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Comparative activities of the enantiomeric $GABA_B$ receptor agonists CGP 44532 and 44533 in central and peripheral tissues

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Abstract

In neocortical slices maintained in Mg^{2+}-free Krebs medium, the γ-aminobutyric acid ($GABA_B$) receptor agonists baclofen, (3-amino-2(*S*)-hydroxypropyl)methylphosphinic acid (CGP 44532), and its (*R*)-enantiomer CGP 44533 depressed the frequency of spontaneous discharges in a concentration-dependent manner (EC_{50} = 10, 6.5, and 50 μM, respectively). These effects were reversibly antagonised by the $GABA_B$ receptor antagonist (+)-(*S*)-5,5 dimethylmorpholinyl-2-acetic acid (Sch 50911) (3, 10, and 30 μM) (average pA_2 value = 6.0 ± 0.2). In neocortical wedges, baclofen, CGP 44532 and CGP 44533 elicited concentration-dependent hyperpolarisations (the EC_{50}s were 14, 7.5 and 16 μM, respectively) sensitive to Sch 50911 (1, 5, 10 μM) (average pA_2 value = 6.0 ± 0.1), whilst they also depressed ileal electrically elicited cholinergic twitch contractions (EC_{50} = 11, 7, and 50 μM) that were antagonised by Sch 50911 (average pA_2 value = 6.0 ± 0.1). In electrically stimulated brain slices preloaded with [^{3}H]GABA, baclofen, CGP 44532 and CGP 44533 decreased [^{3}H]GABA release (IC_{50} = 5, 0.45, and 10 μM); this effect was reversed by Sch 50911 (50 μM). It is concluded that CGP 44532 is a far more potent agonist at $GABA_B$ autoreceptors than at central or peripheral heteroreceptors. © 2001 Published by Elsevier Science B.V.

Keywords: $GABA_B$ receptor agonist; Rat neocortex; Ileum, Guinea-pig; Baclofen; CGP 44532; CGP 44533

1. Introduction

Bicuculline-insensitive γ-aminobutyric $acid_B$ ($GABA_B$) receptors belong to a class of family 3 (C) G-protein-coupled metabotropic receptors for the neurotransmitter GABA that mediates neuronal inhibition. They share sequence similarity with the metabotropic glutamate receptors, the Ca^{2+}-sensing receptors, a family of vomeronasal and putative taste receptors and bacterial periplasmic amino acid-binding proteins (Kaupmann et al., 1997; Bettler et al., 1998; Bowery and Enna, 2000; Ong and Kerr, 2000). Using in situ hybridization, the $GABA_B$ receptor splice variants R1a and R1b are found extensively localized throughout the brain (Lu et al., 1999; Liang et al., 2000), where they appear to be associated with pre- and postsynaptic elements, respectively, in central neurones (Billinton et al., 1999). Similar to many other G-protein-coupled receptors, $GABA_B$ receptors either activate inwardly rectifying K^+ channels or inhibit voltage-gated Ca^{2+} channels, and have been demonstrated in both pre- and postsynaptic components of excitatory and inhibitory neurons in the central nervous system (Kerr and Ong, 1995). Stimulation of presynaptic $GABA_B$ heteroreceptors has been shown to decrease neurotransmitter release, possibly by reducing Ca^{2+} conductance, whilst activation of postsynaptic $GABA_B$ receptors causes a hyperpolarization of postsynaptic neurones by increasing a K^+ conductance responsible for long-lasting inhibitory potentials (for reviews see Bowery, 1993; Misgeld et al., 1995; Mott and Lewis, 1995).

Apart from baclofen (β-4-chlorophenyl-GABA), a well characterised prototypical agonist for $GABA_B$ receptors where the β-4-chlorophenyl substituent on the GABA-

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backbone imparts specificity for these receptors (Bowery et al., 1981; Hill and Bowery, 1981), other selective agonists with considerable potencies have been developed (Froestl et al., 1995). These include (3-amino-2(*S*)-hydroxypropyl)methylphosphinic acid (CGP 44532), and its (*R*)-enantiomer, CGP 44533, which show relatively potent pharmacological and binding activities at $GABA_B$ receptors in a number of preparations (Froestl et al., 1995; Knight and Bowery, 1996; Froestl and Mickel, 1997; Bonanno et al., 1998; Enna et al., 1998; Brebner et al., 1999; Yamada et al., 1999). Structurally, CGP 44532 and CGP 44533 are related to other agonists such as 3-aminopropyl-phosphinic acid, and 3-aminopropyl-methyl-phosphinic acids, which have been shown previously to be more potent than baclofen itself (Froestl et al., 1995). Indeed, the binding affinity to $GABA_B$ receptors is higher when the 2-hydroxy substituent is oriented in the (*S*)-configuration, as in CGP 44532, where the latter is about seven times more potent than the (*R*)-form CGP 44533 in displacing [^{3}H]baclofen binding to $GABA_B$ receptors (Froestl and Mickel, 1997). In rat dorsolateral septal nucleus, CGP 44533 is a selective presynaptic $GABA_B$ heteroreceptor agonist at glutamatergic nerve terminals without affecting postsynaptic receptor sites (Yamada et al., 1999).

In this study, we have evaluated the effects of CGP 44532 and CGP 44533 at presynaptic $GABA_B$ heteroreceptors, in spontaneously discharging rat neocortical slices, and on $GABA_B$ postsynaptic receptor-mediated hyperpolarizations in neocortical wedges. Furthermore, their actions were examined on peripheral $GABA_B$ receptors, and on $GABA_B$ autoreceptors modulating electrically evoked [^{3}H]GABA from neocortical slices. Interestingly, in the latter, we found that CGP 44532 was 22 times more potent than CGP 44533 on autoreceptors in decreasing [^{3}H]GABA release, whilst CGP 44532 was only 2–8 times more potent than CGP 44533 in activating heteroreceptors.

2. Materials and methods

2.1. Rat neocortical slice preparations

The experiments were conducted in strict accordance with the guidelines of the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985), the Australian Code of Practice for the care and use of animals for scientific purposes of the National Health and Medical Research Council and The University of Adelaide Animal Ethics Committee. Rat neocortical slices were prepared from halothane anaesthetized outbred male adult Sprague–Dawley rats (250–350 g), which were decapitated. The brains were rapidly dissected out and immersed for 30 min in ice-cold oxygenated Krebs solution gassed with 95% O_2:5% CO_2 (pH 7.4) of the following composition (in mM): NaCl 118, KCl 2.1, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 11, $MgSO_4$ 1.3. Cerebral cortical slices (400 μM thick) were prepared by cutting coronal sections using a vibraslice microtome (Campden Instruments, UK), and a radial wedge was cut from each side of the dorsal mid-line to yield slices of cingulate cortex and corpus callosum 2–3 mm wide. The slices were subsequently equilibrated in gassed Krebs solution at room temperature (20–23°C) for 60 min prior to experimentation.

