{50}{}^{nH} + [A]^{nH})$, where I_{max} is the maximum current, EC_{50} is the effective concentration that activates 50% of the maximum current produced by a given drug and n_H is the Hill coefficient. EC_{50} values are expressed as mean ± s.e.mean ($n = 3–6$ oocytes) and are determined by fitting data from individual oocytes using PRISM 2.0a (1997). The intrinsic activity of partial agonists, I_m, was calculated as a percentage of the maximum whole cell current produced by a maximum dose of GABA. Estimated K_B values are the binding constants for the antagonists and were determined using the following equation $K_B = [Ant]/\{(A)/(A^*) - 1\}$ where A is the EC_{50} of GABA in the presence of a known antagonist concentration, A* is the EC_{50} of GABA in the absence of the antagonist and [Ant] is the concentration of the antagonist.

Results

Expression of rat ρ3 cRNA in *Xenopus* oocytes generated GABA gated channels similar to those described by Ogurusu *et al*. (1999). The amplitude of the whole cell currents recorded ranged between 50–2000 nA when the cell was clamped at −60 mV. Increasing concentrations of GABA produced a dose dependent effect on oocytes expressing ρ3 $GABA_C$ receptors. The maximal current was achieved by 300 μM GABA (Figure 2).

Figure 3A–D shows sample traces of the activation current produced by muscimol (10 μM), TACA (10 μM), CACA (300 μM) and (±)-CAMP (300 μM), respectively, against the maximal current produced by GABA (300 μM), while Figure 4A,B shows traces of the inhibition of the current produced by GABA (30 μM) by (±)-TAMP (30 μM) and TPMPA (30 μM).

Agonist and partial agonist dose response curves for ρ3 $GABA_C$ receptors expressed in oocytes are shown in Figure 5A,B, respectively. The EC_{50} values, intrinsic activity (I_m, % of the maximal response of the agonist compared to the maximal response of GABA) and Hill coefficients (n_H) of agonists (GABA, muscimol and TACA) and partial agonists ((+)-TACP, thiomuscimol, CACA and (±)-CAMP) are summarized in Table 1. The EC_{50}, I_m and n_H of GABA, TACA, muscimol and CACA were similar to the values reported by Shingai *et al*. (1996) for ρ3 $GABA_C$ receptors expressed in *Xenopus* oocytes.

The potency order of agonists was muscimol ($EC_{50} = 1.9 \pm 0.1$ μM) ≈ (+)-TACP ≈ ($EC_{50} = 2.7 \pm 0.9$ μM) ≈ TACA ($EC_{50} = 3.8 \pm 0.3$ μM) ≈ GABA ($EC_{50} = 4.0 \pm 0.3$ μM) > thiomuscimol ($EC_{50} = 24.8 \pm 2.6$ μM) > (±)-CAMP ($EC_{50} = 52.6 \pm 8.7$ μM) > CACA ($EC_{50} = 139.4 \pm 5.2$ μM). Significance between potencies of agonists was determined using a one-way analysis of variance ($P = 0.0001$; $F = 29.70$; d.f. (5,12)). Furthermore each compound was subjected to Bonferroni's Multiple comparison test. Muscimol ($I_m = 88\%$) and TACA ($I_m = 93\%$) had the highest intrinsic activities, while (+)-TACP ($I_m = 27\%$) had the lowest. The Hill coefficients of most agonists tested ranged between

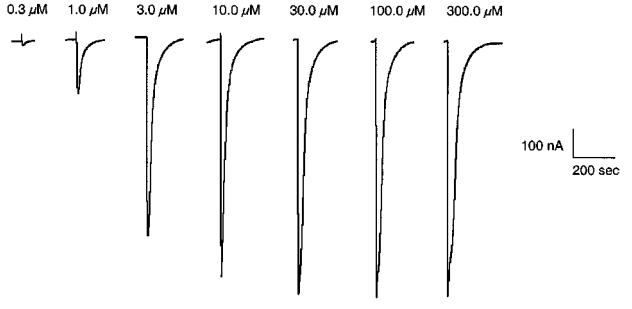

Figure 2 Increasing concentrations of GABA produced a dose dependent effect on oocytes expressing ρ3 $GABA_C$ receptors. The maximal current was achieved by 300 μM GABA.

