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Morpholin-2-yl-phosphinic acids are potent $GABA_B$ receptor antagonists in rat brain

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Abstract

The pharmacological properties of morpholin-2-yl-phosphinic acids were evaluated on $GABA_B$ receptors. In rat neocortical slices maintained in Mg^{2+}-free Krebs medium, baclofen, a $GABA_B$ receptor agonist, produced a concentration-dependent depression of the frequency of spontaneous discharges with an EC_{50} of 14 ± 5.5 μM, which was antagonised reversibly by the morpholin-2-yl-phosphinic derivatives. The order of potency was 3-{(3*S*,6*R*)-6-[(cyclohexylmethyl)hydroxyphosphinoylmethyl]-morpholin-3-yl}benzoic acid (CGP 76290A) ($pA_2 = 7.1 \pm 0.05$) > its enantiomer 3-{(3*R*,6*S*)-6-[(cyclohexylmethyl)hydroxyphosphinoylmethyl]-morpholin-3-yl}benzoic acid (CGP 76291A) ($pA_2 = 6.8 \pm 0.1$) > cyclohexylmethyl-[(2*R*′,5*S*′)-5-(3-nitrophenyl)-morpholin-2-ylmethyl]phosphinic acid (CGP 71978) ($pA_2 = 6.5 \pm 0.05$) > cyclohexylmethyl-[(2*R*,5*S*)-5-phenyl-morpholin-2-ylmethyl]phosphinic acid (CGP 71980) ($pA_2 = 6.3 \pm 0.15$) > its enantiomer cyclohexylmethyl-[(2*S*,5*R*)-5-phenyl-morpholin-2-ylmethyl]phosphinic acid (CGP 71979) ($pA_2 = 5.8 \pm 0.1$). An open chain analogue of CGP 76290A, CGP 56999A (3-{1(*R*)-[(3-cyclohexylmethyl-hydroxyphosphinoyl)-2(*S*)-hydroxypropyl-amino]-ethyl}benzoic acid lithium salt) gave a pA_2 of 6.6 ± 0.2. In $GABA_B$ receptor binding assays, CGP 71982 (the racemic mixture of CGP 76290A and CGP 76291A), CGP 76290A, CGP 76291A, CGP 71978, CGP 71980 and CGP 71979 had IC_{50} values against [^{3}H]CGP 27492 binding of 8, 1.85, 69, 124, 326 and 1460 nM, respectively. In electrically-evoked [^{3}H]GABA release from rat cortical slices, CGP 71982, CGP 71978, CGP 71980 and its enantiomer CGP 71979, antagonised $GABA_B$ autoreceptors with EC_{150} values of 2.5, 33, 181 and 474 nM, respectively. These compounds form a novel class of potent $GABA_B$ receptor antagonists.

Keywords: Baclofen; $GABA_B$ receptor antagonist; Brain slice, rat; Morpholin-2-yl-phosphinic acid; CGP 56999A

1. Introduction

Bicuculline-insensitive $GABA_B$ receptors for the inhibitory transmitter γ-aminobutyric acid (GABA) are activated by baclofen (β-*p*-chlorophenyl GABA), a prototypical agonist for these receptors (Bowery, 1993). Recent expression cloning of $GABA_B$ receptors have revealed two highly conserved isoforms that show sequence similarity to the metabotropic glutamate receptors (Kaupmann et al., 1997). $GABA_B$ receptors belong to the larger superfamily of heptahelical transmembrane receptors that are G-protein-linked to a variety of cellular effectors, including Ca^{2+} and K^+ channels which they regulate (Kerr and Ong, 1995). In general, presynaptic $GABA_B$ receptors modulate synaptic transmission by depressing neurotransmitter release, including that of GABA itself through autoreceptors (Baumann et al., 1990), while postsynaptic $GABA_B$ receptors contribute to the inhibitory control of overall neuronal excitability.

Phaclofen and 2-hydroxy-saclofen, the original $GABA_B$ receptor antagonists, were based on bioisosteric replacement of the carboxylic moiety in baclofen (Kerr et al., 1987, 1988). The synthesis of phosphinic bioisosteres of GABA itself provided a major improvement in antagonist potency (Froestl et al., 1995). Subsequently, the potency of these analogues on $GABA_B$ receptors was greatly enhanced by the introduction of *N*-benzyl and 2-hydroxy substituents into these parent phosphinic analogues (Froestl et al., 1995; Froestl and Mickel, 1997). More recently, a unique series of $GABA_B$ receptor antagonists has been described, based on 2,5-disubstituted-1,4-morpholines (Kuo

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et al., 1994; Blythin et al., 1996). The most potent of these is (*S*)-(+)-5,5-dimethylmorpholinyl-2-acetic acid (SCH 50911), first characterised as a moderately potent, orally-active, selective antagonist at central and peripheral $GABA_B$ receptors (Bolser et al., 1995; Hosford et al., 1995). From a structure-activity viewpoint, such morpholinyl-2-acetic acids are of particular interest, since they represent GABA analogues partially frozen in an antagonist conformation which retains the N-substitution and β-hydroxy of the previous antagonists incorporated into the morpholine ring.