Using a superfusion method based on a grease-gap system as described previously (Horne et al., 1986; Ong et al., 1990), wedge-shaped slices from the neocortex were superfused with gassed Mg^{2+}-free Krebs medium at 25°C delivered by a peristaltic pump at 1 ml/min. $MgSO_4$ was omitted in the Mg^{2+}-free medium. Potentials between the cingulate cortex and underlying corpus callosum were monitored on a chart recorder, using Ag/AgCl electrodes, agar/saline bridges and a high input-impedance preamplifier AC coupled with a 1 s time constant. The neocortical slices generally developed spontaneous paroxysmal discharges after equilibration in Mg^{2+}-free Krebs medium for 15 min.

Compounds such as the $GABA_B$ receptor agonists baclofen, CGP 44532, or CGP 44533 were added to the superfusing medium, and applied to the cortical side of the tissue for 2 min; the preparation was allowed 30 min recovery between drug applications. The antagonist was first superfused for 2 min and then added together with the agonist or test compounds. Results were quantified by counting the number of spontaneous discharges in 10-min epochs, in the absence and presence of test agents, and the values expressed as a percentage depression of the average control discharge rate during the 10 min immediately before the addition of drugs. Concentration–response curves for the agonist were constructed in the absence and presence of the antagonist. The EC_{50} value, the concentration that produced 50% inhibition of the discharge rate, was calculated as the geometric mean from the concentration–response curve. Estimates of apparent pA_2 values were made for the antagonist. The pA_2 value was derived from the relationship $pA_2 = \log (CR - 1) - \log [B]$, where $(CR - 1)$ is the concentration ratio $- 1$, and [B] the antagonist concentration. All numerical data on the concentration–response curves were expressed as means ± S.E.M. Each experiment was repeated on eight slices obtained from at least four different animals.

The method used for recording hyperpolarizing responses to the $GABA_B$ receptor agonists baclofen, CGP 44532, or CGP 44533 was essentially similar to the above, except that each wedge was placed across a septum, separating pools containing the cortex and white matter by a grease seal. Differential recordings (mV) between the cortex and white matter were measured with Ag/AgCl electrodes, and the DC potentials were monitored on a chart recorder using a high input-impedance DC amplifier. The white matter was immersed in a chamber containing

Krebs solution with Mg^{2+} (1.3 mM), whilst the grey matter in the second chamber was superfused with gassed Mg^{2+}-containing Krebs buffer at 25°C delivered by a peristaltic pump at 1 ml/min. Here, Mg^{2+}-containing Krebs medium was used throughout the experiments to eliminate the spontaneous discharges, since the latter tended to complicate the hyperpolarizing responses. After 60 min equilibration, the $GABA_B$ receptor agonists were added to the superfusing medium, and applied to the cortical side of the tissue for 3 min to achieve steady-state concentrations within the recording chamber. Each preparation was allowed a minimum of 30-min recovery between drug applications. The antagonist was first superfused for 10 min and then added together with the agonist or test compounds. Results were quantified, and values expressed as a percentage of the maximum hyperpolarization obtained with each compound, measured from the chart recordings. Concentration–response curves were constructed in the absence and presence of the antagonist. The EC_{50} value was calculated from the concentration–response curve as above, and estimates of apparent pA_2 values were made. All numerical data on the concentration–response curves were expressed as means ± S.E.M. Each experiment was repeated on 8–12 slices obtained from 4–6 different animals.

2.2. Guinea-pig ileal preparations

Male guinea-pigs, weighing between 200 and 400 g, were killed by cervical dislocation. Segments of the terminal ileum, 2–3 cm in length, were quickly removed and mounted in 5 ml organ baths containing modified Krebs–bicarbonate solution of the following composition [mM]: NaCl 125, KCl 3.0, NaH_2PO_4 1.4, $NaHCO_3$ 25, $CaCl_2$ 2.0, $MgSO_4$ 1.0 and glucose 10 (pH 7.4 at 37°C). The Krebs solution was continuously aerated with a gas mixture of 95% O_2 and 5% CO_2 as previously described (Ong and Kerr, 1983). After 60 min equilibration in Krebs solution, pulses (duration 0.5–1 ms, frequency 0.15 Hz, just submaximal voltage) were delivered from a Grass S48 stimulator to give transmural stimulation of cholinergic intrinsic neurones. Effects of drug treatments were examined on repetitive twitch contractions evoked by field stimulation, elicited through ring electrodes positioned around the segments of the ileum. Mechanical activity of the longitudinal muscle was recorded isometrically using Grass FT03 force transducers, and changes in tension were displayed on a Grass Model 79 polygraph.

The agonists were applied at 30-min intervals, and the antagonist added 5 min before the agonists were tested. Control responses to the agonist were routinely re-established after washing out the antagonist. Concentration–response curves to the agonists, in the presence and absence of different doses of the antagonist, were constructed and the inhibitory responses were calculated as % maximum response. By interpolation from the concentration–response curves, the half maximally effective agonist concentrations (EC_{50}) were derived for the agonists alone, and in the presence of the antagonists. Three concentrations of the antagonist were tested, and the pA_2 value was derived as an average from the relationship $pA_2 = \log (CR - 1) - \log [B]$, where $(CR - 1)$ is the concentration ratio − 1, and [B] the antagonist concentration. All numerical data on the concentration–response curves were expressed as means ± S.E.M. Student's *t*-test for paired and unpaired samples was used to assess the significance ($P < 0.05$) of differences between mean values of the concentration–response effects; *n* represents the number of preparations used for each drug treatment. Drug volumes never exceeded 1% of the total bath volume, and all drugs were dissolved in distilled water.

