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3-Chloro,4-methoxyfendiline is a potent $GABA_B$ receptor potentiator in rat neocortical slices

Jennifer Ong[a,*], David A.S. Parker[b], Victor Marino[b], David I.B. Kerr[a], Ni Made Puspawati[c], Rolf H. Prager[c]

[a]*Department of Anaesthesia and Intensive Care, The University of Adelaide, Adelaide, South Australia 5005, Australia*
[b]*Dental School, The University of Adelaide, South Australia 5005, Australia*
[c]*School of Physical Sciences, The Flinders University, Bedford Park, South Australia 5042, Australia*

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Abstract

Using grease-gap recording from rat neocortical slices, the $GABA_B$ receptor agonist baclofen elicited reversible and concentration-dependent hyperpolarizing responses (EC_{50}=18±2.3 μM). The hyperpolarizations were antagonised by the $GABA_B$ receptor antagonist Sch 50911 [(+)-(*S*)-5,5-dimethylmorpholinyl-2-acetic acid). (+)-*N*-1-(3-chloro-4-methoxyphenyl)ethyl-3,3-diphenylpropylamine (3-chloro,4-methoxyfendiline; 3-Cl,4-MeO-fendiline) reversibly potentiated baclofen-induced hyperpolarizing responses, which were reduced by Sch 50911, producing leftward shifts of the baclofen concentration–response curves, with a marked increase in the maximal hyperpolarization (EC_{50}=2±0.5 μM). In slices preincubated with either [^{3}H]GABA or [^{3}H]glutamic acid, 3-Cl,4-MeO-fendiline (1 μM) potentiated the inhibitory effect of baclofen (2 μM) on the electrically evoked release of [^{3}H]GABA and had a similar effect on the release of [^{3}H]glutamic acid at a concentration of 0.5 μM, without affecting the basal release. These effects were blocked by Sch 50911 (10 μM). Our findings suggest that 3-Cl,4-MeO-fendiline is a potent potentiator of pre- and postsynaptic $GABA_B$ receptor-mediated functions.

Keywords: Neocortex; Baclofen; 3-Chloro,4-methoxyfendiline; Pre- and postsynaptic $GABA_B$ receptor; (Rat)

1. Introduction

Metabotropic γ-aminobutyric $acid_B$ ($GABA_B$) receptors belong to Family 3 of the G-protein-coupled receptors which share the characteristic of a large extracellular amino terminal domain that contains a so-called "Venus flytrap" ligand-binding site (Bockaert and Pin, 1999). $GABA_B$ receptors control neuronal excitability and transmission in the nervous system and are of crucial importance in many physiological activities, such as autonomic function, memory, and cognition, as well as motor and sensory control including pain regulation and epilepsy (Kerr and Ong, 1995, 2001; Bowery et al., 2002). Indeed, $GABA_{(B1)}$ subunit knockout mice lacking these receptors exhibit defects in all these processes, resulting in premature death (Prosser et al., 2001). $GABA_B$ receptors can be subdivided into presynaptic heteroreceptors that inhibit synaptic transmission, presynaptic autoreceptors that similarly regulate the release of GABA itself, and postsynaptic receptors that reduce neuronal excitability (Kerr and Ong, 1995, 2001). Stimulation of presynaptic $GABA_B$ heteroreceptors has been shown to decrease neurotransmitter release, possibly by reducing Ca^{2+} conductance, while 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).

Other members of Family 3 that show a degree of similarity to $GABA_B$ receptors include metabotropic

* Corresponding author. Tel.: +61 8 8303 5163; fax: +61 8 8303 3788.
E-mail address: jennifer.ong@adelaide.edu.au (J. Ong).

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glutamate, Ca^{2+}-sensing, some pheromone and taste receptors (Bockaert and Pin, 1999; Couve et al., 2000). Of particular interest with $GABA_B$ receptors is their obligatory heterodimerization from two similar but not identical receptors, $GABA_{(B1)}$ and $GABA_{(B2)}$, that are physically coupled via α-helical coiled-coil domains at their respective intracellular C-termini. Such heterodimer formation is unique to $GABA_B$ receptors and is essential for proper functional expression and maturation, as well as receptor activation and ligand specificity (Kerr and Ong, 2001; Bowery et al., 2002; Vacher and Bettler, 2003). Importantly, however, $GABA_{(B2)}$ modulates $GABA_{(B1)}$ and is the main signalling receptor essential for G-protein coupling of the $GABA_B$ receptor heterodimer (Margeta-Mitrovic et al., 2001; Robbins et al., 2001).

