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European Journal of Pharmacology 383 (1999) 137–141

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Short communication

Pharmacological re-evaluation of a $GABA_B$ receptor antagonist CGP 47332A in rat brain

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Received 20 May 1999; received in revised form 16 August 1999; accepted 20 August 1999

Abstract

In rat neocortical slices maintained in Mg^{2+}-free Krebs medium, the γ-aminobutyric acid ($GABA_B$) receptor agonist baclofen concentration-dependently depressed the frequency of spontaneous discharges ($EC_{50} = 12$ μM). This was reversibly antagonised by (*R,S*)-3-amino-2-hydroxy-propyl-*P*-*n*-butyl-phosphinic acid (CGP 47332A) (25, 100, 300 μM) which produced rightwards shifts of the baclofen concentration–response curves (pA_2 value $= 4.8 \pm 0.1$). In electrically stimulated slices preloaded with [^{3}H]GABA, CGP 47332A increased its release ($EC_{150} = 100$ μM) through antagonism of $GABA_B$ autoreceptors. Although CGP 47332A was some six times weaker on $GABA_B$ auto- than on heteroreceptors, yet its congener lacking the β-hydroxy substituent displays equal potency in both binding ($IC_{50} = 38$ μM) and $GABA_B$ autoreceptor functional studies ($EC_{150} = 38$ μM) as previously reported [Froestl, W., Mickel, S.J., Von Sprecher, G., Diel, P.J., Hall, R.G., Maier, L., Strub, D., Melillo, V., Baumann, P.A., Bernasconi, R., Gentsch, C., Hauser, K., Jaekel, J., Karlsson, G., Klebs, K., Maitre, L., Marescaux, C., Pozza, M.F., Schmutz, M., Steinmann, M.W., Van Riezen, H., Vassout, A., Mondadori, C., Olpe, H.R., Waldmeier, P.C., Bittiger, H., Phosphinic acid analogues of GABA: 2. Selective, orally active $GABA_B$ antagonists. J. Med. Chem. 38 (1995) 3313–3331.]. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: $GABA_B$ receptor antagonist; Neocortical slices, rat; Baclofen; CGP 47332A

1. Introduction

A class of antagonists for γ-aminobutyric $acid_B$ ($GABA_B$) receptors has been developed by replacing the carboxylate moiety in GABA with various P-substituted phosphinic acid residues (Froestl and Mickel, 1997; Froestl et al., 1995). One of these, (*R,S*)-3-amino-2-hydroxy-propyl-*P*-*n*-butyl-phosphinic acid (CGP 47332A) (Fig. 1) inhibits binding of [^{3}H]3-aminopropylphosphinic acid ([^{3}H]CGP 27492) to $GABA_B$ receptors with a moderate potency ($IC_{50} = 29$ μM), yet its relative potency at $GABA_B$ autoreceptors (8% increase in [^{3}H]GABA release at 10 μM; see Table 2 in Froestl et al., 1995) appears lower than might be expected (30% increase at 10 μM), from the results with comparable antagonists in the study by Waldmeier et al. (1994). By contrast, the congener 3-amino-propyl-*P*-*n*-butyl-phosphinic acid (CGP 36742) (Fig. 1), lacking only the β-hydroxy substituent, displays equal potency in both binding ($IC_{50} = 38$ μM) and $GABA_B$ autoreceptor functional studies ($EC_{150} = 38$ μM), as previously reported (Froestl et al., 1995). However, the activity of CGP 47332A at $GABA_B$ receptors has not been completely characterised, and its antagonist potency at $GABA_B$ heteroreceptors has not been studied previously in a functional assay. It therefore seemed of interest to evaluate the comparable antagonist potencies of CGP 47332A at $GABA_B$ heteroreceptors, using spontaneously discharging rat neocortical slices, and at the autoreceptors modulating electrically evoked [^{3}H]GABA release from similar slices. Here, we show that CGP 47332A, when compared to CGP 36742, is a weaker antagonist at $GABA_B$ auto- than at heteroreceptors, although both compounds showed similar binding and antagonist potencies at 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