In the original description of these morpholinyl-acetic acid derived antagonists (Kuo et al., 1994), the *P*-methylphosphinic analogue of SCH 50911 was described. This substituted phosphinic acid was based on the 2-hydroxypropylphosphinic acid derivative 3-amino-2-hydroxypropyl-methylphosphinic acid (CGP 34938) (Froestl and Mickel, 1997) which is a potent agonist at $GABA_B$ receptors. By introducing a methylcyclohexyl substituent on the phosphinic head, CGP 34938 was converted to a potent $GABA_B$ receptor antagonist 3-amino-2-hydroxypropyl-cyclohexylmethylphosphinic acid (CGP 49311A) (Froestl and Mickel, 1997). Following this lead, the antagonist *P*-methylcyclohexyl moiety was incorporated into a series of morpholin-2-yl-phosphinic derivatives bearing 5-phenyl-substitutions. In the present study, we describe the $GABA_B$ receptor antagonist properties of a number of compounds in this series which have not been reported previously, using rat neocortical slice preparations maintained in Mg^{2+}-free Krebs medium. These include 3-{(3*S*,6*R*)-6-[(cyclohexylmethyl)hydroxyphosphinoylmethyl]-morpholin-3-yl}benzoic acid (CGP 76290A), its enantiomer {(3*R*,6*S*)-6-[(cyclohexylmethyl)hydroxyphosphinoylmethyl]-morpholin-3-yl}benzoic acid (CGP 76291-A), as well as cyclohexylmethyl-[(2*S*,5*R*)-5-phenyl-morpholin-2-ylmethyl]phosphinic acid (CGP 71979), its enantiomer cyclohexylmethyl-[(2*R*,5*S*)-5-phenyl-morpholin-2-ylmethyl]phosphinic acid (CGP 71980), and cyclohexylmethyl-[(2*R*′,5*S*′)-5-(3-nitrophenyl)-morpholin-2-ylmethyl] phosphinic acid (CGP 71978) (Fig. 1). In addition, for comparison, we have also used an open chain analogue of opposite stereochemistry to CGP 76290A, 3-{1(*R*)-[(3-cyclohexylmethyl-hydroxyphosphinoyl)-2(*S*)-hydroxypropyl-amino]-ethyl}benzoic acid lithium salt (CGP 56999A) (Fig. 1). We have also evaluated their affinities for binding to $GABA_B$ receptors using radioligand binding assays in

