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Short communication

CGP 36216 is a selective antagonist at $GABA_B$ presynaptic receptors in rat brain

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Abstract

In rat neocortical preparations maintained in Mg^{2+}-free Krebs medium, baclofen depressed the frequency of spontaneous discharges in a concentration-dependent manner ($EC_{50} = 6$ μM), sensitive to (3-aminopropyl)ethylphosphinic acid (CGP 36216) (100, 300 and 500 μM) ($pA_2 = 3.9 \pm 0.1$). By contrast, CGP 36216, up to 1 mM, was ineffective in antagonising baclofen-induced hyperpolarisations, mediated through γ-aminobutyric $acid_B$ ($GABA_B$) postsynaptic receptors. In electrically stimulated brain slices preloaded with [^{3}H]GABA, CGP 36216 increased [^{3}H]GABA release ($IC_{50} = 43$ μM), which was reversed by baclofen (20 μM). While CGP 36216 is ineffective at $GABA_B$ postsynaptic receptors, it is appreciably more active at presynaptic receptors.

Keywords: $GABA_B$ receptor antagonist; Neocortex, rat; Baclofen; CGP 36216

1. Introduction

Previous studies indicate that (3-aminopropyl)phosphinic acid and its homologue (3-aminopropyl)methylphosphinic acid display agonist activities at bicuculline-insensitive γ-aminobutyric $acid_B$ ($GABA_B$) receptors, whereas the higher homologue (3-aminopropyl)ethylphosphinic acid (CGP 36216) shows antagonist properties (Froestl et al., 1995b). In the P-substituted (3-aminopropyl)phosphinic acids, the transition from agonist to antagonist effects occur between a methyl substituent to an ethyl substituent, with CGP 36216 exhibiting antagonist activities at $GABA_B$ autoreceptors in rat cortical slices (Froestl et al., 1995b). The described potency in the latter preparation ($EC_{150} = 118$ μM) was lower than that found in binding studies where CGP 36216 inhibits binding of [^{3}H]3-aminopropylphosphinic acid ([^{3}H]CGP 27492) to $GABA_B$ receptors with an IC_{50} of 2 μM (see Table 1 in Froestl et al., 1995b). However, such a direct comparison between the release and binding data may not correctly reflect its antagonist potencies (Froestl et al., 1995b; but see Waldmeier et al., 1994). In this study, we have evaluated the antagonist effects of CGP 36216 at presynaptic $GABA_B$ heteroreceptors regulating glutamate release in spontaneously discharging rat neocortical slices, and compared its actions on $GABA_B$ postsynaptic receptors mediating K^+-dependent hyperpolarizations in neocortical wedges. In addition, to confirm the earlier study (Froestl et al., 1995b), we have further examined the actions of CGP 36216 on $GABA_B$ autoreceptors modulating electrically evoked [^{3}H]GABA from neocortical slices. Here we show that while CGP 36216 is ineffective at $GABA_B$ postsynaptic receptors, it is appreciably more active as an antagonist at the presynaptic receptors. Furthermore, this compound is some three times more potent in antagonising $GABA_B$ autoreceptors than previously reported (Froestl et al., 1995b).

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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care" (National Institute of Health 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.

The $GABA_B$ receptor agonist baclofen was 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, that is the concentration, which 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 three different animals.

The method used for recording hyperpolarizing responses to the $GABA_B$ receptor agonist baclofen 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), while 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, baclofen was added to the superfusing medium, and applied to the cortical side of the tissue for three 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. Results were quantified, and values expressed as a percentage of the maximum hyperpolarization obtained with the agonist, 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 three to six different animals.

2.2. Release studies

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, rinsed, placed in a small chamber and superfused at 1 ml/min with Krebs solution (95% O_2:5% CO_2, 32°C) as previously described (Ong et al., 1998). The perfusion medium contained the GABA uptake inhibitor 1-(2-(((diphenylmethylene)amino)oxy)-ethyl)-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid (NO-711) (5 μM). 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 10 min and for 150 s at 38 min (S_1), 58 min (S_2), 78 min (S_3) and 98 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 assayed. The effects of CGP 36216 and baclofen on the fractional overflow of [^{3}H] were tested

at either S_2 or S_4. The compounds 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 until the end of the experiment (S_4).