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PII: S0014-2999(99)00608-1

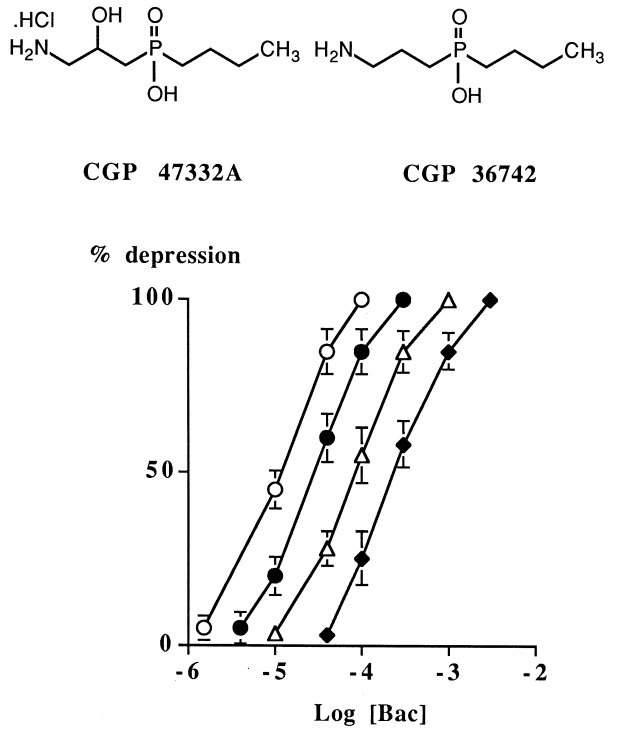

Fig. 1. Chemical structures of (*R,S*)-3-amino-2-hydroxy-propyl-*P*-*n*-butyl-phosphinic acid (CGP 47332A) and its congener 3-amino-propyl-*P*-*n*-butyl-phosphinic acid (CGP 36742). Concentration–response curves for (*R,S*)-baclofen-induced suppression of the frequency of spontaneous discharges in the rat isolated neocortical slices, maintained in Mg^{2+}-free Krebs medium, in the absence and presence of CGP 47332A. The concentration–response curve for baclofen (○) was shifted to the right, in a surmountable manner by the antagonist CGP 47332A (● 25, △ 100 and ◆ 300 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.

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°C–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), 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 equilibration in Mg^{2+}-free Krebs medium for 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. 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 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, and estimates of apparent pA_2 values were made. The pA_2 value was derived from the relationship $pA_2 = \log (CR\text{-}1) - \log [B]$, 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.

2.2. 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 four collections and for 4 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 (2.0 ms duration, 25 mA) for 30 s at 10 min and for 300 s at 48 min (S_1), 68 min (S_2), 88 min (S_3) and 108 min (S_4) 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 min 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 the effects of baclofen and CGP 47332A were tested at either S_2 or S_4. The test

