ELSEVIER

European Journal of Pharmacology 369 (1999) 33–37

ejp

Short communication

Antagonism of $GABA_B$ receptors by morpholino-2-acetic acid derivatives Sch 54679 and Sch 51324 in rat brain

Jennifer Ong [a,*], Victor Marino [b], David A.S. Parker [b], David I.B. Kerr [a], David J. Blythin [c]

[a] *Department of Anaesthesia and Intensive Care, The University of Adelaide, Adelaide, South Australia 5005, Australia*
[b] *Department of Dentistry, The University of Adelaide, Adelaide, South Australia 5005, Australia*
[c] *Chemical Research Department, Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033, USA*

Received 15 October 1998; revised 18 January 1999; accepted 22 January 1999

Abstract

In rat neocortical slices maintained in Mg^{2+}-free Krebs medium, baclofen depressed the rate of spontaneous discharges in a concentration-dependent manner ($EC_{50} = 4.5$ μM). This depression was reversibly antagonised by 5-(*S,R*)-hydroxymethyl-5-methyl-morpholinyl-2-(*R,S*)-acetic acid (Sch 54679) and 2-(*R,S*)-5-[spirocyclopentyl]-morpholinyl-acetic acid (Sch 51324) (respective pA_2 values of 5.8 ± 0.15 and 5.4 ± 0.2). In electrically-stimulated slices preloaded with [^{3}H]γ-aminobutyric acid (GABA), Sch 54679 ($EC_{50} = 3$ μM) was 2.3 times more potent than Sch 51324 ($EC_{50} = 7$ μM) in increasing [^{3}H]GABA release through antagonism of $GABA_B$ autoreceptors. These structurally novel analogues may be pharmacologically useful for elucidating $GABA_B$ receptor functions.

Keywords: Baclofen; $GABA_B$ receptor; $GABA_B$ receptor antagonist; Sch 54679; Sch 51324; Neocortical slice, rat

1. Introduction

Recently, a new class of antagonists for bicuculline-insensitive γ-aminobutyric acid ($GABA_B$) receptors has been described. These belong to a novel series of 2,5-disubstituted-1,4-morpholines (Kuo et al., 1994; Blythin et al., 1996), the most potent of which is (+)-(*S*)-5,5-dimethyl-morpholinyl-2-acetic acid (Sch 50911) that antagonises central and peripheral $GABA_B$ receptor-mediated functions, and suppresses absence seizures by blocking $GABA_B$ receptors in vivo (Bolser et al., 1995; Hosford et al., 1995). Amongst these, 5-(*S,R*)-hydroxymethyl-5-methyl-morpholinyl-2-(*R,S*)-acetic acid (Sch 54679) and the spiro-analogue 2-(*R,S*)-5-[spirocyclopentyl]-morpholinyl-acetic acid (Sch 51324) (Fig. 1) are derivatives of Sch 50911 bearing differing 5-alkyl-substituents on the morpholino-ring, which show binding affinities for $GABA_B$ receptors in the low micromolar range, as does Sch 50911 (Blythin et al., 1996). As yet, the pharmacological actions of these new compounds on $GABA_B$ receptors in brain slices have not been characterised using functional studies.

We have now evaluated the antagonist actions of Sch 54679 and Sch 51324 on baclofen-induced suppression of spontaneous discharges in rat neocortical slices, mediated through $GABA_B$ heteroreceptors. Their effects on $GABA_B$ autoreceptors modulating GABA release in electrically-stimulated neocortical slices preloaded with [^{3}H]GABA have also been examined. Our results indicate that although Sch 54679 and Sch 51324 are relatively potent antagonists at $GABA_B$ receptors, they show no pharmacological discrimination between auto- and heteroreceptor subtypes.

2. Methods

2.1. Preparation of brain slices

Rat neocortical slices were prepared from outbred male adult Sprague–Dawley rats (250–350 g) which were anaesthetized with halothane and decapitated, as previously described (Ong et al., 1990). 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

* Corresponding author. Tel.: +61-8-8303-5163; Fax: +61-8-8303-3788; E-mail: jong@medicine.adelaide.edu.au