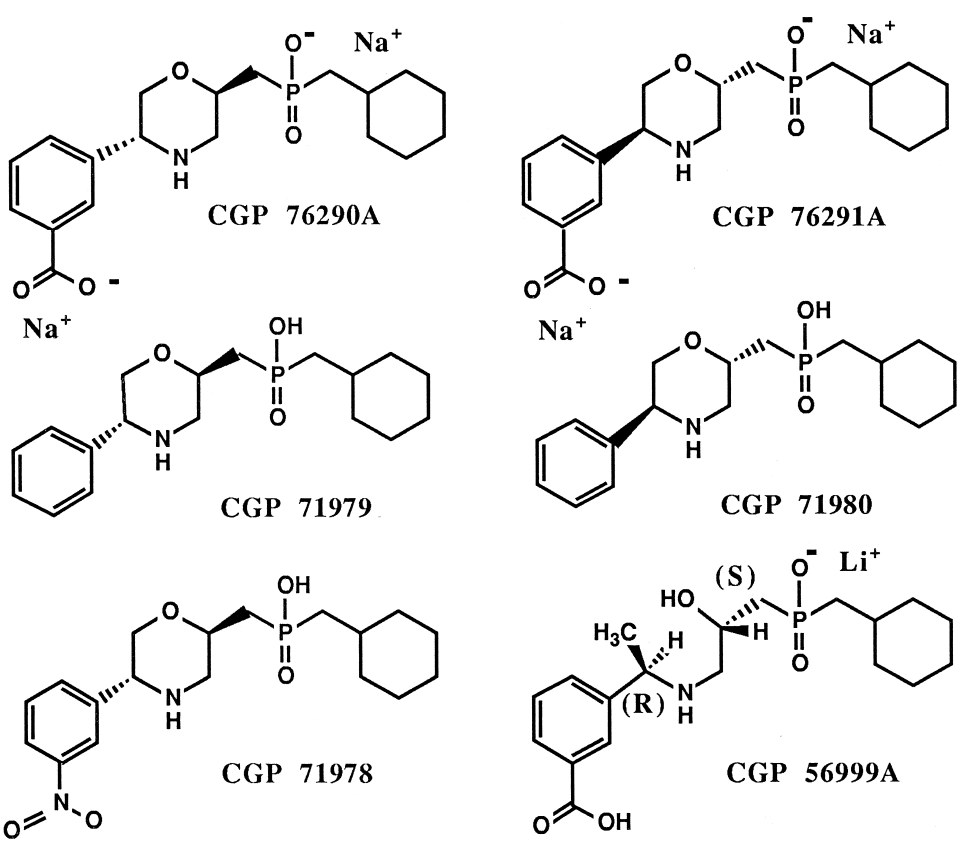

Fig. 1. Chemical structures of a series of morpholin-2-yl-phosphinic acid analogues showing 3-{(3*S*,6*R*)-6-[(cyclohexylmethyl)hydroxyphosphinoylmethyl]-morpholin-3-yl}benzoic acid (CGP 76290A), its enantiomer 3-{(3*R*,6*S*)-6-[(cyclohexylmethyl)hydroxyphosphinoylmethyl]-morpholin-3-yl}benzoic acid (CGP 76291A), cyclohexylmethyl-[(2*S*,5*R*)-5-phenyl-morpholin-2-ylmethyl]phosphinic acid (CGP 71979), and its enantiomer cyclohexylmethyl-[(2*R*,5*S*)-5-phenyl-morpholin-2-ylmethyl]phosphinic acid (CGP 71980), and cyclohexylmethyl-[(2*R*′,5*S*′)-5-(3-nitrophenyl)-morpholin-2-ylmethyl]phosphinic acid (CGP 71978). The open chain analogue of CGP 76290A, 3-{1(*R*)-[(3-cyclohexylmethyl-hydroxyphosphinoyl)-2(*S*)-hydroxypropyl-amino]-ethyl}benzoic acid lithium salt (CGP 56999A) is shown for comparison.

homogenised rat brain tissues, and established their $GABA_B$ autoreceptor antagonist potencies using electrically-evoked [^{3}H]GABA release from rat brain slices.

2. Materials and methods

2.1. Preparation of rat neocortical slices

All studies described here 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.

2.2. Grease-gap recording of brain slices

Using a superfusion method based on a grease-gap system as described previously (Ong et al., 1990), the 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. DC potentials between the cingulate cortex and corpus callosum were monitored on a chart recorder using Ag/AgCl electrodes, agar/saline bridges and a high input-impedance DC amplifier. The neocortical slices developed spontaneous paroxysmal discharges after a period of equilibration in Mg^{2+}-free Krebs medium for 10–15 min. The $GABA_B$ receptor agonist baclofen, added to the superfusing medium, was applied to the cortical side of the tissue for 2 min and the preparation was allowed 30 min recovery between drug applications. Although the agonist was superfused for only 2 min, the effects of the agonist outlasted this for a period depending on the concentration of agonist; at a maximum concentration, the discharge ceased for at least 10 min, with some slowing for a further 5 min. The antagonist was first superfused for 2 min and then added together with the agonist. Results were quantified by counting the number of spontaneous discharges in 10 min epochs, in the absence and presence of test compounds, and the values expressed as a percentage depression of the average control discharge rate for a given slice 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, that is the concentration which produced 50% inhibition of the discharge rate, was calculated from the concentration–response curve. Due to limited amounts of test compounds available, estimates of apparent pA_2 values were made using the relationship $pA_2 = \log (CR - 1) - \log [B]$, where the concentration ratio (CR), relative to corresponding controls, was produced by a single concentration of antagonist $[B]$, assuming competitive antagonism and Schild regression close to unity. Each experiment was repeated on 6–8 slices obtained from at least three different animals and data are expressed as mean ± S.E.M.

2.3. $GABA_B$ receptor radioligand binding assay and electrically-evoked [^{3}H]GABA release from rat cortical slices

The compounds synthesized were tested for their ability to inhibit the binding of the potent $GABA_B$ receptor agonist ligand [^{3}H]CGP 27492, using the method as described previously (Hall et al., 1995). Briefly, well washed cerebral cortical membranes were prepared from male rats (see Olpe et al., 1990), and the radioreceptor binding assay was performed in 2 ml Krebs–Henseleit buffer containing 20 mM Tris (pH 7.4), 200–300 μg membrane protein and 2 nM [^{3}H]CGP 27492 (15 Ci/mmol) ($K_D = 5$ nM), together with the ligand to be tested. After 40 min at 20°C, the incubation was terminated by rapid filtration on glass fiber filters which were rapidly washed with ice-cold buffer. Filter-bound radioactivity was counted in Irgascint A300® scintillant. Incubations were performed in triplicate, and non-specific binding was determined in the presence of 10 μM (*R*)-baclofen. The half maximal concentration (i.e., the IC_{50} values) for inhibition of [^{3}H]CGP 27492 binding to $GABA_B$ receptors of each compound was obtained by computer-aided curve fitting, according to a single-site model.