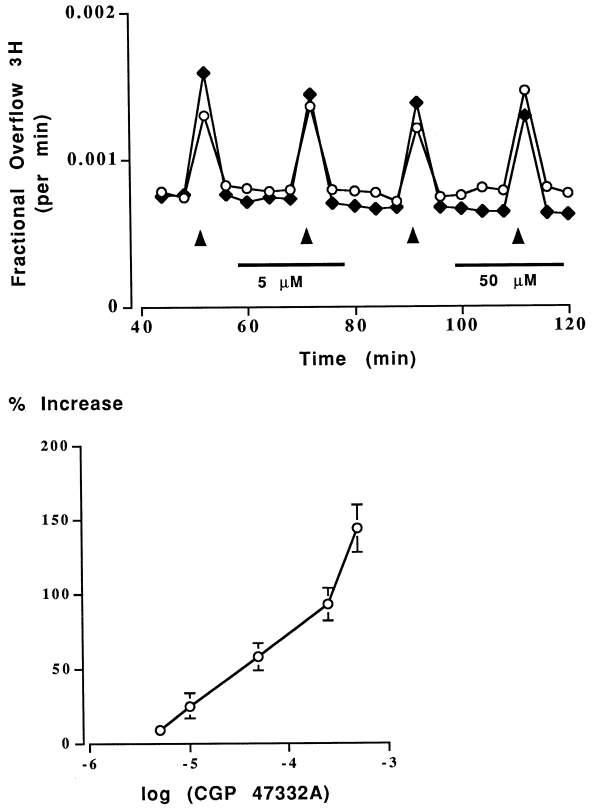

Fig. 2. Upper graph represents the fractional overflow of [^{3}H] per min from pairs of rat neocortical slices pre-incubated in [^{3}H]GABA (0.1 μM) in two typical experiments. In one (♦), the slices were untreated and in the other (○) they were perfused with CGP 47332A (concentrations shown) for the periods represented by the bars. Tissues were stimulated at the arrows at 2 Hz for 300 s. The overflow of [^{3}H] in the 4-min period commencing with the onset of stimulation in the untreated slices was maximal for the first stimulation and declined slightly for each succeeding stimulation. CGP 47332A caused a slight increase in the SI-overflow at 5 μM and a more marked increase at 50 μM. The SIO_2/SIO_1 and the SIO_4/SIO_3 ratios in control conditions are 0.94 ± 0.03 ($n = 12$), and 0.95 ± 0.02 ($n = 12$), respectively. Lower graph is the concentration–response curve of CGP 47332A on the SI-overflow of [^{3}H]. Data presented are the means and standard errors of the means of four to six experiments.

compounds were added 15 min prior to the onset of stimulation and remained in the Krebs solution for 20 min before washout (S_2) or to the end of the experiment (S_4).

In those experiments in which the effects of CGP 47332A were examined in the presence of baclofen, the $GABA_B$ receptor agonist was added to the Krebs solution superfusing the slices 8 min prior to S_1 and remained for the duration of the experiment.

2.3. Resting and stimulation-induced overflows

The resting overflow of ^{3}H is defined as the fractional overflow in the 4 min prior to stimulation. The stimulation-induced (SI) overflow for each stimulation, SIO_1–SIO_4, were calculated by subtracting the relevant resting overflow from the fractional overflow in the 4 min following the onset of stimulation at S_1–S_4, respectively. When CGP 47332A or baclofen was added to the superfusion medium after S_1 and before S_2, the effects of these compounds 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/agonist. Similar techniques were used to determine the effects of the compounds when they were added to the superfusion medium after $S_3$and before $S_4$and to examine the effects of CGP 47332A in baclofen-treated slices. Using this technique, an antagonist of $GABA_B$ autoreceptors would increase the SI-overflow ratio and an agonist decrease it.

2.4. 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).

2.5. Statistical analysis

The significance of the effects of CGP 47332A and baclofen were assessed by unpaired Student's *t*-tests, with significance levels at $P < 0.05$.

2.6. Drugs

Racemic baclofen and CGP 47332A 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 (Missouri, 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 CGP 47332A on baclofen-induced suppression of spontaneous discharges in rat neocortical slices

In neocortical slices superfused with Mg^{2+}-free Krebs medium, baclofen reversibly depressed the frequency of spontaneous depolarisations in a concentration-dependent manner, with an approximate EC_{50} of 12 μM (Fig. 1). The suppression of activity by baclofen generally lasted some 10 min, and returned to baseline levels within 20 min following the initial washout of the drug. Pre-treatment of the slices with the antagonist CGP 47332A (25, 100, 300

μM) alone for 2 min did not affect the discharge rate or amplitude, but when used in combination with baclofen for 2 min, it reversibly antagonised the baclofen-induced suppression of spontaneous discharges. Following washout of the compounds, there was a complete recovery of the spontaneous activity, and of the depressant response to baclofen within 15 min. In order to quantify the antagonist potency of CGP 47332A, the effects of three concentrations of CGP 47332A (25, 100, 300 μM) on the baclofen concentration–response curve were measured. As illustrated in Fig. 1, increasing concentrations of the test compound 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 4.8 ± 0.1 ($n = 8$).