Recently, a number of allosteric modulators which affect members of Family 3 G-protein-coupled receptors, such as extracellular Ca^{2+}-sensing (Nemeth et al., 1998), metabotropic glutamate (Knoflach et al., 2001; Gasparini et al., 2002), and $GABA_B$ receptors (Urwyler et al., 2001, 2003) have been described. In all these Family 3 receptors, the orthosteric binding site for the natural agonist lies within a specialized conserved Venus flytrap region of the amino terminal domain (Couve et al., 2000), whereas the allosteric modulators bind at a site on the seven-transmembrane domain, apart from this specialized agonist binding region of the amino terminal domain. A variety of phenylalkylamines are potent allosteric modulators at extracellular Ca^{2+}-sensing receptors. These include the lead compound fendiline [*N*-(1-phenylethyl)-3,3-diphenylpropylamine] and a number of *N*-[3-phenylpropyl]-methylphenylethylamines bearing various substituents (Nemeth et al., 1998). Recently, we have shown that phenylalkylamines akin to fendiline potentiate and enhance neuronal actions at $GABA_B$ receptors (Kerr et al., 2002, 2004) and have now explored the structure–action profiles of a number of fendiline derivatives.

Using baclofen as the agonist at $GABA_B$ receptor-mediated hyperpolarizations in rat neocortical slice preparations (Ong et al., 2001), we have examined the potentiating effects of the analogue (+)-*N*-1-(3-chloro-4-methoxyphenyl)ethyl-3,3-diphenylpropylamine (3-chloro,4-methoxyfendiline; 3-Cl,4-MeO-fendiline; see chemical structure; Fig. 1), identified as a potent modulator, at these receptors.

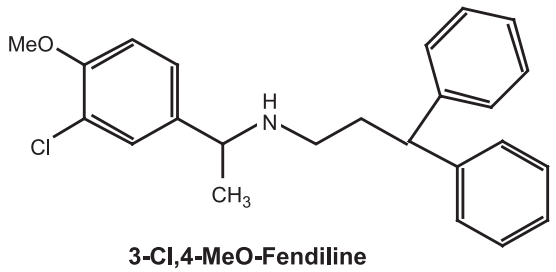

Fig. 1. Chemical structure of the arylalkylamine analogue 3-chloro,4-methoxyfendiline (3-Cl,4-MeO-fendiline).

Furthermore, using neurochemical assays on electrically stimulated release of [^{3}H]GABA and [^{3}H]glutamic acid from rat neocortical slices (Parker et al., 2004), we also examined possible potentiating actions of 3-Cl,4-MeO-fendiline on $GABA_B$ receptor-mediated presynaptic responses induced by balcofen, since these have not been examined previously at presynaptic $GABA_B$ auto- and heteroreceptors modulating transmitter release. Our results show that the potency of fendiline at both pre- and postsynaptic $GABA_B$ receptors is increased by the chloro- and *O*-methyl-substituents on the α-methylbenzyl moiety of the fendiline molecule to give 3-Cl,4-MeO-fendiline.

2. Materials and methods

2.1. Rat neocortical slice preparations

Rat neocortical slices were prepared from halothane-anaesthetized outbred male adult Sprague–Dawley rats (250–350 g), which were decapitated, using established procedures described previously (Horne et al., 1986; Ong et al., 2001). All 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.

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 VIBROSLICE microtome (Campden Instruments, UK), and a radial wedge was cut from each side of the dorsal midline 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 1 h prior to experimentation.

2.2. Hyperpolarizing responses

Following the equilibration period, wedge-shaped slices from the neocortex were placed in a two-compartment perspex perfusion chamber, where each wedge was placed across a septum, separating pools containing the cortex and white matter by a grease seal, using a superfusion method based on a grease–gap system as described previously (Horne et al., 1986). The grey matter was then continuously superfused with gassed Krebs medium at 25 °C delivered by a peristaltic pump at 1 ml/min. The white matter was immersed in a chamber containing Krebs solution. Potential changes induced by $GABA_B$ receptor agonists were recorded during 3-min applications of each agonist. Differ-

ential 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. Here, Mg^{2+}-containing Krebs medium was used throughout the experiments to eliminate spontaneous discharges, since the latter tended to complicate the hyperpolarizing responses.