2.3. Release studies

The methods used were described in detail previously (see Ong et al., 1998). Briefly, pairs of neocortical slices were incubated in Krebs solution (32°C) containing [^{3}H]GABA (0.05 μM) plus GABA (0.05 μM) for 20 min. Each pair was rinsed, placed in a small chamber and superfused at 1 ml/min with Krebs solution (95% O_2:5% CO_2, 32°C). The perfusion medium contained NO-711 (5 μM), an inhibitor of the uptake of GABA. Aliquots of superfusate were collected at 10 min intervals for the first four collections and for 4 min thereafter and assayed by liquid scintillation spectrometry. Slices were stimulated through platinum field electrodes by square wave pulses at 2 Hz (2.0 ms duration, 20 mA) for 30 s at 20 min and for 150 s at 48 min (S_1), 68 min (S_2), 88 min (S_3) and usually 108 min (S_4) after superfusion commenced (see Fig. 6). At the end of each experiment, the residual [^{3}H] content in the slices was extracted in 0.4 M $HClO_4$ (containing EDTA, 3.0 mM and Na_2SO_3, 10 mM) at 4°C for at least 16 h and assayed. The effects of baclofen, CGP 44532 and CGP 44533 on the fractional overflow of [^{3}H] were tested at either S_2 or S_4. The agents were added 12 min prior to the onset of stimulation and remained in the Krebs solution for 20 min prior to washout (S_2), or the end of the experiment (S_4). In those experiments in which the effects of CGP 44532 or CGP 44533 were examined in the presence of the $GABA_B$ receptor antagonist Sch 50911 (Ong et al., 1998), both agents were added to the Krebs solution superfusing the slices 12 min prior to S_2 and were washed out 12 min before S_3.

The resting overflow of [^{3}H] is defined as the fractional overflow of [^{3}H] in the 4 min prior to stimulation and the stimulation-induced overflow as the fractional overflow in the 4 min following the onset of stimulation, minus the resting overflow. The effects of the four agents used on the stimulation-induced overflow (SIO) of [^{3}H] were determined by comparing the ratio of SIO at S_2/SIO at S_1 (or SIO_4/SIO_1) with the same ratio in the absence of the agonist/antagonist. An antagonist of $GABA_B$ autoreceptors would increase the SI-overflow ratio and an agonist

decrease it. A similar technique was used to measure the effect of the agonist/antagonist on the resting overflow of [^{3}H]. The significance of the effects of the agents used were assessed by unpaired Student's t-tests, with significance levels at $P < 0.05$.

Krebs solution used in these experiments was of the following composition (mM): NaCl (120), KCl (4.7), $NaHCO_3$ (25), KH_2PO_4 (1.0), $CaCl_2$ (1.5), $MgSO_4$ (1.3), glucose (11), and contained aminooxyacetic acid (0.05 mM).

2.4. Drugs

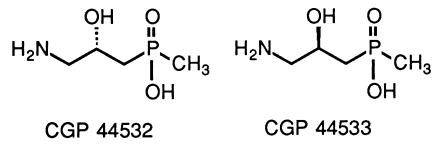
Racemic (±)-baclofen, (3-amino-2(*S*)-hydroxypropyl)-methylphosphinic acid (CGP 44532) and 3-amino-2(*R*)-hydroxypropyl)methylphosphinic acid (CGP 44533) were synthesised at Novartis Pharma (Basel, Switzerland). [2,3-^{3}H(N)]GABA, specific activity 1.06 TBq/mmol, was obtained from New England Nuclear (Boston, MA). Aminooxyacetic acid hemihydrochloride was purchased from Sigma (MO, USA) and the GABA uptake inhibitor, NO-711 (1-(2-(((diphenylmethylene)amino)oxy)ethyl)-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid), was obtained from Research Biochemicals (Natick, MA). (+)-(*S*)-5,5 Dimethylmorpholinyl-2-acetic acid (Sch 50911) was purchased from Tocris (UK).

3. Results

3.1. Effects of baclofen, CGP 44532 and CGP 44533 on spontaneous discharges in rat neocortex

Rat neocortical slices maintained in Mg^{2+}-free medium displayed repetitive paroxysmal discharges within 15 min. Confirming previous results (Ong et al., 1998), the frequency and amplitude of discharges were consistently attenuated by the $GABA_B$ receptor agonist, baclofen, in a concentration-dependent manner over a concentration range of 1–200 μM (Fig. 1A; $n = 8$). Baclofen briefly depressed the amplitude, suppressed the afterpotentials and subsequently reduced the frequency of the discharges, with an EC_{50} of 10 μM. This depressive action of baclofen typically persisted for 10–15 min, with the amplitude and frequency of the discharges returning to control level within 30 min upon baclofen wash-out. As previously reported by Ong et al. (1998), the $GABA_B$ receptor antagonist Sch 50911 (20 μM) reversibly antagonised the baclofen action, confirming that this effect of baclofen was indeed mediated through $GABA_B$ receptors (figure not shown).

Superfusion of CGP 44532 (1–100 μM) and its enantiomer CGP 44533 (6–600 μM) onto the rat neocortical slice similarly reduced the amplitude of the discharges and attenuated the frequency, in a concentration-dependent manner (Fig. 1A; $n = 8$). The EC_{50} values for CGP 44532



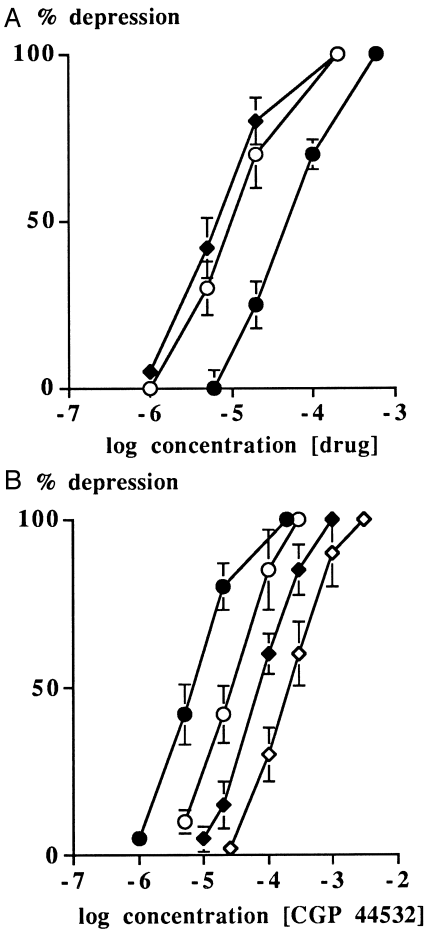


Fig. 1. Chemical structures of (3-amino-2(*S*)-hydroxypropyl)methylphosphinic acid (CGP 44532) and its (*R*)-enantiomer CGP 44533 showing the orientation of the 2-hydroxy substituent. (A) Complete concentration–response curves for (*R,S*)-baclofen (○), CGP 44532 (♦), and CGP 44533 (●)-induced suppression of the frequency of spontaneous discharges in rat isolated neocortical slices, maintained in Mg^{2+}-free Krebs medium. (B) Concentration–response curves for CGP 44532-induced suppression of discharge frequency, in the absence and presence of (+)-(*S*)-5,5 dimethylmorpholinyl-2-acetic acid (Sch 50911). The concentration–response curve for CGP 44532 (●) was subsequently shifted to the right, in a parallel fashion, by Sch 50911 (○ 3 μM; ♦10 μM; ◇ 30 μM). Values are expressed as a percentage depression of the control discharge rate. Each point represents the mean and standard error of the mean of eight determinations.

