**ABSTRACT**

*N,N’-Dicyclopentyl-2-methylsulfanyl-5-nitro-pyrimidine-4,6-diamine (GS39783) and Structurally Related Compounds: Novel Allosteric Enhancers of γ-Aminobutyric Acid\(_B\) Receptor Function*

The receptors for the major inhibitory neurotransmitter in the central nervous system, GABA, are subdivided into ionotropic GABA\(_A\) receptors and metabotropic GABA\(_B\) receptors. Whereas GABA\(_A\) receptors form a chloride-permeable ion channel that elicits short-lasting inhibitory postsynaptic potentials, GABA\(_B\) receptors are G-protein coupled receptors (GPCRs) that inhibit cyclic AMP formation and modulate the activity of inwardly rectifying potassium channels and voltage-sensitive calcium channels. By these mechanisms, they act post- and presynaptically to inhibit neuronal excitability (by producing a late, long-lasting component of inhibitory postsynaptic potentials) and neurotransmitter release, respectively. Whereas benzodiazepines are well established positive allosteric modulators of GABA\(_A\) receptor function, the first examples of such allosteric enhancers for GABA\(_B\) receptors have only recently been described (Urwyler et al., 2001). As a therapeutic principle, positive modulators are expected to have several advantages over compounds acting as agonists, because they are only effective in the presence of the endogenous ligand and therefore act in line with physiological neurotransmission in its temporal and spatial organization. Agonists, on the other hand, activate receptors independently of synaptic activity, possibly leading to unwanted side effects. For these reasons, the search for pos-

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itive modulators not only for ionotropic receptors but also for GPCRs has recently attracted considerable interest (Pin et al., 2001; Christopoulos, 2002; Christopoulos and Kenakin, 2002). In this study, we present a novel class of allosteric enhancers of GABAB receptors, GS39783 and some of its analogs (Fig. 1). Furthermore, we also provide some new insights into the molecular and physiological mechanisms of allosteric GABAB receptor modulation.

**Materials and Methods**

Culture and the preparation of membranes of CHO cell clones stably expressing human GABAB1b/rat GABAB2 were done as described in detail previously (Urwyler et al., 2001). The preparation of rat brain cortical membranes for native receptor assays was also performed as described previously (Olpe et al., 1990).

**[^35S]GTPγS Assay.** The composition of the assay mixtures (in a final volume of 250 μl in 96-well clear-bottom microtiter plates, Isoplates; PerkinElmer Wallac, Gaithersburg, MD) was as follows: 50 mM Tris-HCl buffer, pH 7.7, 10 mM MgCl2, 0.2 mM EGTA, 2 mM CaCl2, 100 mM NaCl, 10 μM guanosine 5′-diphosphate (30 μM with rat cortical membranes; Sigma-Aldrich, St. Louis, MO), membrane suspension (approximately 10–20 μg of protein), 1.5 mg wheat germ agglutinin-coated SPA beads (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK), 0.3 nM[^35S]GTPγS (ca. 1,000 Ci/mmol, stabilized solution; Amersham Biosciences UK, Ltd.), and the test compounds at the appropriate concentrations. Nonspecific binding was measured in the presence of unlabeled GTPγS (10 μM; Sigma Chemical, Buchs, Switzerland). The samples were incubated at room temperature for 60 min, before the SPA beads were sedimented by centrifugation at 2,600 rpm for 10 min. The plates were then counted in a 1450 Microbeta liquid scintillation counter (PerkinElmer Wallac). For data analysis, nonspecific binding was subtracted from all the other values; the effects of GABA and modulators were expressed relative to basal activity, measured in the absence of agonist. Concentration–response curves were analyzed by nonlinear regression. Prism 3.0 software (GraphPad Software Inc., San Diego, CA) was used for all data calculations.

**Radioligand Binding Experiments.** The procedure to measure the binding of [3H]CGP62349 to rat cortical membranes was based on that described by Bittiger et al. (1996); it was, however, conducted in the SPA format. The assay mixture in a final volume of 250 μl contained 20 mM Tris-HCl buffer, pH 7.4, 118 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 5 mM D-glucose, 1 nM[^3H]CGP62349, the test compounds at the desired concentrations, rat cortical membranes (ca. 15 μg of protein), and 1.5 mg of wheat germ agglutinin-coated SPA beads (Amersham Biosciences UK, Ltd.). Nonspecific binding was assessed in the presence of 5 μM CGP56999A. The samples were incubated for 90 min at room temperature, before being counted in a 1450 Microbeta liquid scintillation counter.