After 60 min of equilibration, the $GABA_B$ receptor agonist baclofen was 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. When examining the potentiating effects of a compound, the latter was first superfused for 5 min and then added together with the agonist for a further 3 min before tissue washout. In some experiments where a $GABA_B$ receptor antagonist was used to test the specificity of the potentiators, it was first superfused for 3 min, and then added together with the test compound and agonist. In each experiment, the responses to the agonist were reestablished after drug application to control for the stability of the preparation. Results were quantified, and values expressed as a percentage of the maximum hyperpolarization obtained with the agonist alone, measured from the chart recordings. Concentration–response curves were constructed, in the absence and presence of the test agent. To test the potentiating activity of the compound, it was applied at ascending concentrations with a fixed agonist concentration. The concentration–response profile for the potentiator was constructed by measuring the peak amplitude during application of the compound and a standard concentration of agonist (EC_{50} of the agonist), calculating the percent increase relative to the agonist (alone) response, and plotting the data as a function of potentiator concentration.

In other experiments, the concentration–response curves of the agonist were constructed, in the absence and presence of differing concentrations of the potentiator. The EC_{50} values were then calculated from the concentration–response curves using GraphPad Prism, where the EC_{50} is the concentration giving a response equal to 50% of the maximally effective concentration. All numerical data on the concentration–response curves were expressed as means±S.E.M. Each experiment was repeated on 6–12 slices obtained from 6–12 different animals. Comparison of the data was made using Student's *t*-test with $P<0.05$ being significant.

2.3. Release studies

2.3.1. Incubation in [^{3}H]GABA

Slices of neocortex were equilibrated for at least 40 min in warm, gassed Krebs solution (34 °C; 95% O_2:5% CO_2) containing aminooxy acetic acid, a GABA transaminase inhibitor which prevents GABA degradation (AOAA; 50 μM). Subsequently, they were incubated for 30 min in Krebs solution containing GABA (0.05 μmol/l) and [^{3}H]GABA (0.05 μmol/l). Pairs of slices were rinsed, placed in small chambers, and superfused at 1 ml/min with Krebs solution (34 °C, gassed) containing AOAA and the uptake inhibitor NO-711 (5 μmol/l). Aliquots of superfusate were collected at 10-min intervals for the first four collections and for 4 min thereafter and their ^{3}H contents assayed by liquid scintillation spectrometry. Untreated slices were stimulated at 38 min (S_1) and 58 min (S_2) after superfusion commenced, through platinum field electrodes by square wave pulses at 2 Hz (2.0 ms duration, 50 V, 300 pulses). In all other experiments, S_1 occurred at 48 min, and S_2 at 76 min. The residual ^{3}H content in the slices was extracted at the end of each experiment in 0.4 mol/l $HClO_4$ (containing EDTA, 3.0 mmol/l and Na_2SO_3, 10 mmol/l) and maintained at 4 °C for at least 16 h and then assayed. From these data, the fractional overflow of ^{3}H during each collection period was computed.

When required, the $GABA_B$ antagonist Sch 50911 was added to the superfusion medium 20 min before S_2 and the agonist baclofen and the putative modulator, 3-Cl,4-MeO-fendiline, 10 min prior to S_2.

2.3.2. Incubation in [^{3}H]glutamic acid

Slices of neocortex were equilibrated as above prior to incubation in Krebs solution containing glutamic acid (0.3 μmol/l) and [^{3}H]glutamic acid (0.3 μmol/l) for 45 min. During the equilibration and incubation periods, the Krebs solution contained semicarbazide (100 μmol/l) to prevent metabolism of the glutamic acid. As detailed above, pairs of slices were superfused at 1 ml/min, aliquots of superfusate were collected (8-min intervals), and their ^{3}H contents assayed by liquid scintillation spectrometry. Slices were stimulated through platinum field electrodes by square wave pulses at 2 Hz (2.0 ms duration, 50 V, 500 pulses) at 64 min (S_1) and 104 min (S_2) after superfusion commenced.

When required, the $GABA_B$ antagonists, agonists, and putative modulator described previously were added 10 min prior to S_2.

2.3.3. Resting and stimulation—induced overflows

The resting overflow of ^{3}H (R_1 or R_2) is defined as the fractional overflow in the 8 min prior to stimulation (S_1 or S_2). The effects of agents on the resting overflow of ^{3}H were determined by comparing the R_2/R_1 ratio with that in appropriate control slices. The stimulation-induced overflows of ^{3}H at S_1 and S_2, SIO_1 and SIO_2, respectively, were calculated by subtracting the resting overflow (8 min) from the fractional overflow in the 8 min following the onset of stimulation. The effects of agents were determined by comparing the SIO_2/SIO_1 ratio in the presence of the agent with that in control tissue.