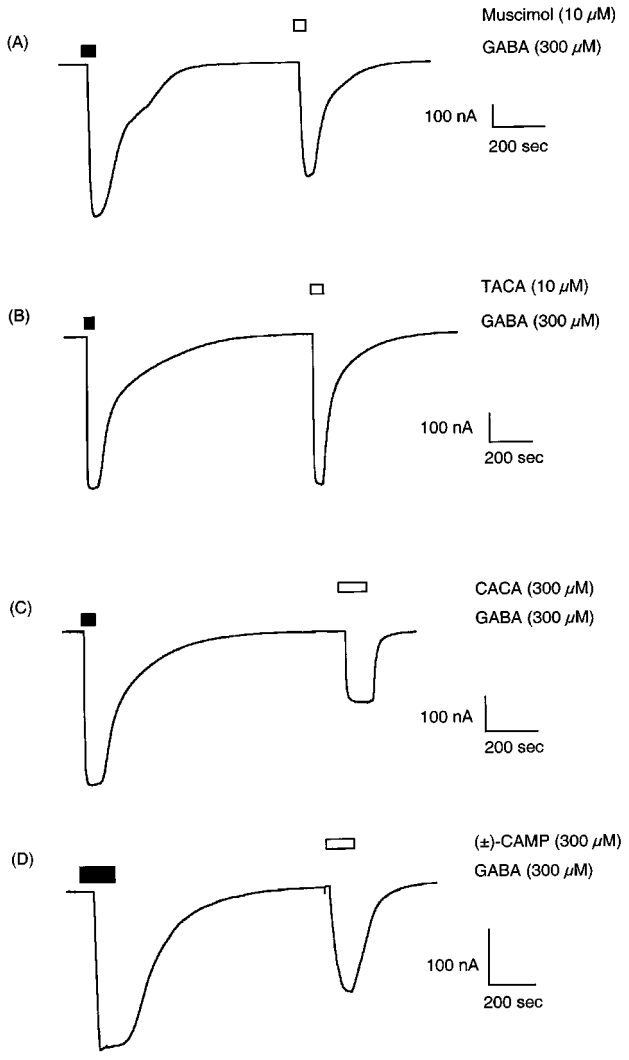

Figure 3 A maximal current is achieved by the addition of GABA (300 μM; duration indicated by solid bar) on *Xenopus* oocytes expressing rat ρ3 $GABA_C$ receptors. This is compared to the activation currents produced by (A) muscimol (10 μM; duration indicated by open bar), (B) TACA (10 μM; duration indicated by open bar), (C) CACA (300 μM; duration indicated by open bar) and (D) (±)-CAMP (300 μM; duration indicated by open bar).

1.5–1.8, with the exception of (±)-CAMP, which had a Hill coefficient of 2.4. The Hill coefficients at ρ3 $GABA_C$ receptors are lower than those found with recombinant ρ1 and ρ2 $GABA_C$ receptors but further analysis of these is needed at the single channel level to further evaluate the number of agonists required to activate the receptor.

Table 1 summarizes the estimated K_B values of antagonists at ρ3 $GABA_C$ receptors. The potency order of antagonists was (±)-TAMP ($K_B = 4.8 \pm 1.8$ μM) ≈ TPMPA ($K_B = 4.8 \pm 0.8$ μM) > P4MPA ($K_B = 10.2 \pm 2.3$ μM) ≈ THIP ($K_B = 10.2 \pm 0.3$ μM) ≈ I4AA ($K_B = 12.6 \pm 2.7$ μM) > 3-APA ($K_B = 35.8 \pm 13.5$ μM). Significance between potencies of antagonists was determined using a one-way analysis of variance ($P = 0.0006$; $F = 10.96$; d.f. (5,11)). Each compound was also subjected to Bonferroni's Multiple comparison test.

TPMPA (30 μM; Figure 6A), THIP (100 μM; Figure 6B) and 3-APA (30 μM; Figure 6C) produced a parallel rightward

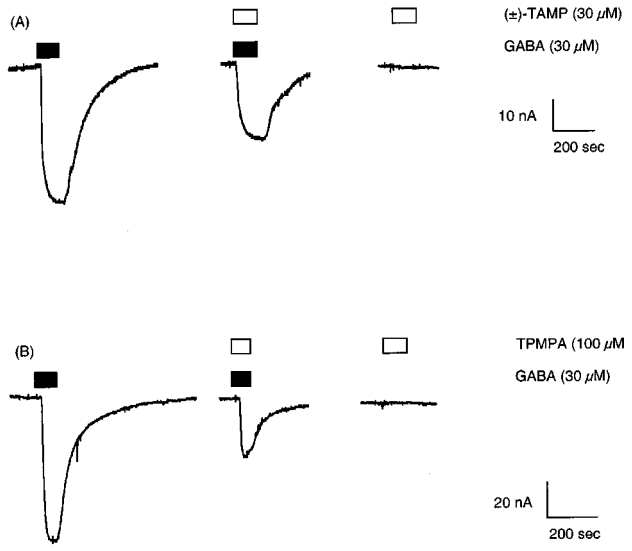

Figure 4 (A) (±)-TAMP (30 μM; duration indicated by open bar) produces no response alone but inhibits the current produced by a submaximal concentration of GABA (30 μM; duration indicated by solid bar) by 43%. (B) TPMPA (100 μM; duration indicated by open bar) produces no response alone but inhibits the current produced by a submaximal concentration of GABA (30 μM; duration indicated by solid bar) by 65%.