Electrically stimulated release of [^{3}H]GABA from rat (male Tif:RAIf (SPF), Tierfarm Sisseln, Switzerland, weighing 160–200 g) preloaded cortical slices was measured as previously described (Baumann et al., 1990). Briefly, cross-chopped rat cortical slices (1000 × 350 × 350 μm) were incubated for 5 min with 100 nmol/l [2,3-^{3}H(*N*)]GABA (25 Ci/mmol; New England Nuclear, Boston, MA, USA) in physiological buffer (composition in mM: NaCl, 188; KCl, 4.8; $CaCl_2$, 2.6; $MgSO_4$, 1.2; KH_2PO_4, 1.2; D-glucose, 10; $NaHCO_3$, 25; aminooxyacetic acid as inhibitor of GABA transaminase, 0.05; SK&F 89976 as inhibitor of GABA uptake, 0.01). Around 25 μl of slice suspension (approximately 1 mg protein)

was transferred to stimulation chambers and superfused at 0.4 ml/min with physiological buffer gassed with 5% CO_2 in O_2. Twelve fractions of 6 min were collected, beginning 50 min after starting the superfusion. The slices were field stimulated at 2 Hz by monophasic pulses (2 ms, 25 mA, 2 min) at the onset of fractions 3 and 9; the test compounds were added to the superfusion medium after fraction 6. Radioactivity in the superfusion medium and in the slices (after solubilization in Irgasolv® and subsequent neutralisation) was counted after addition of Irgascint A300® scintillant. From the raw data, stimulated fractional release ± S.E.M. was calculated and S_2/S_1 ratios determined as described (Baumann et al., 1990). The data obtained with the test compounds were expressed as means ± S.E.M. of the S_2/S_1 ratios in percent of controls, and the concentrations causing a 50% increase in release determined by graphical interpolation.

2.4. Drugs

Baclofen, SK&F 89976, [^{3}H]CGP 27492, CGP 71982, CGP 76290A, CGP 76291A, CGP 71978, CGP 71980, CGP 71979 and CGP 56999A were synthesised at Novartis Pharmac. (Basel, Switzerland). [2,3-^{3}H(*N*)]GABA was obtained from New England Nuclear (Boston, MA, USA). Aminooxyacetic acid hemihydrochloride was purchased from Sigma (MO, USA).

3. Results

3.1. Antagonism of baclofen-induced suppression of spontaneous discharges in rat neocortical slices by morpholin-2-yl-phosphinic acids and CGP 56999A

Neocortical slices when superfused with Mg^{2+}-free Krebs medium exhibited spontaneous depolarisations within 10–15 min. These discharges are suppressed by the $GABA_B$ receptor agonist baclofen (Horne et al., 1986; Ong et al., 1990), an action that is sensitive to selective $GABA_B$ receptor antagonists (Kerr et al., 1988, 1989). In the present experiments, the frequency of occurrence and the duration of the discharges varied between different preparations, but once these were established at a stable rate, baclofen superfused at various concentrations for 2 min reduced the firing rate in a concentration-dependent manner. This reduction was seen at concentrations as low as 1 μM, reaching a maximal effect at 300 μM, with a mean EC_{50} of 14 ± 5.5 μM ($n = 8$). Fig. 2a shows a representative experiment in which application of baclofen (Bac; 30 μM) for 2 min suppressed the firing rate by 60% for 6 min, as indicated by the horizontal bar. The spontaneous discharges returned to baseline levels of about 3–4 per min, within 15 min following the initial wash-out of the drug. Pre-treatment with CGP 76290A (0.5 μM) alone for 2 min did not affect the discharge rate or amplitude,