2.3.4. Solutions

Krebs solution was of the following composition (mmol/l): NaCl (120), KCl (2.1), $NaHCO_3$ (25), KH_2PO_4 (1.2),

$CaCl_2$ (2.0), $MgCl_2$ (1.3), glucose (11), and contained aminooxyacetic acid (AOAA; 0.05 mmol/l) for the [^{3}H]GABA experiments.

2.3.5. Statistical analysis

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

2.4. Drugs and chemicals

2, 3-[^{3}H][N]-GABA, specific activity 1.06 TBq/mmol and L-[3,4-^{3}H]-glutamic acid, specific activity 1.89 TBq/mmol were from New England Nuclear (Boston, MA, USA). Aminooxyacetic acid hemihydrochloride (AOAA), GABA, and semicarbazide were obtained from Sigma (MO, USA), while NO-711 was from Research Biochemicals (Natick, MA, USA). Racemic (±)-baclofen was purchased from Tocris Cookson (Bristol, UK). Sch 50911 [(+)-(*S*)-5,5-dimethylmorpholinyl-2-acetic acid] was a gift from Dr. David Blythin (Schering Plough, USA). 3-Cl,4-MeO-fendiline (Fig. 1), used as the hydrochloride salt, mp 192–195C, was synthesised in house (Puspawati and Prager, The Flinders University) by the reductive alkylation of commercially available 3,3-diphenylpropylamine and 3-chloro-4-methoxyacetophenone with sodium cyanoborohydride in the presence of titanium isopropoxide.

3. Results

3.1. Potentiating effects of 3-Cl,4-MeO-fendiline on $GABA_B$ receptor-mediated hyperpolarizations in neocortical slices

The $GABA_B$ receptor agonist baclofen induced concentration-dependent hyperpolarizing responses which were reversibly antagonised by Sch 50911, a selective $GABA_B$ receptor antagonist. These population hyperpolarizations

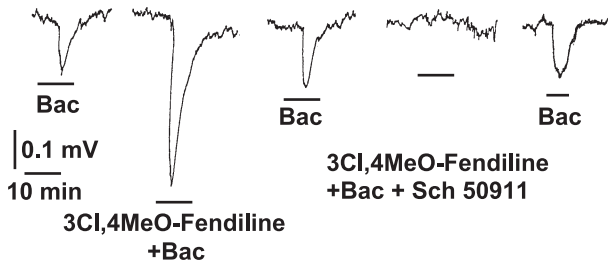

Fig. 2. Discontinuous record of the hyperpolarizing effects of baclofen (10 μM) in a rat neocortical slice preparation and the potentiating effect of 3-chloro,4-methoxyfendiline (3-Cl,4-MeO-fendiline; 60 nM) on baclofen-induced responses. The control responses to baclofen were subsequently reestablished upon tissue washout within 60 min. The enhanced response to baclofen in the presence of 3-Cl,4-MeO-fendiline was completely abolished by the $GABA_B$ receptor antagonist (+)-(*S*)-5,5-dimethylmorpholinyl-2-acetic acid (Sch 50911; 10 μM). The interval between drug applications was 30–60 min.

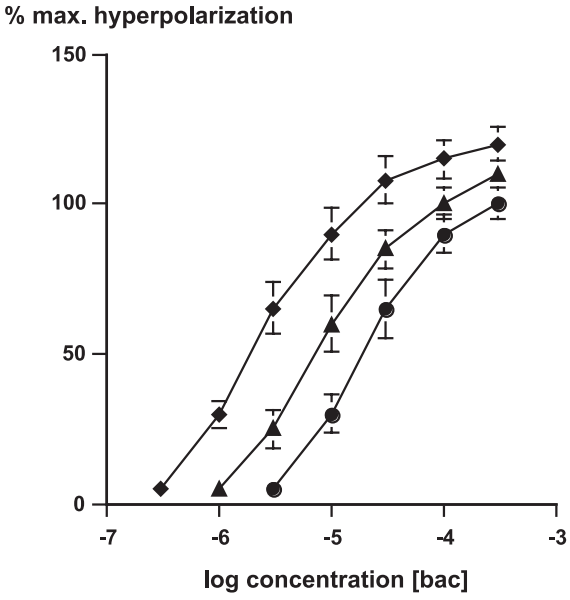

Fig. 3. Concentration–response curves for baclofen-induced hyperpolarizations recorded from the rat neocortical slices, in the absence and presence of the potentiator 3-chloro,4-methoxyfendiline (3-Cl,4-MeO-fendiline). Complete concentration–response curves for baclofen alone (●), and the leftward shifts of the concentration–response curves by two concentrations of 3-Cl,4-MeO-fendiline (▲ 10 nM; ♦ 60 nM). Values are expressed as a percentage of the maximum hyperpolarization achieved by the agonist alone, and each point represents the mean and standard error of the mean of 8–12 determinations.