3.2. Effects of CGP 47332A 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 40 min of commencing superfusion (Fig. 2). In the presence of the GABA uptake inhibitor NO-711 (10 μM), electrical stimulation increased the overflow of [^{3}H] approximately two-fold in the 4 min collection following the onset of stimulation, an increase which returned to the resting level within a further 4 min. In a typical experiment, CGP 47332A enhanced the stimulation-induced overflow by 9 ± 2% at a concentration of 5 μM and by 58 ± 9% at 50 μM relative to untreated slices (Fig. 2). The concentration–response curve derived from these experiments (Fig. 2), shows that CGP 47332A facilitated the overflow of [^{3}H]GABA over the concentration range of 5–500 μM. Maximal facilitation occurred at the highest concentration tested (500 μM) and the EC_{150} value for CGP 47332A was 100 μM. The EC_{150} value was derived by interpolating 50% of the maximal response induced by 500 μM of the test compound. The mean resting overflows were unaffected by CGP 47332A.

3.3. Effect of CGP 47332A on the overflow of [^{3}H]GABA in the presence of baclofen

Baclofen alone inhibited the SI-overflow of [^{3}H]GABA at concentrations greater than 0.2 μM with a maximum inhibition of 48 ± 2% at 200 μM and an EC_{50} of 20 μM. The facilitatory effects of CGP 47332A (10 μM) on the SI-overflow described above were absent when neocortical slices were perfused with Krebs solution containing baclofen (20 μM). That is, the SI-overflow was inhibited by 30.2 ± 10.2% cf. 43.8 ± 5.2% in the presence of baclofen alone (data not shown). However, in two experiments, when the concentration of the antagonist was increased to 50 μM, the SI-overflow increased by at least 30% relative to the baclofen (20 μM) treated slices. This protocol was similar to that previously used by Waldmeier et al. (1994) where the inhibitory effects of the agonist were progressively reduced by increasing concentrations of the antagonist.

4. Discussion

In functional tests, a comparison of the potencies of CGP 47332A on baclofen-induced suppression of spontaneous discharges, and on enhancement of electrically stimulated [^{3}H]GABA release from rat neocortical slices, suggest that this compound is some six times weaker in antagonising $GABA_B$ autoreceptors than heteroreceptors. The concentration of CGP 47332A required to induce a 50% increase in [^{3}H]GABA overflow (EC_{150}) was 100 μM, while the apparent pA_2 value of CGP 47332A in antagonising $GABA_B$ receptor-mediated responses in the spontaneously discharging slices was 4.8 ± 0.1 (16 μM). From its binding data, CGP 47332A was almost four times more potent in inhibiting the binding of the $GABA_B$ receptor agonist ligand [^{3}H]CGP 27492 to $GABA_B$ receptors ($IC_{50} = 29$ μM; see Table 2 in Froestl et al., 1995) than in blocking $GABA_B$ autoreceptors. Although Froestl et al. (1995) has previously reported an 8% increase in [^{3}H]GABA release in the presence of 10 μM CGP 47332A, here, we have established a concentration–response curve for autoreceptor antagonism, reflecting an EC_{150} of 100 μM. Furthermore, we have also confirmed that CGP 47332A is indeed a selective $GABA_B$ receptor antagonist since its facilitatory effects on [^{3}H]GABA release were sensitive to baclofen.

Unlike CGP 47332A, its congener CGP 36742, lacking a β-hydroxy-substituent, displayed equipotent affinities at $GABA_B$ receptor binding sites ($EC_{50} = 38$ μM), and at autoreceptors ($EC_{150} = 38$ μM) (Froestl et al., 1995), as well as in the rat spontaneously discharging brain slices. In the latter, CGP 36742 antagonised $GABA_B$ receptor-mediated effects with an apparent pA_2 value of 4.5 (35 μM; Ong and Kerr, unpublished). Although the β-hydroxy substituent in racemic CGP 47332A evidently imparts a weaker affinity at $GABA_B$ autoreceptors than the heteroreceptors, it is not known whether the (*R*)- or (*S*)-enantiomer is responsible for this selectivity which is somewhat unique among established $GABA_B$ receptor antagonists.

Acknowledgements

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

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