and CGP 44533 were 6.5 and 50 μM, respectively; CGP 44532 was about 1.5 times more potent than baclofen, whilst CGP 44533 was five times less potent than the latter but 7.7 times less potent than CGP 44532. These depressive effects, lasting some 10–15 min, were also competitively antagonised by Sch 50911 (3, 10, and 30 μM), again in a reversible manner, with a recovery of the

responses to various concentrations of CGP 44532 and CGP 44533 within 30 min ($n = 8$). As Fig. 1B illustrates, increasing concentrations of the antagonist Sch 50911 (3, 10, and 30 μM) caused a progressive shift of the CGP 44532 concentration–response curve to the right, without depression of the maximum response. Using the ratio method and averaging, this yielded an apparent pA_2 value of 6.0 ± 0.2 ($n = 8$). In a typical experiment, the CGP 44532 (20 μM)-induced suppression of spontaneous activity in the rat neocortical slice was effectively antagonised by Sch 50911 (30 μM), the latter being readily washed-out within 30 min, after which the response to the agonist was re-established (Fig. 2). Sch 50911 also caused similar parallel rightward shifts of the CGP 44533 concentration–response curve ($pA_2 = 6.0 \pm 0.1$; data not shown). However, prior exposure to Sch 50911 itself (3, 10 and 30 μM) for 5 min did not affect the frequency or amplitude of the spontaneous discharges. Neither baclofen, CGP 44532 nor CGP 44533 showed any detectable tissue desensitization when superfused onto the preparations.

3.2. Baclofen, CGP 44532 and CGP 44533-induced hyperpolarizations in neocortical wedges

When the wedges were superfused with Krebs solution containing either Ba^{2+} (0.1 mM) or Cs^{+} (1 mM) to block K^{+} conductances, the hyperpolarizing effects to the $GABA_B$ receptor agonists baclofen-, CGP 44532- and CGP 44533-induced hyperpolarizations (each at 50 μM) were effectively abolished, suggesting that the agonists activate $GABA_B$ receptors that are coupled to inwardly rectifying Ba^{2+}-sensitive K^{+} channels (data not shown). Superfusion of neocortical wedges, with baclofen for 3 min over a concentration range of 2–200 μM, in Mg^{2+}-containing Krebs solution to suppress spontaneous discharges, consistently induced concentration-dependent hyperpolarizing responses (Fig. 3A; $n = 12$). The onset of the baclofen-evoked hyperpolarization was reached after 2 min of drug application, and the maximal effect occurred within 3–5 min. This persisted for a variable period of 5–10 min, after which repolarization of the membrane potential occurred within 15–20 min following reintroduction of drug-free Krebs solution. Responses obtained by using different concentrations of the $GABA_B$ receptor agonists were normalized to those obtained by using a maximum concentration of each agonist. Here, as shown in Fig. 3A, the concentration–response curve for baclofen was plotted as a percentage of the maximal response elicited by baclofen at 200 μM (100% response; $n = 12$). This curve yielded the half-maximal effect (EC_{50} value) for baclofen which occurred at 14 μM. Furthermore, the threshold for inducing a hyperpolarization response was around 2 μM, resulting in a 10% response, and the maximal effect was elicited by 200 μM baclofen (100% response). In general, full recovery to baclofen-induced responses was obtained only after 30 min of wash-out, although in some preparations, up to 60 min wash-out was required.

In a representative experiment, baclofen (20 μM) induced a significant hyperpolarization that was reversibly antagonised by Sch 50911 (5 μM) (Fig. 4A). After 10 min pretreatment with Sch 50911, which on its own had no effect on the membrane potentials, baclofen was then co-applied with Sch 50911. During application of baclofen and Sch 50911, the hyperpolarizing response to baclofen was substantially reduced; subsequently upon tissue wash-out within 30 min, there was a full recovery of the control response to baclofen (Fig. 4A). In this preparation, Sch 50911 (1, 5, and 10 μM) caused a progressive shift of the baclofen concentration–response curve to the right, without depression of the maximum response. Using the ratio method and averaging, this again yielded an apparent pA_2 value of 6.0 ± 0.1 for this antagonist (Fig. 3A; $n = 12$).

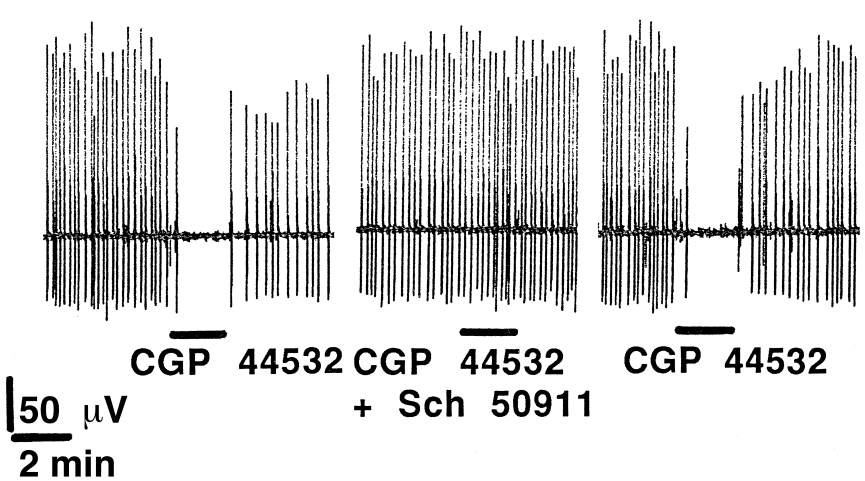


Fig. 2. Representative records from a typical experiment showing the antagonism of (+)-(*S*)-5,5 dimethylmorpholinyl-2-acetic acid (Sch 50911) on the depressant responses to CGP 44532 in the rat neocortical slice preparation, maintained in Mg^{2+}-free Krebs medium. CGP 44532 (20 μM) induced a suppression of spontaneous discharges, reversibly antagonised by Sch 50911 (30 μM) and the control response to CGP 44532 was subsequently re-established upon wash-out of the test compounds.