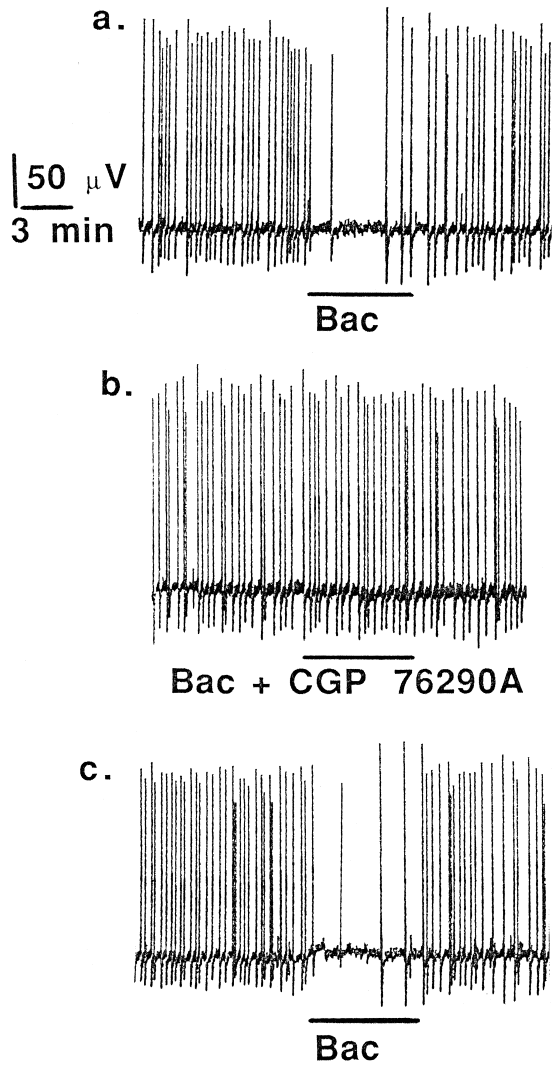

Fig. 2. Representative records from a typical experiment showing the effects of 3-{(3*S*,6*R*)-6-[(cyclohexylmethyl)hydroxyphosphinoylmethyl]-morpholin-3-yl}benzoic acid (CGP 76290A) (0.5 μM) on the responses to baclofen (Bac) in the rat neocortical slice preparation, maintained in Mg^{2+}-free Krebs medium. (a) Baclofen (Bac; 30 μM) induced a suppression of spontaneous discharges (b) reversibly antagonised by CGP 76290A (0.5 μM) and (c) the control response to baclofen was subsequently re-established upon wash-out of the test compounds. In (a) and (c), the action of baclofen persisted for 5 min following removal of baclofen, while in (b), this effect was abolished by CGP 76290A over the same period.

but in combination with baclofen (30 μM) for 2 min, effectively antagonised the baclofen-induced suppression of spontaneous discharges (Fig. 2b). Following wash-out of the test compounds, there was a complete recovery of the spontaneous activity and the depressant response to baclofen (30 μM) within 30 min (Fig. 2c).

As can be seen from the baclofen concentration–response curve in Fig. 3a, the threshold concentration for the reduction in frequency of spontaneous activity by baclofen was 3 μM and the estimated half-maximally effective concentration was 20 μM. Over the 10-min period, the

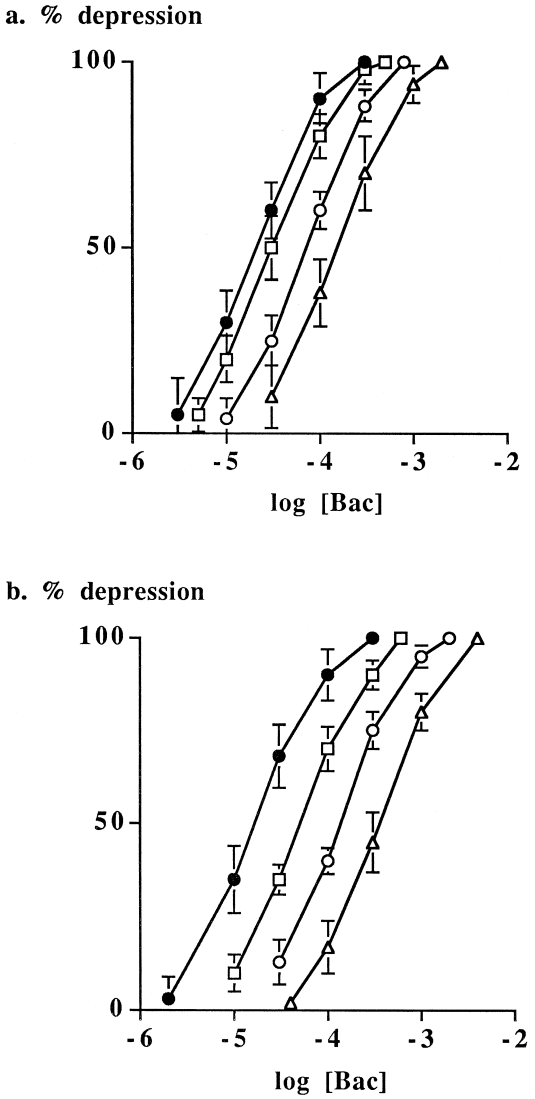

Fig. 3. Concentration–response curves for (*R,S*)-baclofen-induced suppression of the frequency of spontaneous discharges in the rat isolated neocortical slices, maintained in Mg^{2+}-free Krebs medium, in the absence and presence of 3-{(3*S*,6*R*)-6-[(cyclohexylmethyl)hydroxyphosphinoylmethyl]-morpholin-3-yl}benzoic acid (CGP 76290A) and its enantiomer 3-{(3*R*,6*S*)-6-[(cyclohexylmethyl)hydroxyphosphinoylmethyl]-morpholin-3-yl}benzoic acid (CGP 76291A). The concentration–response curve for baclofen (●) was subsequently shifted to the right, in a parallel fashion by (a). CGP 76290A (open squares 0.05, ○ 0.3 and △ 0.5 μM) and by (b). CGP 76291A (open squares 0.3, ○ 1 and △ 3 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.