were mediated through activation of inwardly rectifying K^+ channels, being sensitive to Ba^{2+} (0.1 mM) or Cs^+ (1 mM; Ong et al., 2001). The onset of the baclofen-evoked hyperpolarization occurred 2 min after baclofen reached the slice, and the maximal effect was reached within 3–5 min. The control response to baclofen could be reestablished after 30 min drug washout. A typical example of this baclofen response is represented in Fig. 2, where the concentration of baclofen used was 10 μM.

Over a concentration range of 3–300 μM, baclofen consistently induced concentration-dependent hyperpolarizing responses (Fig. 3; $n=12$). Responses obtained by using different concentrations of this $GABA_B$ receptor agonist were normalized to those obtained by using a maximum saturating concentration of the agonist. The concentration–response curve for baclofen was then plotted as a percentage of the normalized maximal hyperpolarizing response elicited by baclofen at 300 μM (100% response; $n=12$). The threshold concentration for inducing a hyperpolarization response was around 3 μM, resulting in a 5% response, and the maximal effect was elicited by 300 μM baclofen (100% response). An EC_{50} value of 18±2.3 μM for baclofen was calculated. In general, full recovery to baclofen-induced responses was obtained only after 30 min of tissue washout.

Application of 3-Cl,4-MeO-fendiline (10 and 60 nM) alone for 5 min had no detectable effect, but when cosuperfused with baclofen, it potentiated the baclofen-

induced hyperpolarizing responses (Figs. 2 and 3; n=8). As shown in Fig. 2, 3-Cl,4-MeO-fendiline (60 nM) substantially increased the $GABA_B$ receptor-mediated hyperpolarizing activity induced by baclofen (10 μM), by nearly fourfold. The hyperpolarizing response elicited by coapplication of 3-Cl,4-MeO-fendiline and baclofen was significantly more pronounced than the response recorded during the application of baclofen alone. This effect, in the presence of 3-Cl,4-MeO-fendiline, was completely reversible as the hyperpolarization to baclofen was reestablished by washing for 60 min. Furthermore, this potentiating effect of 3-Cl,4-MeO-fendiline was abolished by the competitive $GABA_B$ receptor antagonist Sch 50911 (10 μM; Fig. 2; n=8), and thus was mediated through $GABA_B$ receptors. Pretreatment with Sch 50911 (1 and 10 μM) alone for 3 min did not affect the resting potential, but in combination with varying concentrations of baclofen (3–300 μM) and 3-Cl,4-MeO-fendiline (10 and 60 nM) for 3 min, reversibly reduced the 3-Cl,4-MeO-fendiline-induced enhancement of baclofen hyperpolarizations and caused a progressive shift of the 3-Cl,4-MeO-fendiline-baclofen concentration–response curves to the right (data not shown; n=8 for each concentration of Sch 50911). Following washout of the compounds, there was a complete recovery of the response to baclofen within 60 min.

3-Cl,4-MeO-fendiline-induced enhancement of the hyperpolarizing response to baclofen was concentration-dependent, since the concentration–response curves for baclofen at two fixed concentrations of 3-Cl,4-MeO-fendiline (10 and 60 nM) revealed an increase of agonist potency as well as maximal efficacy (Fig. 3; n=8–12 for each drug concentration). The baclofen concentration–response curves were significantly displaced to the left by 3-Cl,4-MeO-fendiline; in particular, at the highest concentration of 60 nM, 3-Cl,4-MeO-fendiline also increased the maximal effect of baclofen at 300 μM to 120% of the control response (Fig. 3). In the latter figure, the concentration–response profile for baclofen alone had an EC_{50} value of 18±2.3 μM (n=12); however, in the presence of two concentrations of 3-Cl,4-MeO-fendiline (10 and 60 nM), the potency of baclofen increased 2.6-fold and 9-fold, such that the EC_{50} values were 7±2.5 and 2±0.5 μM, respectively (Fig. 3; n=8).