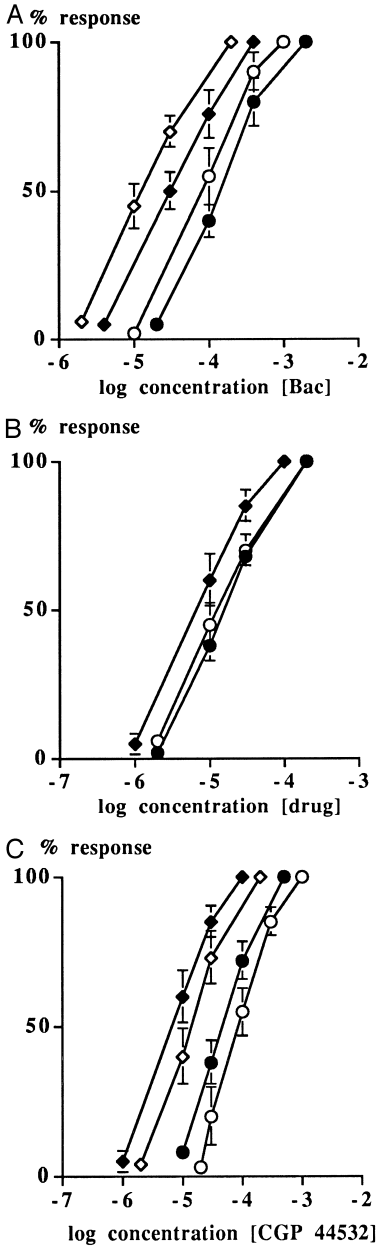


Fig. 3. Concentration–response curves for hyperpolarizing responses induced by (A) baclofen alone (◇), or in the presence of the antagonist (+)-(*S*)-5,5 dimethylmorpholinyl-2-acetic acid (Sch 50911) (◆ 1 μM; ○ 5 μM; ● 10 μM) in rat neocortical slices, maintained in Mg^{2+}-containing Krebs solution. (B) A comparison of the concentration–response curves for baclofen (○), as well as (3-amino-2(*S*)-hydroxypropyl)methylphosphinic acid (CGP 44532) (◆) and its (*R*)-enantiomer CGP 44533 (●). (C) Concentration–response curves for CGP 44532, in the absence (◆), and presence of the antagonist (+)-(*S*)-5,5 dimethylmorpholinyl-2-acetic acid (Sch 50911) (◇ 1 μM; ● 5 μM; ○ 10 μM). Values are expressed as a percentage of the maximum hyperpolarization (expressed as a 100%) induced by the agonists, and each point represents the mean and standard error of the mean of 12 determinations.

These baclofen-induced hyperpolarizing effects were mimicked by both CGP 44532 (1–100 μM) and CGP 44533 (2–200 μM), reversibly inhibited by Sch 50911 (1, 5, and 10 μM). The antagonism was surmountable by application of higher concentrations of the agonists. In approximately 60% of the preparations, this action to CGP 44532, but not CGP 44533, far outlasted that of baclofen by 5 or more minutes; intriguingly, upon wash-out of a high concentration of CGP 44532 (100 μM), and during recovery of the resting potential, large magnitude spontaneous discharges appeared for some 20 min, after which the quiescent baseline resumed. This effect was not observed with either baclofen or CGP 44533 application under the same recording conditions. From the concentration–response curves, the EC_{50} values for CGP 44532 and CGP 44533 were 7.5 and 16 μM, respectively (Fig. 3B; $n = 12$). When their EC_{50}s were compared to that of baclofen, CGP 44532 was about twice more potent than baclofen, whilst CGP 44533 was virtually equipotent to baclofen but twice less potent than CGP 44532 itself. Sch 50911 caused parallel rightwards shifts of the concentration–response curves to CGP 44532 (Fig. 3C; $n = 12$) and CGP 44533 (Fig. not shown, $n = 12$) without altering the maximal responses, producing a calculated pA_2 value of 6.0 ± 0.1. At the concentrations employed in these experiments, Sch 50911 itself did not affect the membrane potentials. Traces displayed in Fig. 4B illustrate hyperpolarizations elicited by CGP 44532 (10 μM) equipotent and comparable to that of baclofen (20 μM), before and during application of Sch 50911 (5 μM) in the same preparation. Subsequent application of Sch 50911 (5 μM) induced a clear inhibition of the CGP 44532 effect after 3 min. This inhibitory action of Sch 50911 was easily reversible 30 min after its removal where full recovery of the response

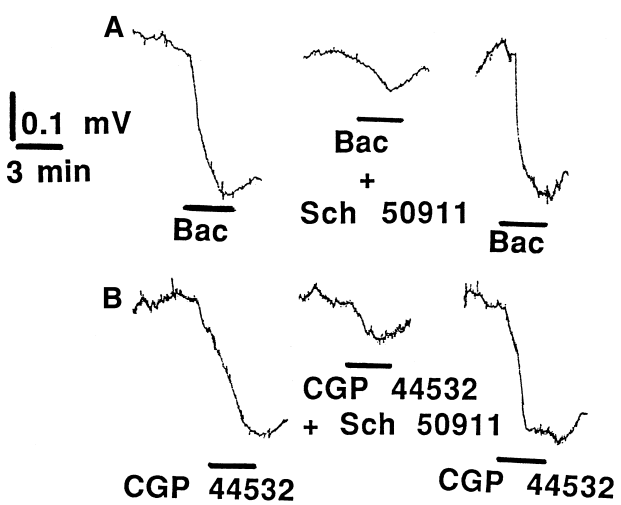


Fig. 4. Discontinuous records of the hyperpolarizing effects of baclofen and (3-amino-2(*S*)-hydroxypropyl)methylphosphinic acid (CGP 44532) on membrane potentials in rat neocortical wedges, and their antagonism by (+)-(*S*)-5,5 dimethylmorpholinyl-2-acetic acid (Sch 50911). (A) Baclofen (Bac; 20 μM) induced a hyperpolarization response that was reversibly antagonised by Sch 50911 (5 μM). The control response to baclofen was subsequently re-established upon tissue wash-out. (B) CGP 44532 (10 μM) also induced a comparable hyperpolarization response to that of baclofen, reversibly antagonised by Sch 50911 (5 μM), with a recovery of the control response to CGP 44532 upon tissue wash-out.