0014-2999/99/$ - see front matter
PII: S0014-2999(99)00047-3

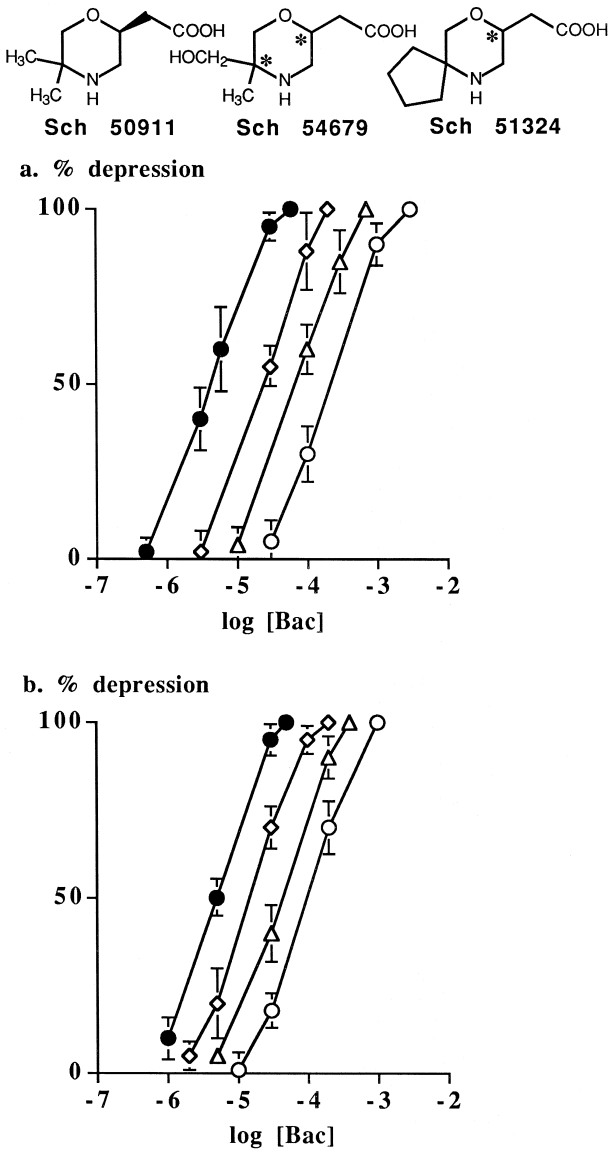

Fig. 1. Concentration–response curves for (*R,S*)-baclofen-induced suppression of the frequency of spontaneous discharges in rat isolated neocortical slices maintained in Mg^{2+}-free Krebs medium, in the absence and presence of 5-(*S,R*)-hydroxymethyl,5-methyl-morpholinyl-2-(*R,S*)-acetic acid (Sch 54679) and 2-(*R,S*)-5-[spirocyclopentyl]-morpholinyl-acetic acid (Sch 51324). The concentration–response curve for baclofen (●) was shifted to the right, in a parallel fashion, (a) by Sch 54679 (◇ 5 μM; △ 20 μM; ○ 50 μM), and (b) by Sch 51324 (◇ 5 μM; △ 20 μM; ○ 50 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 8 determinations. The chemical structures of (+)-(*S*)-5,5 dimethylmorpholinyl-2-acetic acid (Sch 50911), and its analogues Sch 54679 and Sch 51324 are shown.

Council, and The University of Adelaide Animal Ethics Committee. Briefly, the brains were rapidly dissected out and immersed for 30 min in ice-cold oxygenated Krebs solution (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, pH 7.4. Cerebral cortical slices (400 μm thick) were prepared by cutting coronal sections using a vibraslice microtome (Campden Instruments, UK), and equilibrated in gassed Krebs solution (95% O_2:5% CO_2) for approximately 1 h prior to experimentation.

2.2. Grease-gap recording of neocortical slices

Using a superfusion method based on a grease-gap system (Ong et al., 1990), the neocortex was initially superfused with gassed Mg^{2+}-containing Krebs medium at 28°C delivered by a peristaltic pump at 1 ml/min, and allowed to equilibrate for 30 min, followed by Mg^{2+}-free medium. For the Mg^{2+}-free medium, $MgSO_4$ was omitted. DC potentials between the cingulate cortex and corpus callosum were monitored by Ag/AgCl electrodes via agar/saline bridges with a high-input impedance DC amplifier, and responses displayed on a chart recorder.

After a period of equilibration for 60 min under Mg^{2+}-free conditions, the neocortical slices developed spontaneous paroxysmal discharges. The $GABA_B$ receptor agonist baclofen, added to the superfusing medium, was applied to the cortical side of the tissues for 2 min at 30 min intervals, and washed out before the recovery of the responses to control level. The antagonist was first superfused for 2 min and then added together with the agonist. Data were analysed 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 during the 10 min immediately before the addition of drugs. Concentration–response curves for the agonist, in the absence and presence of the antagonist, were constructed. The EC_{50} value that is the concentration that produced 50% inhibition of the discharge rate was graphically estimated, and estimates of apparent pA_2 values were determined 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*]. Each experiment was repeated on 8 slices from 4 different animals. All results are expressed as mean value ± S.E.M. Statistical significance was determined by Student's *t*-test for unpaired samples (significance level $P < 0.05$).