A further series of experiments were performed with increasing concentrations of 3-Cl,4-MeO-fendiline on a fixed concentration of the agonist baclofen. Here, from the concentration–response curves for the potentiation of baclofen (10 μM) by 3-Cl,4-MeO-fendiline (3 nM to 1μM) at the $GABA_B$ receptor, the EC_{50} value for 3-Cl,4-MeO-fendiline potentiation was 30±4.0 nM (Fig. 4; n=6–12). On its own, 3-Cl,4-MeO-fendiline did not have any effect on the membrane potential or on $GABA_A$ receptor-mediated depolarizing responses to GABA (100 μM; data not shown; n=8). Such depolarizing responses were of immediate onset, before any $GABA_B$ receptor-mediated hyperpolarizing component.

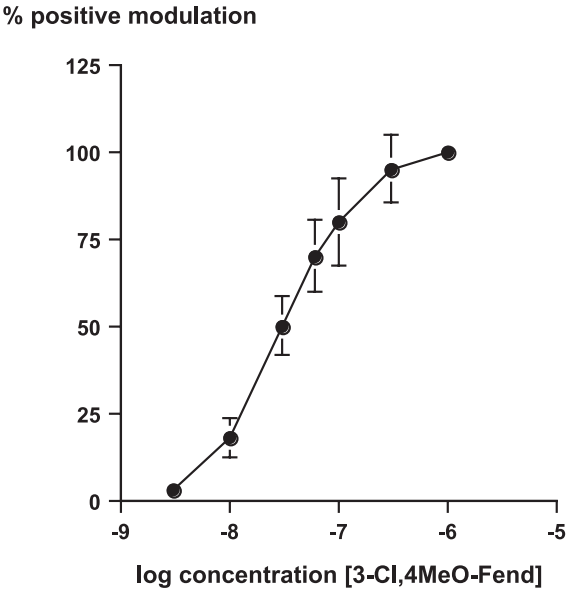

Fig. 4. Concentration–response profile for the compound 3-chloro,4-methoxyfendiline (3-Cl,4-MeO-fendiline) in potentiating the hyperpolarizing responses induced by a fixed concentration of baclofen (10 μM), in rat neocortical slices. Values are expressed as a percentage of the hyperpolarization induced by baclofen alone, and each point represents the mean and standard error of the mean of 6–12 determinations.

3.2. Release of ^3H from neocortical slices incubated in [^3H]GABA

The SIO_2/SIO_1 ratio in untreated neocortical slices was 0.94±0.01 (n=12), and the effects of the various agents tested were standardised against a ratio of 1.0, expressed as a percentage (Fig. 5). As reported previously, baclofen (2 μM) had little or no effect on the stimulation-induced overflow of ^3H, whereas at a concentration of 20 μM, it reduced the overflow by 27% (Ong et al., 2001). In the present experiments, while 3-Cl,4-MeO-fendiline (1 μM) alone had no effect on the SI-overflow, in the presence of baclofen (2 μM), the overflow was reduced by 22% (Fig. 5). Sch 50911 (10 μM) reversed this effect and enhanced the SI-overflow relative to that in untreated slices by 96%. Similar increases in the overflow were produced by Sch 50911 alone (124%) and in the presence of 3-Cl,4-MeO-fendiline (61%).

Baclofen had no effect on the resting overflow of ^3H; however, the overflow was increased by Sch 50911 and/or 3-Cl,4-MeO-fendiline, by between 18% and 27%, in the presence and absence of baclofen.

3.3. Release of ^3H from neocortical slices incubated in [^3H]glutamic acid

As described above, the SIO_2/SIO_1 ratios in the presence of the agents were standardised against that in the absence of agents and expressed as a percentage. The actual ratio in untreated tissue was 0.90±0.05 (n=8).

Like their actions in the GABA series of experiments, baclofen (0.5 μM) and 3-Cl,4-MeO-fendiline (1 μM) alone