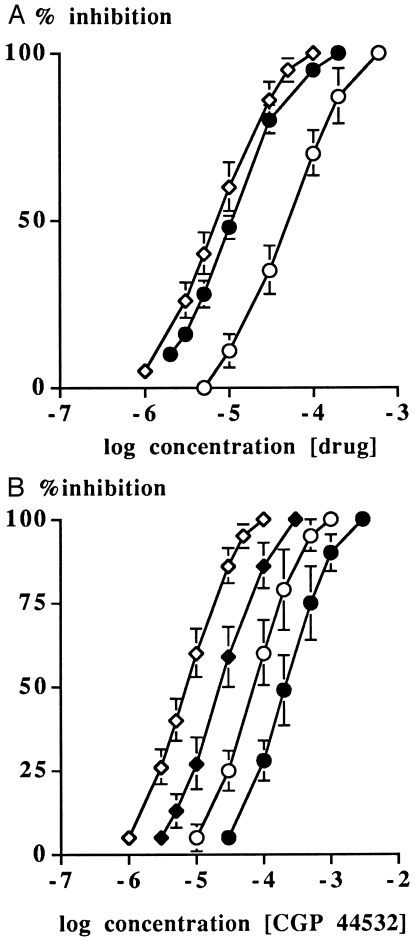


Fig. 5. Concentration–response curves for the inhibitory effects of the $GABA_B$ receptor agonists on cholinergic twitch contractile responses in the guinea-pig isolated ileum. (A) Baclofen (●), (3-amino-2(*S*)-hydroxypropyl)methylphosphinic acid (CGP 44532) (◇) and its (*R*)-enantiomer CGP 44533 (○) elicited concentration-dependent depression of ileal twitch contractions. (B) Rightward parallel shift of the concentration–response curves of CGP 44532 alone (◇), or in the presence of three concentrations of (+)-(*S*)-5,5 dimethylmorpholinyl-2-acetic acid (Sch 50911) (◆ 3 μM; ○ 10 μM; ● 30 μM). The concentration–response curves are represented as a percentage of the maximal inhibition (expressed as a 100%) of the twitch contractions induced by the agonists. Each point represents the mean and standard error of the mean of 8–16 separate determinations.

to CGP 44532 was seen (Fig. 4B). Baclofen, CGP 44532 and CGP 44533 generally did not exhibit tissue desensitization.

3.3. Effects of baclofen, CGP 44532 and CGP 44533 on ileal cholinergic twitch contractions

Baclofen (2–100 μM), CGP 44532 (1–100 μM) and CGP 44533 (5–600 μM) each elicited concentration-dependent inhibition of cholinergic twitch contractions in the guinea-pig ileum, reversibly antagonised in a competitive manner by Sch 50911 (3, 10, and 30 μM), which itself had no discernible actions on the twitch responses. The estimated EC_{50} values for baclofen, CGP 44532 and CGP 44533 in depressing twitch contractions were 11, 7, and 50 μM, respectively (Fig. 5A; $n = 16$). Thus, CGP 44532 was about 1.6 times more potent than baclofen in depressing twitch contractions, whilst CGP 44533 was 4.5 times less potent than baclofen, and seven times less potent than CGP 44532. Preincubation of Sch 50911 alone for 5 min, and then in combination with each of the agonists for 2 min, produced parallel shifts in the concentration–response curves for baclofen, CGP 44532 and CGP 44533 without a significant change in the maximal responses (average pA_2 value for Sch $50911 = 6.0 \pm 0.1$; $n = 8$). The estimated pA_2 values for the antagonist were the same when using either baclofen, CGP 44532 or CGP 44533 as the agonist, there being no significant difference between these pA_2 values at the 0.05 confidence level. As shown in Fig. 5B, there was a rightwards displacement of the concentration–response curve for CGP 44532 by Sch 50911 (3, 10, and 30 μM) in a parallel manner with no reduction in the maximal response to CGP 44532 ($n = 8$). In these preparations, Sch 50911 was effective within 5 min of its application, and the responses to the agonists returned to their control levels within 30 min following tissue wash-out. No desensitization to baclofen, CGP 44532 or CGP 44533 occurred, since the depressive responses induced by the agonists were readily re-established after wash-out in agonist-free Krebs solution.

3.4. Effects of baclofen, CGP 44532 and CGP 44533 on the release of [^{3}H]GABA

As shown by the results of a typical experiment, the overflow of [^{3}H] into the Krebs solution superfusing neo-

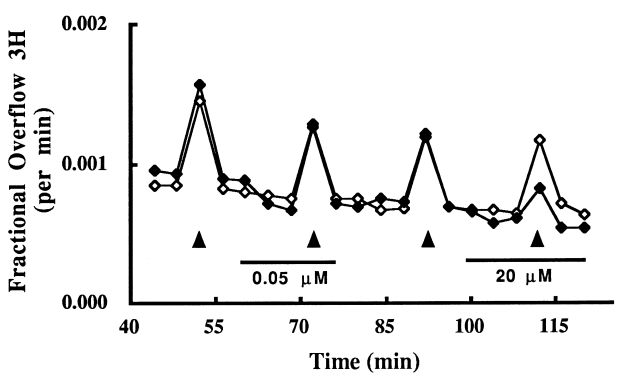


Fig. 6. The fractional overflow per min of [^{3}H]GABA from rat neocortical slices pre-incubated in [^{3}H]GABA (0.1 μM) and superfused with Krebs solution containing NO-711 (5 μM) in two typical experiments. In one (◇), the slices were untreated and in the other (◆), perfused with (3-amino-2(*S*)-hydroxypropyl)methylphosphinic acid (CGP 44532) (0.05 and 20 μM) for the periods represented by the bars. Tissues were stimulated (▲) at 2 Hz for 150 s. In untreated slices, the overflow of [^{3}H] in the four 4-min collections, each commencing with a period of stimulation, was maximal for the first stimulation and declined slightly with each succeeding stimulation. In this experiment, CGP 44532 (0.05 μM) had little effect on the stimulated overflow, but at a concentration of 20 μM, the overflow was inhibited strongly.

cortical slices, prelabelled with [^{3}H]GABA (0.1 μmol/l), reached a near steady-state within 40 min of commencing superfusion (Fig. 6). In the presence of the GABA uptake inhibitor NO-711 (5 μM), electrical stimulation increased the overflow of [^{3}H] by approximately 75% in the 4 min collection following the onset of stimulation, an increase which returned to the resting level within a further 4 min. In this experiment, CGP 44532 had little effect on the stimulation-induced overflow at a concentration of 0.05 μM, but inhibited the overflow markedly when the concentration was increased to 20 μM (Fig. 6). From the concentration–response curve derived from these experiments (Fig. 7), CGP 44532 inhibited the stimulation-induced overflow of [^{3}H]GABA over the concentration range of 0.05–200 μM, with maximal facilitation near 20 μM and an IC_{50} value of 0.45 μM.