2.3. Release studies

Pairs of neocortical slices were incubated in Krebs solution containing [^{3}H]GABA (0.1 μM) for 20 min. Each pair was rinsed, placed in a small chamber and superfused at 1 ml/min with oxygenated Krebs solution (37°C). As previously described (Ong et al., 1998), aliquots of superfusate were collected at 10 min intervals for the first 5 collections and for 5 min thereafter, and their [^{3}H] contents were assayed by liquid scintillation spectrometry. Slices were stimulated through platinum field electrodes by square wave pulses at 2 Hz (300 pulses, 2.0 ms duration, 25 mA) at 10 min, 65 min (S_1) and 100 min (S_2) after superfusion commenced. 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 then assayed. From this data the fractional overflow of ^{3}H during each collection period was computed and the overflow per 5 min collection plotted (Fig. 2a). The GABA uptake inhibitor, NO-711 (10 μM), was added to the perfusion medium at the beginning of superfusion and remained throughout each experiment, while test compounds were added 15 min prior to the second stimulation.

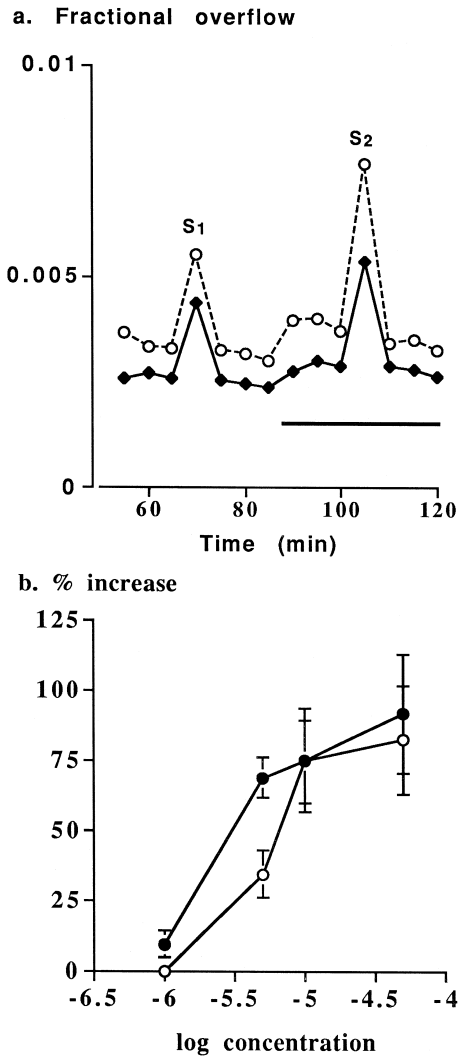

Fig. 2. The fractional overflow of ^{3}H per minute from rat neocortical slices in a typical experiment in which slices were stimulated at 2 Hz for 300 pulses at S_1 and S_2. Slices were pre-incubated with [^{3}H]GABA (0.1 μM) and superfused with Krebs solution containing NO-711 (10 μM). (a) Prior to and during the second period of stimulation, either 5-(*S,R*)-hydroxymethyl,5-methyl-morpholinyl-2-(*R,S*)-acetic acid (Sch 54679) or 2-(*R,S*)-5-[spirocyclopentyl]-morpholinyl-acetic acid (Sch 51324) was added to the perfusion medium superfusing one pair of slices (○ Sch 54679; ♦ Sch 51324) at a concentration of 5 μM for the period represented by the bar. (b) Concentration–response curves to Sch 54679 (○) and Sch 51324 (●) for electrically-stimulated release of [^{3}H]GABA from rat neocortical slices. The data presented are means ± S.E.M. from 4–6 experiments calculated as a percentage of the increase above the same ratio in the absence of the antagonist, the SIO_2/SIO_1 ratio being 0.9 in the absence of the antagonist.