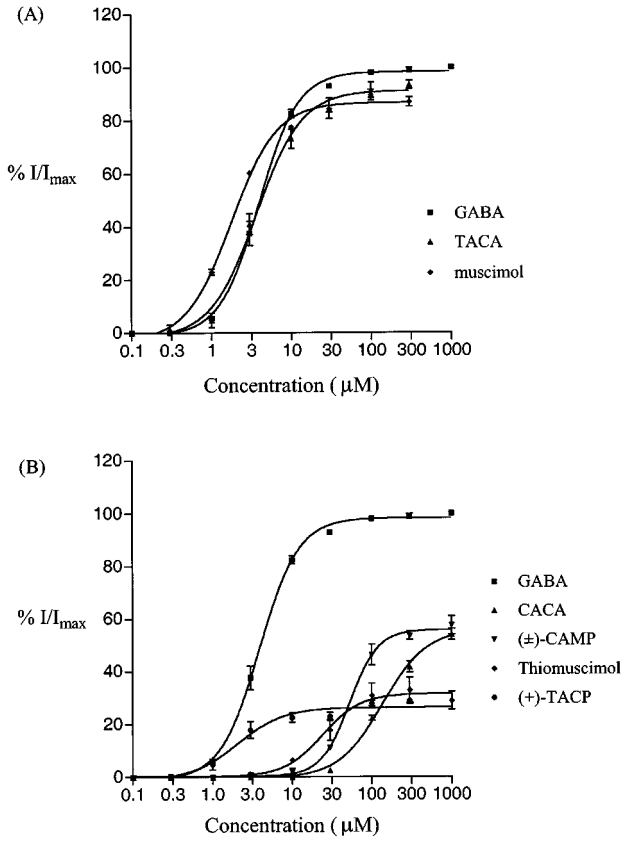

Figure 5 Dose response curves for (A) the agonists GABA, TACA and muscimol and (B) the partial agonists, CACA, (±)-CAMP, thiomuscimol and (+)-TACP compared to GABA at rat ρ3 $GABA_C$ receptors expressed in *Xenopus* oocytes. Data are the mean ± s.e.mean. ($n = 3$–6 oocytes) of the percentage of I/I_{max} (% I/I_{max}) where I/I_{max} is the percentage ratio of current generated by the compound divided by the current produced by a maximal dose of GABA (300 μM).

Table 1 The effects of GABA analogues on rat ρ3 $GABA_C$ receptors expressed in *Xenopus* oocytes

Compound	EC_{50} (μM)[a]	K_B (μM)[b]	m_H[c]	I_m (%)[d]
GABA	4.0±0.3		1.7±0.1	100
Muscimol	1.9±0.1		1.5±0.1	88
(+)-TACP	2.7±0.9		1.5±0.2	27
TACA	3.8±0.3		1.5±0.2	93
Thiomuscimol	24.8±2.6		1.8±0.6	32
(±)-CAMP	52.6±8.7		2.4±0.3	57
CACA	139.4±5.2		1.5±0.1	57
(±)-TAMP		4.8±1.8		
TPMPA		4.8±0.8		
P4MPA		10.2±2.3		
THIP		10.2±0.3		
14AA		12.6±2.7		
3-APA		35.8±13.5		

[a]EC_{50} is the effective dose that activates 50% of the I_{max}, where I_{max} is the maximum current produced by the agonists. Data are mean±s.e.mean ($n=3-6$ oocytes). [b]K_B is the estimated binding constant of the antagonist. Data are mean±s.e.mean ($n=3-6$ oocytes). [c]n_H is the Hill coefficient. Data are mean±s.e.mean ($n=3-6$ oocytes). [d]I_m 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±s.e.mean ($n=3-6$ oocytes).

shift of the GABA dose response curve with minimum reduction in the maximal response of GABA indicating that TPMPA, THIP and 3-APA are competitive antagonists over the concentration tested. In contrast, (±)-TAMP (30 μM; Figure 7A), I4AA (30 μM; Figure 7B) and P4MPA (30 μM; Figure 7C) produced a non-parallel rightward shift of the GABA dose response curve with minimum reduction in the maximal response of GABA indicating that (±)-TAMP, I4AA and P4MPA may be non-competitive antagonists at ρ3 $GABA_C$ receptors.