Also shown in Fig. 7, CGP 44533 and baclofen inhibited the SI-overflow of [^{3}H]GABA. For CGP 44533, inhibition occurred at concentrations of 2 μM and above (maximal inhibition at 200 μM), with an IC_{50} of 10 μM, whereas for baclofen the concentration range was 0.2–200 μM (maximal inhibition at 20 μM) and the IC_{50} 5 μM.

The inhibitory effects of CGP 44532 (1.0 μM) and CGP 44533 (10 μM) on the stimulation-induced overflow of [^{3}H]GABA were reversed in the presence of Sch 50911 (50 μM). In these experiments, the overflow increased by $184 \pm 33\%$ in tissue treated with CGP 44532 and Sch 50911 ($n = 5$) and by $184 \pm 18\%$ ($n = 3$) in slices superfused with CGP 44533 and the antagonist, relative to the overflow in tissue treated with the agonist alone.

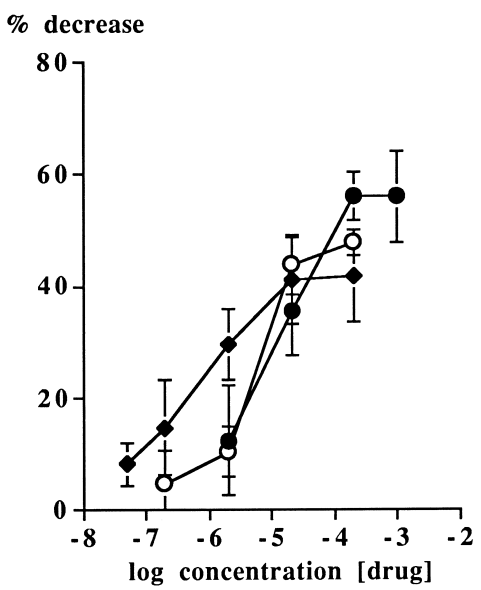


Fig. 7. Concentration–response curves for the $GABA_B$ receptor agonists baclofen (○), (3-amino-2(*S*)-hydroxypropyl)methylphosphinic acid (CGP 44532) (◆) and its (*R*)-enantiomer CGP 44533 (●). Values shown are the decreases in the stimulation-induced overflows in the presence of each agent expressed as a percentage of the overflow in the absence of the agents. Data are the means and standard errors of the means of at least four experiments. All three agents inhibited the release of [^{3}H]GABA.

Baclofen did not affect the resting overflow of [^{3}H] at any of the concentrations used; however, at higher concentrations CGP 44532 (2 and 20 μM) and CGP 44533 (1.0 mM) depressed the overflow by between 18% and 20%. This inhibitory effect was reversed in the presence of Sch 50911 (50 μM), such that the resting overflow was increased by between 45% and 50% relative to that with each of the agonists alone.

4. Discussion

In the present study, the $GABA_B$ receptor agonists baclofen, CGP 44532 and CGP 44533 induced $GABA_B$ receptor-mediated responses in central and peripheral preparations, sensitive to the antagonist Sch 50911. Overall, the 2-hydroxy-substituted phosphinic acid derivative CGP 44532 where the 2-hydroxy group is oriented in the (*S*)-configuration (Fig. 1), was more potent than either its (*R*)-enantiomer CGP 44533, or (*R,S*)-baclofen in the preparations examined. In the spontaneously discharging neocortical slices maintained in Mg^{2+}-free Krebs medium, the frequency and amplitude of the repetitive paroxysmal discharges were attenuated by baclofen, mediated through $GABA_B$ heteroreceptors, and also reversibly antagonised by the $GABA_B$ receptor antagonist Sch 50911 (Ong et al., 1998). Here, CGP 44532 was near 1.5 times more potent than baclofen in depressing the discharge frequency, but was some eight times more potent than CGP 44533, whilst CGP 44533 was five times less potent than baclofen. Likewise, Sch 50911 reversibly blocked the depressant effects induced by CGP 44532 and CGP 44533 with similar pA_2 values to baclofen (average pA_2 of 6.0). In the guinea-pig isolated ileum, $GABA_B$ receptor activation by baclofen inhibited electrically evoked cholinergic twitch contractions, reducing acetylcholine output through presynaptic receptors, as previously shown (Bowery et al., 1981; Ong and Kerr, 1983). The (*S*)-enantiomer CGP 44532 was 1.6 times more potent than baclofen in depressing twitch contractions, whilst the (*R*)-enantiomer CGP 44533 was 4.5 times less potent than baclofen, and seven times less potent than its isomer CGP 44532. The inhibitory actions of the three agonists on twitch responses were antagonised by Sch 50911 in a competitive manner, with a rightward parallel shift of the dose–response curves in the presence of Sch 50911, with an average pA_2 value of 6.0 ± 0.1.

These results indicate that on presynaptic $GABA_B$ heteroreceptors, CGP 44532 and CGP 44533 exhibit a similar range of potency difference when compared to other functional assays (Froestl et al., 1995). For example, in binding experiments, CGP 44532 shows a threefold higher binding affinity to $GABA_B$ receptors than CGP 44533 using [^{3}H]3-aminopropylphosphinic acid ([^{3}H]CGP 27492) (see Table 3 in Froestl et al., 1995), whilst in the hemisected rat spinal cord preparation, it is about three times more effec-

tive than the (*R*)-enantiomer in blocking monosynaptic reflexes. Furthermore, CGP 44532 is significantly more potent than either baclofen or CGP 44533 in inducing muscle relaxation (Froestl et al., 1995), where CGP 44532 is thought to have a larger therapeutic window, eliciting more effective muscle relaxant actions without inducing the sedation, reduced vigilance, vomiting or other side-effects associated with baclofen. Moreover, in vivo, the duration of action of CGP 44532 markedly exceeds that of baclofen. It also exerts a substantially more potent analgesic effect than baclofen in a rodent model of chronic pain, and unlike baclofen, tolerance does not occur during CGP 44532 administration, rendering it a potentially useful compound for clinical use (Enna et al., 1998). In the latter model, using formalin hindpaw injection, it is proposed that $GABA_B$ receptor stimulation produces analgesia, in part, by blocking tachykinin NK_1 receptor gene expression in the spinal cord. Recently, baclofen has been shown to produce a selective decrease in the motivation of rats to self-administer cocaine (Roberts et al., 1996; Roberts and Andrews, 1997). Similarly, CGP 44532 at low doses with less sedative effects can specifically attenuate the reinforcing effects of cocaine (Brebner et al., 1999). When tested for their potencies in modulating forskolin and noradrenaline-stimulated adenylyl cyclase activity, they were marginally more potent in inhibiting the forskolin-stimulated adenylyl cyclase activity than in inhibiting the noradrenaline effect; however, CGP 44532 was equipotent to CGP 44533 in the forskolin assay, but was four times more potent in modulating noradrenaline-stimulated cyclic AMP production (Knight and Bowery, 1996).