maximal baclofen effect with complete cessation of discharges was obtained with 300 μM baclofen. No desensitization of the tissues to baclofen was detected since continuous exposure to baclofen induced a consistent effect, with no resultant decrease in tissue responsiveness in all slices studied. In order to quantify the antagonist potency of CGP 76290A, the effects of three concentrations of CGP 76290A (0.05, 0.3 and 0.5 μM) on the baclofen concentration–response curve were measured. Increasing concentrations of CGP 76290A 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 yielded an apparent pA_2 value of 7.1 ± 0.05 (Fig. 3a; $n = 8$). In a similar series of experiments, 0.3, 1 and 3 μM CGP 76291A ($pA_2 = 6.8 \pm 0.1$), 0.3, 1 and 3 μM CGP 71978 ($pA_2 = 6.5 \pm 0.05$), 0.5, 1 and 5 μM CGP 71980 ($pA_2 = 6.3 \pm 0.15$) and 2, 5 and 10 μM CGP 71979 ($pA_2 = 5.8 \pm 0.1$) ($n = 8$ for each compound) reversibly antagonised baclofen-responses, and shifted the baclofen concentration–response curves to the right in a parallel manner (Fig. 3b Figs. 4 and 5; $n = 8$). In a further series of experiments, CGP 56999A (0.5, 1 and 5 μM), an open chain analogue of CGP 76290A, also antagonised the depression of discharge rate induced by baclofen, yielding a pA_2 value of 6.6 ± 0.2 ($n = 8$, Table 1). On their own, none of these compounds affected or modified the frequency of the spontaneous discharges at concentrations ranging from 0.05 to 10 μM.

3.2. Inhibition by morpholin-2-yl-phosphinic acids of [^{3}H]CGP 27492 binding, and antagonism of GABA$_B$ autoreceptors in rat brain preparations

Several structural analogues of morpholin-2-yl-phosphinic derivatives were tested for their binding affinities at $GABA_B$ receptors, as well as for their antagonism of $GABA_B$ autoreceptors regulating [^{3}H]GABA release. The data for the potency of such morpholin-2-yl-phosphinic derivatives on inhibition of [^{3}H]CGP 27492 binding in rat

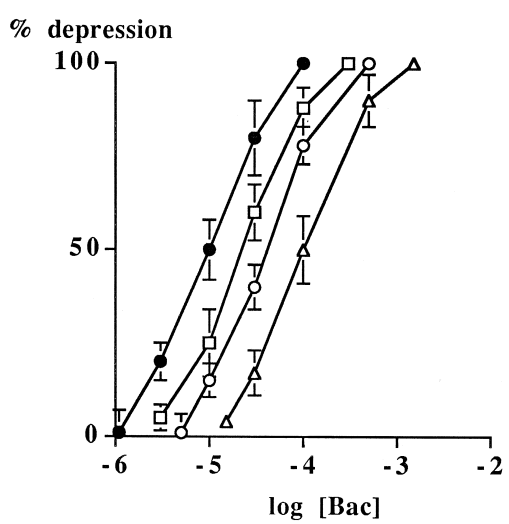

Fig. 4. Concentration–response curves for (*R,S*)-baclofen-induced suppression of the frequency of spontaneous discharges in the rat isolated neocortical slices, maintained in Mg^{2+}-free Krebs medium, in the absence and presence of cyclohexylmethyl-[(2*R*′,5*S*′)-5-(3-nitrophenyl)-morpholin-2-ylmethyl]phosphinic acid (CGP 71978). The concentration–response curve for baclofen (●) was subsequently shifted to the right, in a parallel fashion by CGP 71978 (open squares 0.3, ○ 1 and △ 3 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.

synaptosomes, and on electrically-evoked [^{3}H]GABA release in rat cortical slices, are summarized in Table 1. As shown in the latter, the order of potency of the compounds in the radioreceptor binding assays correlated well with the data from the release studies. From the binding data, the IC_{50} values range between 1.85 nM and 1460 nM, while in the release paradigm, the EC_{150} values range between 2.5 nM and 490 nM. CGP 76290A, CGP 76291A and their racemic mixture CGP 71982, as well as CGP 71978, CGP 71980 and CGP 71979 all inhibited binding of [^{3}H]CGP 27492 to $GABA_B$ receptors, each having IC_{50} values of 1.85, 69, 8, 124, 326, and 1460 nM, respectively. Here, CGP 76290A was approximately 37 times more potent than its enantiomer CGP 76291A, and CGP 71980 was in turn 4.5 times more potent than its enantiomer CGP 71979 in displacing [^{3}H]CGP 27492 binding. In electrically-evoked [^{3}H]GABA release from rat cortical slices, the compounds antagonised $GABA_B$ autoreceptors, enhancing evoked overflow to 150% (Table 1). The most potent compound from this series was CGP 71982 (the racemic mixture of CGP 76290A and CGP 76291A, no release data being available for the latter enantiomers) showing an EC_{150} of 2.5 nM, followed by CGP 71978, CGP 71980, and CGP 71979, with EC_{150} values of 33, 181 and 474 nM, respectively; CGP 71980 was 2.6 times more potent than its enantiomer CGP 71979.