Interestingly, 2-MeTACA was inactive at ρ3 $GABA_C$ receptors. 2-MeTACA (300 μM) produced no response on its own nor did it significantly shift the dose response curve of GABA to the right ($P>0.05$; Figure 8A,B).

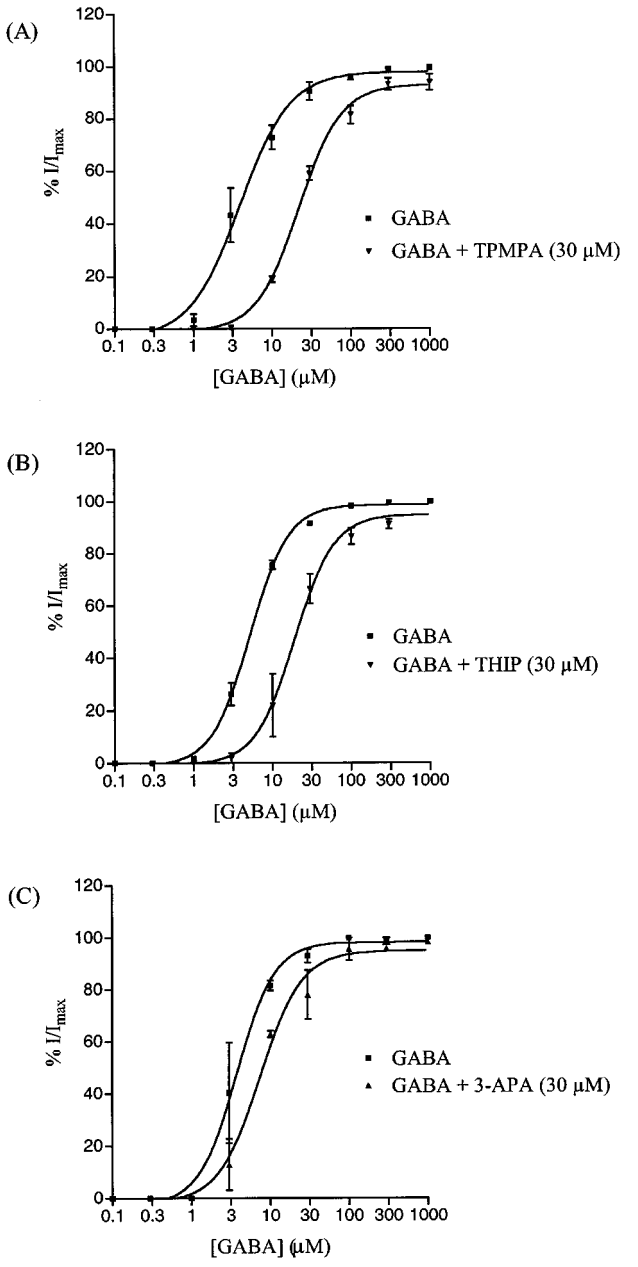

Figure 6 Dose response curves of (A) GABA alone and GABA in the presence of TPMPA (30 μM), (B) GABA alone and GABA in the presence of THIP (30 μM) and (C) GABA alone and GABA in the presence of 3-APA (30 μM) at rat ρ3 $GABA_C$ receptors expressed in *Xenopus* oocytes. Data are the mean±s.e.mean ($n=3-6$ oocytes) of the percentage of I/I_{max} (% I/I_{max}) where I/I_{max} is the percentage ratio of current generated by the compound divided by the current produced by a maximal dose of GABA (300 μM).

Discussion

To date, only a few GABA analogues have been studied on rat ρ3 $GABA_C$ receptors. In this study, a variety of GABA analogues were examined for their effects rat ρ3 $GABA_C$ receptors expressed in *Xenopus* oocytes using 2-electrode voltage clamp methods. Our study showed that the pharmacological profiles of ρ1, ρ2 and ρ3 $GABA_C$ receptors were different. The pharmacological profiles of GABA, TACA, (±)-CAMP, 2-MeTACA and (±)-TAMP at ρ3 $GABA_C$ receptors differed significantly to those at ρ1 and ρ2 $GABA_C$ receptors whereas muscimol, (+)-TACP, CACA, TPMPA and P4MPA showed similar pharmacological profiles.

The different pharmacological profiles at $GABA_C$ receptor subtypes may be due to a number of reasons. Firstly, differences in the amino acid residues in the agonist/antagonist-binding site of these receptors will contribute to the different pharmacological profiles of these receptors. Amino acids involved in the binding of GABA have been identified in ρ1 $GABA_C$ receptors (Amin & Weiss, 1994). To date, none have been identified which confer to differences in the pharmacology of $GABA_C$ receptor subtypes.