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 compounds used on the stimulation-induced overflow of [^{3}H] (SIO) 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. A similar technique was used to measure the effect of the compounds 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 (in mM): NaCl (120), KCl (4.7), $NaHCO_3$ (25), KH_2PO_4 (1.0), $CaCl_2$ (1.5), $MgCl_2$ (1.0), glucose (11), and contained aminooxyacetic acid (50 μM).

2.3. Drugs

Racemic (±)-baclofen and (3-aminopropyl)ethylphosphinic acid (CGP 36216) 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, 1-(2-(((diphenylmethylene)-amino)oxy)ethyl)-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride (NO-711), 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. Antagonism of baclofen-induced suppression of spontaneous discharges by CGP 36216 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 10–15 min, and was washed out completely within 30 min of application. As shown in Fig. 1(A) ($n = 8$), baclofen, over a concentration range of 1–100 μM, depressed the frequency of spontaneous discharges, with a mean estimated EC_{50} value of 6 μM. CGP 36216 (100, 500 and 1000 μM) alone did not have any appreciable effect on the discharge rate or amplitude, but 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. Increasing concentrations of CGP 36216 produced surmountable concentration-dependent rightward shifts in the baclofen concentration–response curves, yielding an apparent pA_2 value of 3.9 ± 0.1 (Fig. 1(A); $n = 8$).

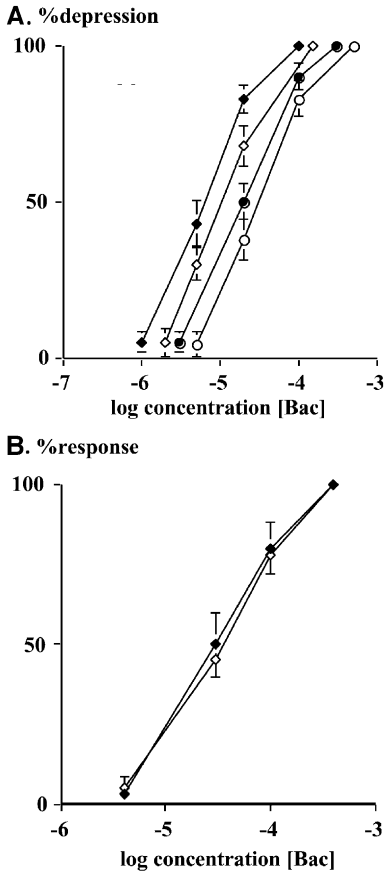

Fig. 1. Effects of (3-aminopropyl)ethylphosphinic acid (CGP 36216) on (*R,S*)-baclofen-induced responses in rat neocortical preparations. (A) Concentration–response curves for baclofen-induced suppression of the frequency of spontaneous discharges in rat isolated neocortical slices maintained in Mg^{2+}-free Krebs medium, in the absence and presence of CGP 36216. The concentration–response curve for baclofen (♦) was shifted to the right, in a parallel fashion, (a) by CGP 36216 (◇, 100 μM; ●, 300 μM; ○, 500 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. (B) Concentration–response curves for hyperpolarizing responses induced by baclofen alone (♦), or in the presence of 1 mM concentration of CGP 36216 (◇) in rat neocortical wedges, maintained in Mg^{2+}-containing Krebs solution. Values are expressed as a percentage of the maximum hyperpolarization (expressed as 100%) induced by baclofen, and each point represents the mean and standard error of the mean of 8–12 determinations.