2.4. Resting and stimulation-induced overflows

The resting overflow of ^{3}H is defined as the fractional overflow in the 5 min prior to stimulation. The stimulation-induced (SI) overflows for each stimulation, SIO_1 and SIO_2 were calculated by subtracting the relevant resting overflow from the fractional overflow in the 5 min following the onset of stimulation at S_1 and S_2, respectively. As putative $GABA_B$ autoreceptor antagonists were added to the superfusion medium after S_1 and before S_2, their effects on the resting and SI-overflows of ^{3}H were determined by comparing the R_2/R_1 and SIO_2/SIO_1 ratios with the same ratios in the absence of the antagonist. Hence, an antagonist of these autoreceptors would increase the SI-overflow ratio.

2.5. Solutions

Krebs solution was of the following composition (mM): NaCl (120), KCl (4.7), $NaHCO_3$ (25), KH_2PO_4 (1.0), $CaCl_2$ (2.5), $MgCl_2$ (1.0), glucose (5.5), and contained aminooxyacetic acid (0.05 mM). Incubation medium comprised [^{3}H]GABA (0.05 μM) and GABA (0.05 μM) in Krebs solution containing aminooxyacetic acid (0.05 mM).

2.6. Statistical analysis

The significance of the effect of each antagonist was assessed by unpaired Student's *t*-test, with significance levels at $P < 0.05$.

2.7. Drugs

Baclofen was a gift from Ciba-Geigy (Basel, Switzerland). Sch 54679 and Sch 51324 which are both racemic, were synthesised in the Chemical Research Department at the Schering-Plough Research Institute (Kenilworth, NJ). 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).

3. Results

3.1. Antagonism of Sch 54679 and Sch 51324 on baclofen-induced suppression of spontaneous discharges in rat neocortical slices

Superfusion of Mg^{2+}-free Krebs medium over the rat neocortical slices resulted in the appearance of repetitive

spontaneous discharges within 30 min. Application of baclofen caused a concentration-dependent reduction in discharge rate that generally lasted within the limit of 10 min, and was washed out completely within 20 min of application. As the baclofen concentration–response curves illustrate, the mean estimated EC_{50} value for baclofen was 4.5 μM (Fig. 1a,b). Neither Sch 54679 nor Sch 51324 (5, 20 and 50 μM) alone had any appreciable effect on the discharge rate or amplitude, but both reversibly antagonised the depressant effects of baclofen, with a complete recovery of the spontaneous activity, and the inhibitory response to baclofen after drug wash-out. Fig. 1 shows the effects of increasing concentrations of Sch 54679 and Sch 51324 against the baclofen concentration–response relationship; they produced surmountable concentration-dependent rightward shifts in the baclofen concentration–response curves, yielding apparent pA_2 values of 5.8 ± 0.15 for Sch 54679, and 5.4 ± 0.2 for Sch 51324, respectively ($n = 8$).

3.2. Effects of Sch 54679 and Sch 51324 on the overflow of [^{3}H]GABA

The overflow of ^{3}H into the Krebs solution superfusing neocortical slices, prelabelled with [^{3}H]GABA (0.1 μmol/l), reached a near steady-state within 60 min of commencing superfusion (Fig. 2a). In the presence of the GABA uptake inhibitor NO-711 (10 μM), electrical stimulation increased the overflow of ^{3}H nearly 2-fold in the 5 min collection following the onset of stimulation, an increase which returned to the resting level within a further 5 min. In a typical experiment, Sch 54679 (5 μM) and Sch 51324 (5 μM) enhanced the stimulation-induced overflow (Fig. 2a). From the concentration–response curves (Fig. 2b), both Sch 54679 and Sch 51324 facilitated the overflow of [^{3}H]GABA over the concentration range of 1–50 μM. Maximal facilitation occurred at the highest concentration tested (50 μM) for both compounds and the EC_{50} values for Sch 54679 and Sch 51324 were 3 and 7 μM, respectively. Neither compound affected the mean resting overflows.