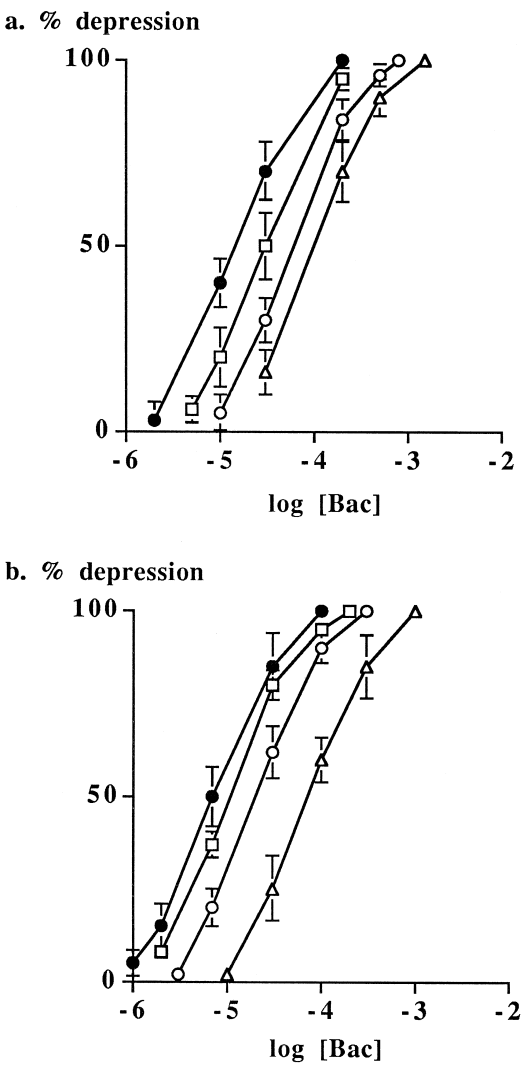

Fig. 5. Concentration–response curves for (*R,S*)-baclofen-induced suppression of the frequency of spontaneous discharges in the rat isolated neocortical slices, maintained in Mg^{2+}-free Krebs medium, in the absence and presence of cyclohexylmethyl-[(2*S*,5*R*)-5-phenyl-morpholin-2-ylmethyl]phosphinic acid (CGP 71979) and cyclohexylmethyl-[(2*R*,5*S*)-5-phenyl-morpholin-2-ylmethyl]phosphinic acid (CGP 71980). The concentration–response curve for baclofen (●) was subsequently shifted to the right, in a parallel fashion by (a). CGP 71979 (open squares 2, ○ 5 and △ 10 μM) and by (b). CGP 71980 (open squares 0.5, ○ 1 and △ 5 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.

Table 1
Summary of the potencies of morpholin-2-yl-phosphinic derivatives as antagonists at $GABA_B$ receptors

	pA_2[a]	IC_{50} (nM)[b]	EC_{150} (nM)[c]
CGP 71982	n.a.	8	2.5
CGP 76290A	7.1 ± 0.05	1.85	n.a.
CGP 76291A	6.8 ± 0.1	69	n.a.
CGP 71978	6.5 ± 0.05	124	33
CGP 71980	6.3 ± 0.15	326	180
CGP 71979	5.8 ± 0.1	1460	474
CGP 56999A	6.6 ± 0.2	2[d]	3[d]

[a] pA_2 values are estimates of antagonist potencies in the spontaneously discharging rat neocortical slices using the relationship $pA_2 = \log(CR-1) - \log[B]$, where CR is the concentration ratio (CR) and [*B*] is the concentration of the antagonist. [b] IC_{50} is the estimated half maximal concentration of inhibition of [^{3}H]CGP 27492 binding to $GABA_B$ receptors in rat cerebral cortical membranes. [c] EC_{150} is the concentration causing a 50% increase in electrically-stimulated [^{3}H]GABA release from rat cortical slices (stimulation frequency 2 Hz). [d] Waldmeier et al. (1994). The binding and release data for CGP 76290A and CGP 76291A are not available (n.a.).

4. Discussion

In the present study, the 5-substituted morpholin-2-yl-methyl-phosphinic acid derivatives were all $GABA_B$ receptor antagonists that reversibly blocked baclofen-induced depressant effects in rat neocortical slices. The most potent antagonist was the (3*S*,6*R*)-benzoic acid derivative CGP 76290A, displaying an apparent pA_2 value of 7.1 ± 0.05, while its (3*R*,6*S*) enantiomer CGP 76291A, with the opposite configuration, was half as potent ($pA_2 = 6.8 \pm 0.1$). Indeed, these are the most potent of the $GABA_B$ receptor antagonists so far found to be active in the discharging rat neocortex maintained in Mg^{2+}-free Krebs medium. Both of these benzoic acid substituted derivatives were more potent than the corresponding analogues lacking a 3-carboxyl on the phenyl attachment to the parent morpholine ring. Of these latter, the unsubstitued (2*R*,5*S*)-phenyl derivative CGP 71980 ($pA_2 = 6.3 \pm 0.15$)

was 3 times more potent than its (2*S*,5*R*) enantiomer CGP 71979 ($pA_2 = 5.8 \pm 0.1$). This is unexpected as the (2*R*,5*S*) configuration of CGP 71980 is opposite to that of the most potent agent CGP 76290 with a (2*S*,5*R*) configuration. In the (2*R*′,5*S*′)-nitrophenyl analogue CGP 71978 ($pA_2 = 6.5 \pm 0.05$) with a similar stereochemistry, the carboxylate moiety of the benzoic acid substituent in CGP 76291 is replaced by a nitro-group.