3.2. Antagonism of baclofen-induced hyperpolarizations by CGP 36216 in neocortical wedges

Superfusion of neocortical wedges, with baclofen for 3 min over a concentration range of 4–400 μM, in Mg^{2+}-containing Krebs solution to suppress spontaneous dis-

charges, consistently induced concentration-dependent hyperpolarizing responses (Fig. 1(B); $n = 12$). 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. Here, as shown in Fig. 1(B), the concentration–response curve for baclofen was plotted as a percentage of the maximal response elicited by baclofen at 400 μM (100% response), with a threshold concentration of around 4 μM, and a half-maximal effect of 30 μM for baclofen. Full recovery to baclofen-induced responses was obtained only after 30–60 min of wash-out. CGP 36216 (100, 300, 500 μM) did not affect the baclofen concentration–response curve (data not shown; $n =$ 8–12), and at the highest concentration of 1 mM CGP 36216, the concentration–response curve for baclofen was not significantly different from that of baclofen alone (Fig. 1(B); $n = 8$). At the concentrations employed in these experiments, CGP 36216 alone did not affect the membrane potentials.

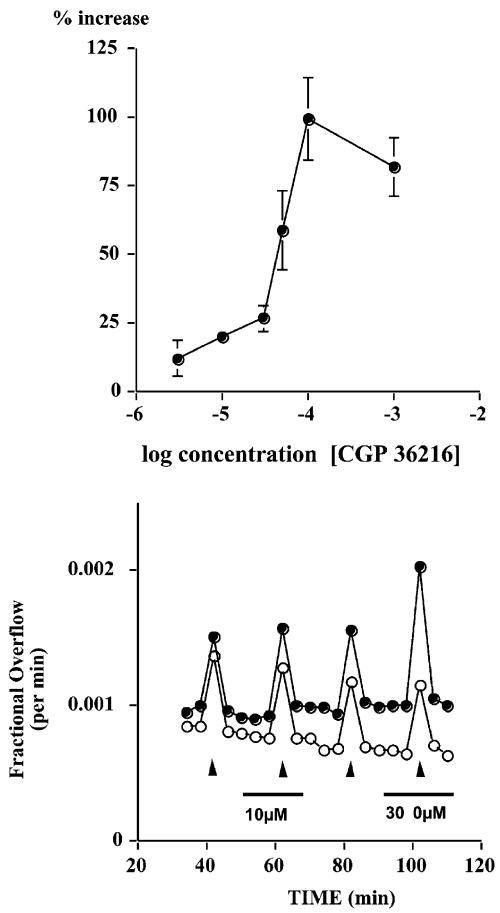

Fig. 2. Concentration–response curve for CGP 36216. Values shown are the increases in the stimulation-induced overflows of [^{3}H]GABA expressed as a percentage of the overflow in the absence of the agents. Data are the means and standard errors of the means of at least four experiments. CGP 36216 facilitated the release of [^{3}H]GABA at concentrations above 3 μM (unpaired Student's *t*-tests, $P < 0.05$). The fractional overflow per minute of [^{3}H]GABA from rat neocortical is also shown for a typical experiment in the presence and absence of the agent CGP36216. These slices were pre-incubated in [^{3}H]GABA (0.1 μM) and superfused with Krebs solution containing 1-(2-(((diphenylmethylene)amino)-oxy)ethyl)-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid (NO-711)NO-711 (5 μM). In one (-○-), the slices were untreated and in the other (-●-) perfused with CGP 36216 (10 and 300 μM) for the periods represented by the bars. Tissues were stimulated (arrows) at 2 Hz for 150 s. In untreated slices, the overflow of [^{3}H] in the 4-min collections commencing with a period of stimulation was maximal for the first stimulation and declined slightly with each succeeding stimulation. In this experiment, CGP 36216 (10 and 300 μM) produced an increase in the stimulated induced overflow of 25.4% and 114.1%, respectively.

3.3. Effects of CGP 36216 on the overflow of [^{3}H]GABA

CGP 36216 at concentrations of 10 μM and above increased the stimulation-induced overflow of [^{3}H]GABA relative to neocortical slices superfused with CGP 36216-free Krebs (Fig. 2). Maximum potentiation occurred at a concentration of 300 μM and although the potentiation was less at 1.0 mM, the difference was statistically insignificant. The IC_{50} for CGP 36216 was 43 μM.