For kinetic experiments with the agonist radioligand[^3H]APPA (Hall et al., 1995), incubation mixtures were made up in a total volume of 25 ml in the same buffer, containing rat brain cortical membranes at the same concentration, as described above for [3H]CGP62349 binding experiments. For association experiments, the samples were preincubated for 15 min at room temperature with or without 30 μM GS39783 before the addition of [3H]APPA (3 nM). Aliquots (1 ml) were then withdrawn from the incubation mixture at different times for vacuum filtration through GF/B filters (What-

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**Fig. 1.** Chemical structures of GS39783 (center) and some of its analogs.
man, Maidstone, UK), followed by rapid rinsing with 2 × 5 ml of ice-cold incubation buffer. For dissociation experiments, the mixtures containing 3 nM [3H]APPA (with or without 30 μM GS39783) were preincubated for 40 min at room temperature to reach equilibrium, before L-baclofen (final concentration, 10 μM) was added in 1.5 ml to initiate the dissociation of the radioligand from the receptor. Aliquots (1 ml) were withdrawn from the incubation mixture at different times after the addition of L-baclofen for separating bound and free radioligand by filtration as in the association experiments. In both types of experiments, nonspecific binding was determined in the presence of 10 μM L-baclofen and measured in separate samples. The results from radioligand binding assays (equilibrium displacement and kinetic experiments) were analyzed using standard procedures and GraphPad Prism software.

Measurement of Change in Intracellular Calcium Concentration by Fluorometry. For the measurement of changes in intracellular calcium concentrations, HEK293 cells were transiently transfected with human GABAB(1a/2) or GABAB(1b/2). All transfections included Gsrα to couple GABAB receptors to PLC (Franek et al., 1999) and were made as described in detail previously (Pagano et al., 2001). Transfected HEK293 cells were plated into poly-D-lysine-coated 96-well plates (BD Biosciences, San Jose, CA). Post-transfection (24–72 h), cells were loaded for 45 min with 2 μM fluo-4 AM (Molecular Probes, Eugene, OR) in Hanks’ balanced salt solution (Invitrogen, Basel, Switzerland) containing 20 mM HEPES and 50 μM probenecid (Sigma Chemical). Plates were washed and transferred to a fluorescence imaging plate reader (FLIPR; Molecular Devices, Crawley, UK). GS39783 was added 5 min before recording. Twenty seconds after the start of the readings, 0.3 μM or 3 μM GABA was added to all wells and their fluorescence was measured for 160 s. Relative fluorescence changes over baseline (F/F) were adjusted so that the size of Ucond was 90% maximal under control conditions. Records were captured and averaged (average of four responses) using an oscilloscope (LeCroy 9430; Le Croy SA, Geneva, Switzerland) and plotted on a Hewlett Packard plotter (HP 7475). Slices were accepted for experiments if the evoked maximum orthodromic population spike during the control period was at least 6 mV and free from multiple spikes.

Chemicals. GS39783 and its analogs were synthesized in house (Fischer, 1972, 1974, 1975). Stock solutions were usually prepared in dimethyl sulfoxide and subsequently diluted in the respective assay buffers. The final dimethyl sulfoxide concentrations usually did not exceed 0.3% and did not interfere with the measured parameters. [3H]APPA (40 Ci/mmol) and [3H]CGP62349 (85 Ci/mmol) were from American Radiolabeled Chemicals (St. Louis, MO). The sources of other chemicals used are given above.