Secondly, different activation equilibria between the $GABA_C$ receptor subtypes may contribute to the pharmacological profiles of these receptors. Such differences may be attributed to different amino acid residues between the ρ1, ρ2 and ρ3 subunits. Thirdly, pK_A effects between the different acidic bioisosteres will also contribute to the activity of the

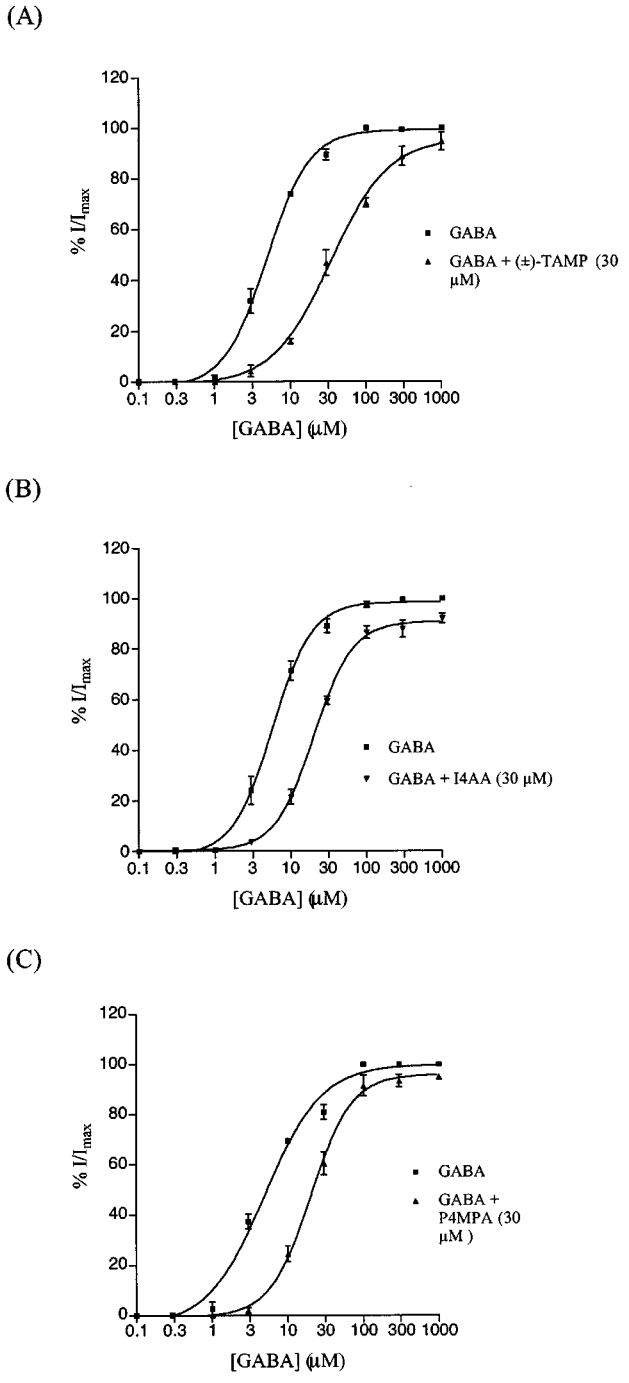

Figure 7 Dose response curves of (A) GABA alone and GABA in the presence of (±)-TAMP (30 μM), (B) GABA alone and GABA in the presence of I4AA (30 μM) and (C) GABA alone and GABA in the presence of P4MPA (30 μM) at rat ρ3 GABA$_C$ receptors expressed in *Xenopus* oocytes. Data are the mean±s.e.mean (n=3–6 oocytes) of the percentage of I/I$_{max}$ (% I/I$_{max}$) where I/I$_{max}$ is the percentage ratio of current generated by the compound divided by the current produced by a maximal dose of GABA (300 μM).