In the presence of baclofen (20 μM), the facilitatory effect of CGP 36216 (50 μM) on the stimulation-induced overflow of [^{3}H] was prevented. Relative to untreated tissue, the combination of baclofen and CGP 36216 inhibited the overflow by $18 \pm 8\%$ ($n = 4$), while baclofen alone inhibited the overflow by $39 \pm 8\%$ ($n = 5$).

At concentrations of 300 μM and 1.0 mM, CGP 36216 increased the resting overflow of [^{3}H] by $25 \pm 7\%$ ($n = 6$) and $26 \pm 6\%$ ($n = 5$), respectively, whereas baclofen (20 μM) and the combination of baclofen and CGP 36216 (50 μM) inhibited the overflow relative to untreated slices by $13 \pm 3\%$ ($n = 5$) and $20 \pm 7\%$ ($n = 4$).

4. Discussion

We have investigated the antagonist effects of CGP 36216 on $GABA_B$ receptors in rat neocortical slices. In spontaneously discharging neocortical slices maintained in Mg^{2+}-free Krebs medium, the baclofen-induced depression of discharge amplitude and rate, mediated through $GABA_B$ heteroreceptors was reversibly antagonised by CGP 36216, with an apparent pA_2 value of 3.9 ± 0.1. However, when compared to the latter, CGP 36216, up to a concentration of 1 mM, was relatively ineffective in blocking the hyperpolarizing effects of baclofen in neocortical wedges maintained in Mg^{2+}-containing Krebs solution. Such hyperpolarizations are mediated through $GABA_B$ receptors, sensitive to the $GABA_B$ receptor antagonist (+)-(*S*)-5,5 dimethylmorpholinyl-2-acetic acid (Sch 50911), and most likely involve the opening of K^+ channels as these effects were sensitive to Ba^{2+} and Cs^+ (Ong and Kerr, unpublished data). From these results, we

suggest that CGP 36216 appears to be inactive at $GABA_B$ postsynaptic receptors while exhibiting appreciable antagonist activities at heteroreceptors. 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, based on our results, it is possible that CGP 36216 could prove to be a useful lead compound to differentiate between pre- and postsynaptic receptors. Interestingly, in the rat dorsolateral septal neurons, the phosphinic acid analogue (3-amino-2(*R*)-hydroxypropyl)methylphosphinic acid (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).

In electrically stimulated slices, CGP 36216 enhanced the overflow of [^{3}H] in a concentration-dependent manner ($EC_{50} = 43$ μM) while having little or no effect on the resting overflow. The facilitatory actions at $GABA_B$ autoreceptors are specific, given that baclofen reversed the effects, suggesting that CGP 36216 is a $GABA_B$ autoreceptor antagonist. Although the antagonist potency of CGP 36216 at autoreceptors in our preparations is some three times more potent than that previously reported (EC_{150} = 118 μM; see Table 1 in Froestl et al., 1995b) using a similar paradigm, nevertheless, its affinity in displacing [^{3}H]CGP 27492 binding is significantly more potent with an IC_{50} of 2 μM; yet on postsynaptic receptors in neocortical wedges, CGP 36216 up to 1 mM concentrations did not antagonise the effects of baclofen. As previously reported, (3-aminopropyl)phosphinic acid and (3-aminopropyl)methylphosphinic acid display agonist activities at $GABA_B$ receptors, whereas the ethyl homologue (3-aminopropyl)ethylphosphinic acid (CGP 36216) shows antagonist properties (Froestl et al., 1995b). Here, the transition from agonist to antagonist effects occurred between a methyl substituent to an ethyl substituent in the P-substituted (3-aminopropyl)phosphinic acids. In keeping with this, CGP 47656, the CHF_2 phosphinic acid analogue, with a substituent of a size between ethyl and methyl is a $GABA_B$ receptor partial agonist (Froestl et al., 1995a). CGP 36216 is thus of interest, showing no effects at $GABA_B$ postsynaptic receptor sites while having appreciable antagonist affinities at presynaptic hetero- and autoreceptors.

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