In neocortical wedges, baclofen, CGP 44532 and CGP 44533 induced concentration-dependent hyperpolarizing responses, reversibly antagonised by Sch 50911 with an apparent pA_2 value of 6.0 ± 0.1. Such hyperpolarizations are mediated through $GABA_B$ receptors, and most likely involve the opening of K^+ channels, as these effects were sensitive to Ba^{2+} and Cs^+. These responses appear to be similar to that described in other brain areas including hippocampus, septum and the brain stem (Inoue et al., 1985; Lacey et al., 1988; Seabrook et al., 1990; Bon and Galvan, 1996). The concentration-dependent curves for baclofen, CGP 44532 and CGP 44533 were parallel, and comparison of the EC_{50} values indicates that CGP 44532 was twice more potent than baclofen, whilst CGP 44533 was virtually equipotent to baclofen and twice less potent than CGP 44532 itself. Yet, in the rat dorsolateral septal neurons, CGP 44533 did not induce any postsynaptic effects at $GABA_B$ receptors at all, but was an effective presynaptic $GABA_B$ heteroreceptor agonist on glutamatergic nerve terminals, depressing the amplitude of excitatory postsynaptic currents (Yamada et al., 1999). Several studies have indicated that pre- and postsynaptic $GABA_B$ receptors in central neurones may be pharmacologically distinct (Deisz et al., 1993, 1997; Dutar and Nicoll, 1988; Thompson and Gahwiler, 1992). Thus, it is possible that in the septum, CGP 44533 could prove to be a useful tool to differentiate between pre- and postsynaptic receptors.

Actions of CGP 44532 but not CGP 44533, outlasted the effect of baclofen in approximately 60% of the preparations, by 5 or more minutes, similar to that found in the spinal cord (Froestl et al., 1995). Overall, CGP 44532, where the 2-hydroxy-substituent is in the (*S*)-configuration, displays a higher potency than the (*R*)-enantiomer (CGP 44533). This is reminiscent of the relative potencies of the hydroxylated GABA derivatives 4-amino-3-(*R*)-(−)-hydroxy-butyric acid ((*R*)-(−)-GABOB) as against the (*S*)-(+)-isomer, which is a partial agonist eight times less potent than the (*R*)-enantiomer (Falch et al., 1986). Indeed, in the presence of bicuculline to block $GABA_A$ receptors, (*R*)-(−)-GABOB was three times more potent than (*S*)-(+)-GABOB in eliciting hyperpolarizing responses in brain slices, with the latter being a partial agonist at $GABA_B$ receptors, reaching only 40% of the maximum response achieved with (*R*)-(−)-GABOB (Ong and Kerr, unpublished data). Despite opposite suffixes for the (*R*)- and (*S*)-enantiomers in these two series of agonists, the absolute stereochemical orientations of the hydroxy-substituents of the more potent ligands are identical in each, due to the higher priority of the phosphinic acid moiety in CGP 44532 and CGP 44533 in the Cahn Ingold Prelog nomenclature. Interestingly, the 2-hydroxy-derivative of the phosphinic acid analog of baclofen is twice less potent than the corresponding unsubstituted analog (Froestl et al., 1995), as is found with baclofen itself, where (*R*,*S*)-3-hydroxy-baclofen is three times less potent than the parent baclofen (Kerr and Ong, 1995). In the same way, the (*R*,*S*)-2-hydroxy derivative of 3-aminopropylsulphinic acid is some 20 times less potent than the parent 3-aminopropylsulphinic acid (Carruthers et al., 1998), presumably because one stereoisomer has an unfavourable configuration for binding to $GABA_B$ receptors.

$GABA_B$ receptor agonists interact with GABA autoreceptors, inhibiting depolarization or electrically evoked [^{3}H]GABA release from brain tissues (Raiteri et al., 1989; Baumann et al., 1990). In the present study, our findings that baclofen, CGP 44532 and CGP 44533 decreased the electrically evoked release of [^{3}H]GABA from rat neocortical slices implies that these compounds are agonists at $GABA_B$ autoreceptors. The inhibitory actions at autoreceptors are specific, given that Sch 50911 reversed the effects; Sch 50911 alone facilitated [^{3}H]GABA overflow by antagonising autoreceptors, as we have previously shown (Ong et al., 1998). In the present experiments, the EC_{50} values for baclofen, CGP 44532 and CGP 44533 in decreasing the stimulation-induced overflow of [^{3}H]GABA were 5, 0.45, and 10 μM, CGP 44532 being twice more potent than baclofen, and 22 times more potent than CGP 44533. In keeping with this, the large magnitude spontaneous discharges, seen during the recovery of the hyperpolarization to a high concentration of CGP 44532, evidently arise from the latter inhibiting endogenous GABA

release, which in turn induced hyperexcitability reflected in these discharges. Such effects were not observed with either baclofen or CGP 44533, presumably because these compounds are considerably less potent at autoreceptors. By contrast, Froestl et al. (1995) found that CGP 44532 was only 1.5 times more potent than the (*R*)-enantiomer (EC_{50} = 0.4 μM as against 0.6 μM in the latter) using a similar paradigm. We have no plausible explanations for such differences. In the spinal cord, CGP 44533 but not baclofen, is a selective agonist at autoreceptors (EC_{50} = 0.81 μM), and is marginally less potent in inhibiting GABA release than reducing glutamate release at heteroreceptors (EC_{50} = 0.5 μM) (Bonanno et al., 1998); however, when compared to baclofen, the latter was virtually inactive against GABA release.

To conclude, in rat neocortical and guinea-pig ileal preparations, the $GABA_B$ receptor agonists baclofen, CGP 44532 and its (*R*)-enantiomer CGP 44533 attenuate the frequency of spontaneous discharges by depressing glutamate release, induce K^+-dependent hyperpolarizing responses, decrease electrically evoked [^{3}H]GABA release, and inhibit cholinergic ileal twitch contractions. The effects of baclofen are consistently less potent than that mediated by CGP 44532. However, the potency difference between CGP 44532 and CGP 44533 in activating $GABA_B$ autoreceptors is considerably larger than the potency differences found at hetero- and postsynaptic $GABA_B$ receptors. Since it has been earlier proposed that the heteroreceptors may be pharmacologically distinct from autoreceptors (Bonanno and Raiteri, 1993; but see Waldmeier et al., 1994), it remains to be seen whether CGP 44532 and CGP 44533 may be of use as pharmacological tools in further discriminating between these receptor subtypes.

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