From receptor binding studies, shown in Table 1, where these analogues were tested for inhibition of [^{3}H]CGP 27492 binding to $GABA_B$ receptors, CGP 76290A was again the most potent in the series, some 37 times more active than its enantiomer CGP 76291A, and about 4 times more potent than the racemic mixture CGP 71982. The other compounds in the series CGP 71978, CGP 71980, and CGP 71979 all displayed lower binding affinities than the benzoic acid analogues. These morpholine-2-yl-methyl-phosphinic acid compounds also antagonised $GABA_B$ autoreceptors, increasing electrically-evoked overflow of [^{3}H]GABA in rat cortical slices. No [^{3}H]GABA release data are available for the individual benzoic acid enantiomers, but their racemate CGP 71982 was the most potent on $GABA_B$ autoreceptors, being approximately 13 times more effective than the nitro-derivative CGP 71978, while CGP 71980 and its enantiomer CGP 71979 were the least active on the evoked overflow, although still effective in the nanomolar range.

Originally, in the prototypical 2,5-disubstituted-1,4-morpholines (Blythin et al., 1996), several of the more active 2-acetic acid derivatives have a (2*S*,5*R*) configuration which shows some 10-fold improvement in antagonist potency over the (2*R*,5*S*)-form. However, although in the most active 5,5-dimethyl derivative (SCH 50911) only the two substituent is chiral, with the (2*S*) configuration showing some 100-fold higher antagonist potency over that of the relatively inactive (2*R*)-enantiomer. Other morpholin-2-yl-acetic acid derivatives also point to the (5*R*) configuration being preferred when the 5-substituents are asymmetric. This is congruent with the stereochemical preferences in the present 5-benzoic-acid-2-(methylcyclohexanyl)-methyl-phosphinic acids (CGP 76290 and CGP 76291) where the (*S*,*R*)-enantiomer is more potent than the (*R*,*S*)-enantiomer, but does not hold for CGP 71979 and CGP 71980 where the latter (*R*,*S*)-enantiomer is the more potent. Nevertheless, between each member of these enantiomeric pairs there is only a 2–3-fold difference in potency at the $GABA_B$ receptors, which suggests that the more bulky *P*-methyl-cyclohexyl-substituent of their acidic functionalities interacts with some secondary binding site on the receptor, with less stereo-selectivity than that of the carboxylate in the morpholino-acetic acids. In the same way, the 5-phenyl or benzoic acid substituents, also likely bind at some additional accessory site, adjacent to the ammonium recognition site on the $GABA_B$ receptor. The improved binding provided by these accessory sites evidently imparts the marked improvement in antagonist potency seen in the present study. In keeping with this, the two most potent $GABA_B$ receptor antagonists previously found, CGP 55845A (3-[1-(*S*)-(3,4-dichlorophenyl)-ethyl]amino-2(*S*)-hydroxy-propyl-benzyl-phosphinic acid) and CGP 56999A, which represent open-chain analogues of the present morpholine series, also exhibit nanomolar affinities. These are some 500–1000 times more potent than earlier antagonists such as CGP 35348 (*P*-(3-aminopropyl)-*P*-di-ethoxymethyl-phosphinic acid) (Olpe et al., 1990; Froestl et al., 1992; Waldmeier et al., 1993, 1994; Froestl et al., 1996) which lack the *N*-benzyl substituent characteristic of CGP 55845A and CGP 56999A. For instance, CGP 55845A is relatively potent in blocking hyperpolarizations elicited by baclofen in dorso-lateral septal neurones, with a pA_2 value of 8.3 (Bon and Galvan, 1996). Similarly, CGP 56999A is among the most potent antagonists found so far in binding and release assays using rat brain tissues (Waldmeier et al., 1994). However, CGP 56999A was less active here against baclofen-induced suppression of discharge rate in the rat neocortex with a pA_2 of 6.6, as against 7.1 for CGP 76290A.

Previously, Waldmeier et al. (1994), using a series of P-substituted phosphinic analogues of GABA, found an almost perfect correlation between their potencies for inhibition of [^{3}H]CGP 27492 binding and for antagonism of $GABA_B$ autoreceptor-mediated suppression of GABA release, suggesting that the latter $GABA_B$ receptor antagonists do not discriminate between $GABA_B$ receptor subtypes (but see Teoh et al., 1996). In the current study, the 5-substituted morpholin-2-yl-phosphinic acids display similar profiles in spontaneously discharging neocortical preparations, binding assays and release studies, which indicates that they also do not select between $GABA_B$ receptor subtypes. Nevertheless, they provide further insight into the requirements for high affinity binding at $GABA_B$ receptors in general.

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