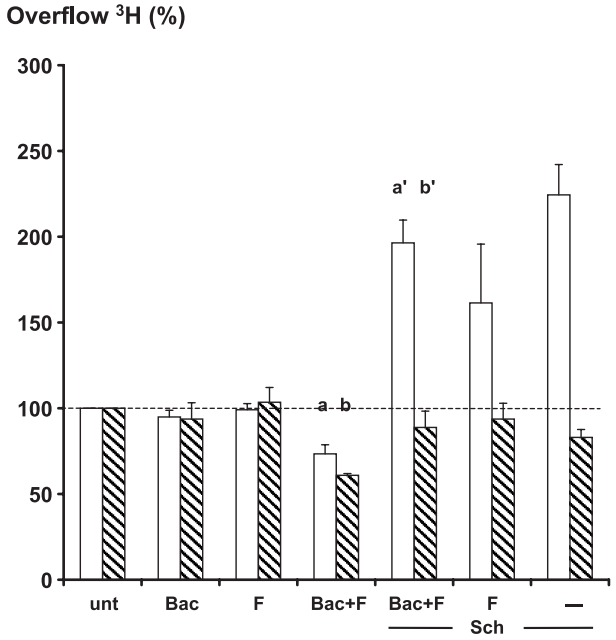

Fig. 5. The effects of baclofen (Bac; 2 μM, GABA series; 0.5 μM, glutamic acid series), 3-Cl,4-MeO-fendiline (F; 1 μM), and Sch 50991 (10 μM) on the stimulation-induced overflow of ^{3}H from rat neocortical slices preincubated with either [^{3}H]GABA (0.1 μM, open columns) or [^{3}H]glutamic acid (0.6 μM, hatched columns). Results are expressed as a percentage of the overflow in untreated slices (100%; broken line). Neither baclofen nor 3-Cl,4-MeO-fendiline alone had an effect on the stimulation-induced overflow of ^{3}H from slices incubated in either [^{3}H]GABA or [^{3}H]glutamic acid, whereas in both series of experiments, the overflows were reduced by the combination of these two agents. Sch 50911 reversed these inhibitory effects; in the GABA, but not glutamic acid, series the overflow of ^{3}H was increased markedly. *n*=at least 5 experiments. ^{a,b}3-Cl,4-MeO-fendiline inhibited the overflow of ^{3}H in baclofen-treated slices; unpaired Student's *t*-test; $P<0.05$. $^{a',b'}$ The inhibitory effects of 3-Cl,4-MeO-fendiline in baclofen-treated slices were blocked by Sch 50911; unpaired Student's *t*-test; $P<0.05$.

had no effect on the SI-overflow of ^{3}H, but when the two agents were combined, the overflow was inhibited by 39% (Fig. 5). In contrast to its effect on the release of [^{3}H]GABA, while Sch 50911 (10 μM) reversed this inhibitory effect, it did not enhance the overflow relative to that in untreated tissue, nor did it affect the SI-overflow in the presence and absence of 3-Cl,4-MeO-fendiline.

The resting overflow of ^{3}H was unaffected by any of the agents used in these experiments.

4. Discussion

The results presented in this study show that 3-Cl,4-MeO-fendiline is a very potent potentiator of $GABA_B$ receptor-mediated actions in rat brain slices, increasing the efficacy of the receptor with an EC_{50} value of 30±4.0 nM. So far, it appears to be more potent than any of the modulators active at these receptors (Urwyler et al., 2001, 2003; Kerr et al., 2002, 2004). The positive allosteric modulators 2,6-di-*tert*-butyl-4-(3-hydroxy-2,2-dimethyl-propyl)-phenol (CGP 7930) and *N*,*N*′-dicyclopentyl-2-methylsulfanyl-5-nitro-pyrimidine-4,6-diamine (GS 39783) both potentiate $GABA_B$ receptor function in a variety of biochemical and cellular assays, effective in the low micromolar concentration range in native and recombinant receptor preparations (Urwyler et al., 2001, 2003; Onali et al., 2003). Compared to 3-Cl,4-MeO-fendiline used in this study, CGP 7930 or GS 39784 is about 10- to 100-fold less potent. 3-Cl,4-MeO-fendiline did not induce any hyperpolarizing response in the absence of the $GABA_B$ receptor agonist baclofen; yet, it potentiated responses to baclofen and produced a leftward shift of the baclofen concentration–response curve, with a marked increase in the maximal hyperpolarization obtained with baclofen alone, indicative of positive allosteric modulation at these receptors.

Here, the 3-Cl,4-MeO-fendiline-induced enhancement of the postsynaptic hyperpolarizing response to baclofen was concentration-dependent, and in the presence of the highest concentration of 3-Cl,4-MeO-fendiline (60 nM), the potency of baclofen increased ninefold. Moreover, the potentiated responses induced by 3-Cl,4-MeO-fendiline on baclofen were depressed by the $GABA_B$ receptor antagonist Sch 50911 (Ong et al., 1998), and therefore dependent on activation and modulation of $GABA_B$ receptors in the neocortex. Furthermore, the potentiating actions of 3-Cl,4-MeO-fendiline were selective to $GABA_B$ receptors since it did not modulate $GABA_A$ receptor-mediated responses induced by GABA itself.