4. Discussion

In the present study, we have investigated the effects of Sch 54679 and Sch 51324 on $GABA_B$ hetero- and autoreceptors in rat neocortical slices. Both compounds exhibited moderately potent, reversible, and competitive antagonism of $GABA_B$ heteroreceptors, producing parallel rightward shifts in the concentration–response curves to baclofen, with pA_2 values of 5.8 ± 0.15 and 5.4 ± 0.2, respectively. In electrically-stimulated slices, Sch 54679 and Sch 51324 enhanced the overflow of ^{3}H in a concentration-dependent manner (Sch 54679, $EC_{50} = 3$ μM; Sch 51324, $EC_{50} = 7$ μM) whilst having little or no effect on the resting overflow. These results show that both Sch 54679 and Sch 51324 possess comparable antagonistic actions at $GABA_B$ hetero- and autoreceptors. Since the potency of Sch 54679 was only 2.3 times that of Sch 51324 at the autoreceptors and 2.5 times that at the heteroreceptors, it appears that these compounds do not discriminate between the two receptor subtypes. Our results are consistent with a previous finding that Sch 54679 has a 5-fold higher binding affinity ($IC_{50} = 2$ μM) for $GABA_B$ receptors than Sch 51324 ($IC_{50} = 11$ μM) (Blythin et al., 1996), although there is less discrimination between the potency of the two analogues in the present functional tests. When compared to the related analogue Sch 50911, Blythin et al. (1996) reported that Sch 54679 and Sch 51324 were less active than Sch 50911 itself in inhibiting GABA binding to $GABA_B$ receptors. From a structure-action viewpoint, Sch 54679 bears a 5-methyl, 5-hydroxymethyl substitution where hydrogen bonding may occur, whilst Sch 51324 has a butyl chain linked across the attached (5)-carbon atom of the morpholine ring to form a larger hydrophobic (5)-spiro-cyclopentyl substitution (Fig. 1). Although these quite differing properties of the substituents at the 5-position, Sch 54679 as against Sch 51324, might perhaps be expected to provide a possible basis for distinguishing $GABA_B$ heteroreceptors from autoreceptors, clearly this was not so. In view of the less bulky 5,5-di-methyl substitution found in Sch 50911, which is optimal for antagonism in this series, provided the 2-acetic acid moiety is in the 2(*S*)-configuration (Blythin et al., 1996; Frydenvang et al., 1997), it is perhaps surprising that the spiro compound Sch 51324 is as active as found here, particularly since the bulky spiro-substituent might well hinder the flexibility of the morpholine ring which must assume a chair conformation for optimal binding at the $GABA_B$ receptor. In general, depending on the nature of the acidic head and its chirality, the 5-substituent(s) appear to govern antagonist potencies in this series of 2,5-disubstituted-1,4-morpholines. In this regard, the structural analogues of the morpholinyl-2-acetic acid Sch 50911, particularly Sch 54679 and Sch 51324, form a new class of novel $GABA_B$ receptor antagonists.

Acknowledgements

The authors wish to thank the Australian Research Council (ARC) for financial support. Jennifer Ong is an ARC Senior Research Fellow.

References

Blythin, D.J., Kuo, S.-C., Shue, H.-J., McPhail, A.T., Chapman, R.W., Kreutner, W., Rizzo, C., She, H.S., West, R., 1996. Substituted morpholine-2*S*-acetic acid derivatives: SCH 50911 and related com-

pounds as novel $GABA_B$ antagonists. Bioorg. Med. Chem. Lett. 6, 1529–1534.

Bolser, D.C., Blythin, D.J., Chapman, R.W., Egan, R.W., Hey, J.A., Rizzo, C., Kuo, S.-C., Kreutner, W., 1995. The pharmacology of SCH 50911: a novel, orally-active GABA-B receptor antagonist. J. Pharmacol. Exp. Ther. 274, 1393–1398.

Frydenvang, K., Enna, S.J., Krogsgaard-Larsen, P., 1997. (−)-(*R*)-5,5-Dimethylmorpholinyl-2-acetic acid ethyl ester hydrochloride, $C_{10}H_{20}NO_3^+ \cdot CL^-$. Acta Crystallogr., Sect. C: Cryst. Struct. Commun. 53, 1088–1091.

Hosford, D.A., Wang, Y., Liu, C.C., Carter Snead, O., 1995. Characterization of the antiabsence effects of SCH 50911, a $GABA_B$ receptor antagonist, in the lethargic mouse, γ-hydroxybutyrate, and pentylenetetrazole models. J. Pharmacol. Exp. Ther. 274, 1399–1403.

Kuo, S.-C., Blythin, D.J., Kreutner, W., 1994. 2-Substituted morpholine and thiomorpholine derivatives as $GABA_B$ antagonists. WO 22843; prior: 26 March 1993.

Ong, J., Kerr, D.I.B., Johnston, G.A.R., 1990. Effects of potassium channel blockers on baclofen-induced suppression of paroxysmal discharges in rat neo-cortical slices. Neurosci. Lett. 112, 216–222.

Ong, J., Marino, V., Parker, D.A.S., Kerr, D.I.B., 1998. Differential effects of phosphonic analogues of GABA on $GABA_B$ autoreceptors in rat neocortical slices. Naunyn-Schmiedeberg's Arch. Pharmacol. 357, 408–412.