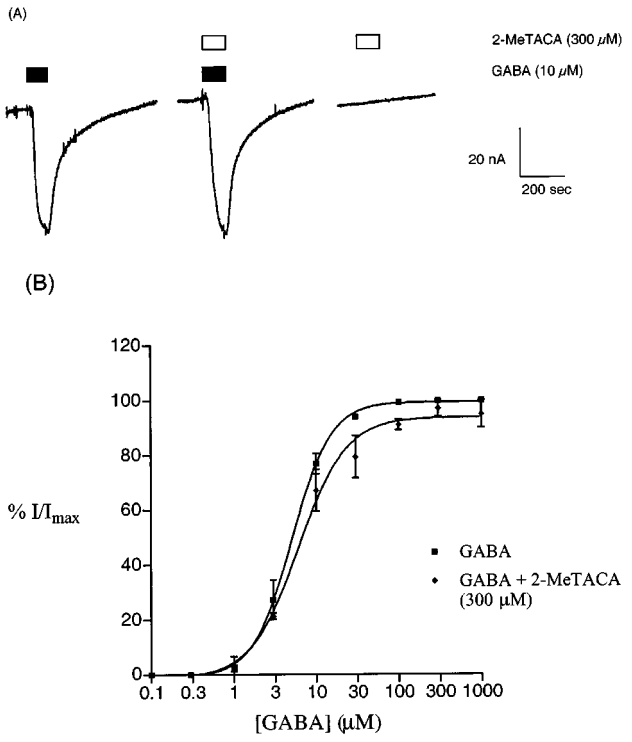

Figure 8 (A) 2-MeTACA (100 μM; duration indicated by open bar) produces no response alone nor does it inhibit the current produced by a submaximal concentration of GABA (30 μM; duration indicated by solid bar). (B) Dose response curve of GABA alone and GABA in the presence of 2-MeTACA (300 μM) at rat ρ3 GABA$_C$ receptors expressed in *Xenopus* oocytes. Data are the mean±s.e.mean (n=3–6 oocytes) of the percentage of I/I$_{max}$ (% I/I$_{max}$) where I/I$_{max}$ is the percentage ratio of current generated by the compound divided by the current produced by a maximal dose of GABA (300 μM).

compounds as exemplified by GABA, TPMPA, muscimol, thiomuscimol and 3-APA.

Finally, steric interaction between the ligand and the ligand binding site appears to be one of the major factors which contribute to the pharmacology of compounds at GABA$_C$ receptors. 2-MeTACA is an analogue of TACA with a methyl substituent in the C2 position. At ρ1 receptors, 2-MeTACA was shown to be a moderately potent antagonist while at ρ2 GABA$_C$ receptors it was a partial agonist with moderate intrinsic activity (Chebib *et al.*, 1997; 1998). At ρ3 GABA$_C$ receptors, 2-MeTACA was shown to have no effect as an agonist or an antagonist even when tested at 300 μM. Alkyl substituents at the C2 position of TACA produced ligands whose interactions with the receptor can be tolerated at ρ1 and ρ2 GABA$_C$ receptors but, in contrast, cannot be tolerated at ρ3 GABA$_C$ receptor. This is an important finding because 2-MeTACA may functionally differentiate ρ3 from ρ1 and ρ2 GABA$_C$ receptors.

Steric effects may also be contributing to the pharmacological profile of (±)-TAMP and (±)-CAMP. At rat ρ3 GABA$_C$ receptors, (±)-TAMP is a potent antagonist, while it is a partial agonist at both human ρ1 and ρ2 GABA$_C$ receptors (Duke *et al.*, 2000). Thus, (±)-TAMP can functionally differentiate rat ρ3 from ρ1 and ρ2 GABA$_C$ receptors.

At ρ3 GABA$_C$ receptors, (±)-CAMP is a partial agonist but, at ρ1 and ρ2 GABA$_C$ receptors, it is a full agonist (Duke *et al.*, 2000). (±)-CAMP and (±)-TAMP are conformationally restricted analogues of GABA held rigidly by a

methylene bridge between positions C2 and C3. The methylene bridge may interact sterically with the receptor protein producing an intrinsic activity less than 100% in the case of (±)-CAMP and antagonism in the case of (±)-TAMP.

Furthermore, (±)-CAMP and (±)-TAMP were tested as racemates, that is a 50 : 50 mixture of (+)- and (−)-CAMP and a 50 : 50 mixture of (+)- and (−)-TAMP. Therefore, the partial agonism of (±)-CAMP may also be due to opposing effects of the enantiomers of (±)-CAMP, one acting as a full agonist and the other acting as an antagonist. Similarly, the antagonist effects of (±)-TAMP may be due to opposing effects of the enantiomers of (±)-TAMP, one acting as an agonist/partial agonist and the other acting as an antagonist. Such pharmacological differences between the enantiomers of (±)-CAMP were reported by Duke *et al*. (2000) at human recombinant ρ1 and ρ2 GABA$_C$ receptors, where (+)-CAMP is a full agonist, while its enantiomer, (−)-CAMP, is a weak antagonist. Such effects were not observed with the enantiomers of (±)-TAMP. Both (+)- and (−)-TAMP were partial agonists with similar intrinsic activities at both ρ1 and ρ2 GABA$_C$ receptors. However, further experiments are required to establish whether the pharmacological effects of (±)-CAMP and (±)-TAMP at the ρ3 GABA$_C$ receptor are due to the opposing effects of enantiomers or unfavourable steric interactions.