$GABA_B$ receptor agonists, such as baclofen interact with presynaptic $GABA_B$ autoreceptors, inhibiting electrically evoked [^{3}H]GABA, or with presynaptic $GABA_B$ heteroreceptors, reducing the release of [^{3}H]glutamic acid from rat brain slices. The $GABA_B$ receptor antagonist, Sch 50911, increases markedly the release of GABA, but has no effect on the release of glutamic acid (Parker et al., 2004). The action of Sch 50911 on release of GABA is attributed to its inhibition of $GABA_B$ autoreceptors responsible for regulating release of the transmitter, whereas its lack of effect on glutamic acid release reflects its inactivity on $GABA_B$ heteroreceptors.

In the present experiments, 3-Cl,4-MeO-fendiline alone had no effect on the release of [^{3}H]GABA or [^{3}H]glutamic acid; however, in the presence of baclofen, at a concentration that did not inhibit the release of either GABA or glutamic acid, these agents together inhibited the stimulation-induced release of both transmitters. Since Sch 50911 reversed these effects, it is proposed that 3-Cl,4-MeO-fendiline potentiated the agonistic effects of baclofen on the release of GABA and glutamic acid through its actions on presynaptic autoreceptors and heteroreceptors, respectively.

3-Cl,4-MeO-fendiline is far more potent than the other arylalkylamine analogues related to fendiline and its derivatives in enhancing $GABA_B$ receptor-mediated hyperpolarizations (Kerr et al., 2002, 2004). The compound originally developed by Nemeth et al. (1998), *N*-(3-phenylpropyl)-α-methyl-3-methoxybenzylamine (NPS 467), although an effective Ca^{2+}-sensing receptor modulator, is found to be

less potent than 3-Cl,4-MeO-fendiline as a modulator of $GABA_B$ receptors; NPS 467 has an EC_{50} value of 0.6 μM (Kerr et al., 2004), as compared to an EC_{50} value of 30 nM for 3-Cl,4-MeO-fendiline in the current experiments. Modification of just the *N*-α-methylbenzyl moiety of fendiline effectively alters the modulatory activity at $GABA_B$ receptors, increasing its potentiating potency, yet with less action at Ca^{2+}-sensing receptors. For instance, *N*-[3,3-diphenylpropyl)-α-methyl-3-methoxybenzylamine (F551), which incorporates a 3-*O*-methyl substituent on the phenyl ring of the α-methylbenzyl moiety although having modulatory activity on postsynaptic $GABA_B$ receptors (Kerr et al., 2002), is found to be more prone to stimulate neuronal Ca_{2+}-sensing receptors and is less potent than 3-Cl,4-MeO-fendiline. The latter is the most potent potentiator of its class at $GABA_B$ receptors so far found, since the corresponding 3,4-dichloro, 3,4-dimethoxy, or 3,4-methylenedioxy derivatives are all less active, with potencies ranging between 10 μM and 100 nM (Kerr et al., unpublished data).

In general, the arylalkylamines allosterically modulate Ca^{2+}-sensing receptors by an action at the seven-transmembrane domain (Hammerland et al., 1998). Given that there is a degree of homology between Ca^{2+}-sensing receptors and $GABA_B$ receptors, at the junction of the extracellular region of TM7 helix and the third extracellular loop, it is therefore not surprising that the arylalkylamines act at both receptor types; this is particularly true of the $GABA_{(B2)}$ subunit, where the arylalkylamines might be expected to act. In a recent study, it has been reported that the possible site of action of the positive allosteric modulator CGP 7930 is on the heptahelical domain of $GABA_{(B2)}$ subunit which is directed activated by CGP 7930 (Binet et al., 2004). In this scenario, the $GABA_{B2}$ subunit modulates the $GABA_{(B1)}/GABA_{(B2)}$ heterodimer, but whether 3-Cl,4-MeO-fendiline might also act in this way, as a $GABA_B$ receptor modulator, remains to be seen.

In conclusion, 3-Cl,4-MeO-fendiline has been shown to modulate both pre- and postsynaptic $GABA_B$ receptors in rat brain slices. Since by nature, the pre- or postsynaptic receptor activities differ in their effector mechanisms, it is unlikely that such modulatory actions induced by 3-Cl,4-MeO-fendiline are mediated through downstream signaling cascades that would differ between the two receptor subtypes. Thus, the possible effects of 3-Cl,4-MeO-fendiline on these receptors are more likely to be due to an increase in G-protein coupling, as a result of an action at the seven-transmembrane region. Nevertheless, 3-Cl,4-MeO-fendiline is by far the most potent $GABA_B$ receptor enhancer found to date.

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