The pharmacological evaluation of the various GABA analogues at GABA$_C$ receptors has contributed towards the SAR profiles for ρ3 GABA$_C$ receptors. The finding that 2-MeTACA has no effect as an agonist or antagonist and that (±)-TAMP is a potent antagonist at rat ρ3 GABA$_C$ receptors highlights the pharmacological differences between GABA$_C$ receptor subtypes. These compounds could be important pharmacological tools because they may functionally differentiate between ρ1, ρ2 and ρ3 GABA$_C$ receptors *in vitro*. Although many of these compounds cannot cross the blood brain barrier, the results of this study may lead to the design and development of selective ρ3 GABA$_C$ receptor ligands, which would aid in studying the role these receptors play in the central nervous system.

The authors wish to thank Mr Kong Li and Dr Hue Tran for their excellent technical assistance and National Health and Medical Research Council of Australia and Circadian Technologies Pty Ltd for financial support.

References

ALLAN, R.D., JOHNSTON, G.A.R., & KAZLAUSKAS, R. (1985). Synthesis of analogues of GABA. XIII. An alternate route to (*Z*)-4-aminocrotonic acid. *Aust. J. Chem.,* **38,** 1647 – 1650.

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

ALLAN, R.D. & TWITCHIN, B. (1980). Synthesis of analogues of GABA. IV Three unsaturated derivatives of 3-aminocyclopentane-1-carboxylic acid. *Aust. J. Chem.,* **33,** 599 – 604.

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

BAILEY, M.E., ALBRECHT, B.E., JOHNSON, K.J. & DARLISON, M.G. (1999). Genetic linkage and radiation hybrid mapping of the three human GABA(C) receptor rho subunit genes: GABRρ1, GABRρ2 and GABRρ3. *Biochim. Biophys. Acta,* **1447,** 307 – 312.

BLEIN, S., HAWROT, E. & BARLOW, P. (2000) The metabotropic GABA receptor: Molecular insights and their functional consequences. *Cell. Mol. Life Sci.,* **57,** 635 – 650.

BORMANN, J. (2000). The 'ABC' of GABA receptors. *TiNS,* **21,** 16 – 19.

BOWERY, N.G. & ENNA, S.J. (2000). γ-Aminobutyric acid$_B$ receptors: first of the functional metabotropic heterodimers. *J. Pharmacol. Exp. Ther.,* **292,** 2 – 7.

BOUE-GRABOT, E., ROUBARAKI, M., BASCLES, L., TRAMU, G., BLOCH, B. & GARRET, M. (1998). Expression of GABA receptor rho subunits in rat brain. *J. Neurochem.,* **70,** 899 – 907.

CHEBIB, M. & JOHNSTON, G.A.R. (2000). GABA activated ion channels: medicinal chemistry and molecular biology, Perspective. *J. Med. Chem.,* **43,** 1427 – 1447.

CHEBIB, M., MEWETT, K.N. & JOHNSTON, G.A.R. (1998). GABA$_C$ receptor antagonists differentiate between human ρ1 and ρ2 receptors expressed in Xenopus oocytes. *Eur. J. Pharmacol.,* **357,** 227 – 234.

CHEBIB, M., VANDENBERG, R.J. & JOHNSTON, G.A.R. (1997). Analogues of γ-aminobutyric acid (GABA) and trans-4-aminocrotonic acid (TACA) substituted in the 2-position as GABA$_C$ receptor antagonists. *Br. J. Pharmacol.,* **122,** 1551 – 1560.

CUTTING, G. R., CURRISTIN, S., ZOGHIBIM H., O'HARA, B., SELDIN, M.F. & UHL, G. R. (1992). Identification of a putative gamma-aminobutyric acid (GABA) receptor subunit ρ2 cDNA and colocalisation of the genes encoding ρ1 (GABRρ2) and ρ1 (GABRρ1) to human chromosome 6ql4-q21 and mouse chromosome 4. *Genomics,* **12,** 801 – 806.

CUTTING, G. R., LU, L., O'HARA, B. F., KASCH, L.M., MONTOSER-AFIZADEH, C., DONOVAN, D.M., SHIMADA, S., ANTONARAKIS, S. E., GUGGINO, W. B., UHL, G. R. & KAZAJIAN JR, H. H. (1991). Cloning of the γ-aminobutyric acid (GABA) ρ1 cDNA: a GABA receptor subunit highly expressed in the retina. *Proc. Natl. Acad. Sci. U.S.A.,* **88,** 2673 – 2677.

DUKE, R.K., CHEBIB, M., ALLAN, R.D., MEWETT, K.N. & JOHNSTON, G.A.R. (2000). (+)- and (−)- CAMP (*cis*-2-aminomethylcyclopropanecarboxylic acid), show opposite pharmacology at recombinant ρ1 and ρ2 GABA$_C$ receptors. *J. Neurochem.,* **75,** 2602 – 2610.

ENZ, R. & BORMANN, J. (1995). A single point mutation decreases picrotoxinin sensitivity of the human GABA receptor ρ1 subunit. *NeuroReport,* **6,** 1569 – 1572.

ENZ, R. & CUTTING, G.R. (1998). Molecular composition of GABA$_C$ receptors. *Vis. Res.,* **38,** 1431 – 1441.

FEIGENSPAN, A., WASSLE, H. & BORMANN, J. (1993). Pharmacology of GABA receptor chloride channels in rat retinal bipolar cells. *Nature,* **361,** 159 – 161.

HACKAM, A.S., WANG, T.L., GUGGINO, W.B. & CUTTING, G.R. (1998). Sequences in the amino termini of GABA ρ and GABA$_A$ subunits specify their selective interaction in vitro. *J. Neurochem.,* **70,** 40 – 46.

JOHNSTON, G.A.R, BURDEN, P.M., MEWETT, K.N. & CHEBIB, M. (1998). Neurologically-active compounds, The University of Sydney and Polychip Pharmaceuticals. International Patent Publication No. WO 98/58939.

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.

KUSAMA, T., SPIVAK, C.E., WHITING, P., DAWSON, V.L., SCHAEFFER, J.C. & UHL, G.R. (1993a). Pharmacology of GABA rho 1 and GABA alpha/beta 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, GR. (1993b). GABA rho 2 receptor pharmacological profile: GABA recognition site similarities to rho 1. *Eur. J. Pharmacol.,* **245,** 83 – 84.

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,** 964 – 968.

OGURUSU, T. & SHINGAI, R. (1996). Cloning of a putative γ-aminobutyric acid (GABA) receptor subunit ρ3 cDNA. *Biochim. Biophys. Acta,* **1305,** 15 – 18.

OGURUSU, T., TAIRA, H. & SHINGAI, R. (1995). Identification of $GABA_A$ receptor subunits in rat retina: cloning of the rat retina $GABA_A$ receptor ρ2 subunit cDNA. *J. Neurochem.,* **65,** 964 – 968.

OGURUSU, T., YANAGI, K., WATANABE, M., FUKAYA, M. & SHINGAI, R. (1999). Localization of GABA receptor ρ2 and ρ3 subunits in rat brain and functional expression of homooligomeric ρ3 receptors and heterooligomeric ρ2ρ3 receptors. *Recept. Channels,* **6,** 463 – 475.

ONG, J. & KERR, D.I.B. (2000). Recent advances in $GABA_B$ receptors: from pharmacology to molecular biology. *Acta. Pharmacol. Sin.,* **21,** 111 – 123.

QIAN, H. & RIPPS, H. (1999). Response kinetics and pharmacological properties of heteromeric receptors formed by coassembly of GABA rho- and gamma 2-subunits. *Proc. Royal Soc. London – Series B: Biol. Sci.,* **266,** 2419 – 2425.

RAGOZZINO, D., WOODWARD, R.M., MURARA, Y., 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.

SHINGAI, R., YANAGI, K., FUKUSHIMA, T., SAKATA, K. & OGURUSU, T. (1996). Functional expression of GABA rho 3 receptors in *Xenopus* oocytes. *Neurosci. Res.,* **26,** 287 – 390.

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.

WEGELIUS, K., PASTERNACK, M., HILTUNEN, J.O., RIVERA, C., KAILA, K., SAARMA, M. & REEBEN, M. (1998). Distribution of GABA receptor rho subunit transcripts in the rat brain. *Eur. J. Neurosci.,* **10,** 350 – 357.

WOODWARD, R.M., POLENZANI, L. & MILEDI, R. (1993). Characterization of bicuculline/baclofen-insensitive (ρ-like) gamma-aminobutyric acid receptors expressed in *Xenopus* oocytes. 2. Pharmacology of gamma-aminobutyric $acid_A$ and gamma-aminobutyric $acid_B$ receptor agonists and antagonists. *Mol. Pharmacol.,* **43,** 3609 – 3625.

ZHANG, D., PAN, Z.H., ZHANG, X., BRIDEAU, A. & LIPTON, S.A. (1995). Cloning of a GABA 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 29, 2001
Revised October 2, 2001
Accepted October 9, 2001)