Results

GS39783 Enhances the Stimulation of [35S]GTPγS Binding by Agonists Activating Native and Recombinant GABAB Receptors. As shown in Fig. 2, GABA stimulated the binding of [35S]GTPγS to membranes from CHO cells stably expressing GABA_BD1,BD2 receptors by close to a 3-fold of basal activity at a maximally active concentration (100 μM). At 1 μM GABA, a stimulation above baseline corresponding to about 20% of the maximal GABA effect was obtained, which was strongly amplified by GS39783 (1 and 10 μM; Fig. 2). In the presence of 10 μM GS39783, the stimulation of [35S]GTPγS binding produced by 1 μM GABA exceeded the effect observed with GABA alone at a saturating concentration (100 μM). Similar amplifications of [35S]GTPγS binding were observed when APPA or L-baclofen were used as agonists instead of GABA (Fig. 2). On the other hand, in the absence of an agonist, GS39783 (1 and 10 μM) did not produce any stimulation of [35S]GTPγS binding above baseline levels (Fig. 2). Also, in the presence or in the absence of GS39783, no increase above basal levels was observed when the activation produced by GABA (1 μM) was blocked by the competitive GABAB receptor antagonist CGP56999A (10 μM; Fig. 2). In membranes from CHO cells stably expressing metabotropic glutamate receptor 1B instead of GABA_BD1,BD2 receptors, GS39783 did not enhance the stimulation of [35S]GTPγS binding produced by glutamate (data not shown).

Figure 2, bottom, also shows similar modulating effects of a number of analogs of GS39783 (Fig. 1) in the [35S]GTPγS assay. None of these compounds stimulated [35S]GTPγS binding in the absence of GABA (data not shown). Because GS39783 showed the most marked effects of them all, further characterization was done with this compound only.

Figure 3 shows concentration-response curves for the enhancing effect of GS39783 at two different fixed concentra-
tions of GABA (1 and 20 μM). It can be seen that the amplifying effect of GS39783 is observed with both recombinant (top) and native (bottom) GABAB receptor preparations, with very similar EC50 values in the low micromolar range (Fig. 3; Table 1).

GS39783 Enhances Both Agonist Affinity and Efficacy at Recombinant GABA<sub>1b/2</sub> Receptors in the [35S]GTPγS Assay. Concentration-response curves for GABA at different fixed concentrations of GS39783 reveal that a dual mechanism of GABA<sub>1b/2</sub> receptor modulation results in an increase of both the potency (maximally about an 8-fold increase) as well as the maximal intrinsic efficacy (about a 2.2-fold increase) of GABA. These effects are dependent on the concentration of the positive modulator GS39783 (Fig. 4; Table 2). The 8-fold increase in the potency of GABA produced by GS39783 represents the effect of a maximally active concentration of the modulator, compared with a control obtained with GABA alone. On the other hand, it is not possible to measure a potency of GS39783 in the absence of GABA, because the compound has no effect on its own. Therefore, the effects of GS39783 on GABA potency (Table 2) cannot be compared with the reciprocal effects of GABA on the potency of the modulator, obtained at two nonsaturating GABA concentrations (Table 1).

Effects of GS39783 on Agonist Affinities and Kinetic Rate Constants in Radioligand Binding Assays. To determine the effects of GS39783 on the affinities of different agonists for native GABA<sub>1b</sub> receptors, binding experiments with [3H]CGP62349 (a competitive antagonist radioligand) using membranes from rat brain cortex were performed. As shown in Fig. 5, displacement curves obtained with GABA, L-baclofen, and APPA revealed enhanced affinities for all three agonists in the presence of 30 μM GS39783. In all cases, a two-site model yielded a significantly better curve fit than a one-site model. As shown in Table 3, the affinities of both the high- and low-affinity components of agonist binding were increased by 30 μM GS39783. (It should be noted that the pK<sub>i</sub> value found for the high-affinity component of APPA was considerably higher than expected from that found earlier in saturation experiments (Urwyler et al., 2001). However, this is due to different assay conditions (the earlier
Effects of GS39783 on the potency and efficacy of GABA to stimulate

**TABLE 1**

Characteristics of the potentiation of the effect of GABA on \[^{35}S\]GTP\(\gamma\)S binding by GS39783

Concentration-response curves for GS39783 were measured with recombinant (expressed in CHO cells) or native receptor preparations in the presence of a lower (1 \(\mu\)M) and higher (20 \(\mu\)M) concentration of GABA, as shown in Fig. 3. The data shown are means \(\pm\) S.E.M. from three independent experiments. Maximal effects are expressed in percentage of the effect produced by a saturating concentration of GABA in the absence of GS39783. Hill coefficients were not significantly different from one in all cases.

<table>
<thead>
<tr>
<th>GABA(_b) Receptor</th>
<th>[GABA]</th>
<th>GS39783 (p)EC(_{50})</th>
<th>EC(_{50})</th>
<th>Maximal Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native (rat cortex membranes)</td>
<td>(0.3) (\mu)M</td>
<td>3.59</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>Recombinant (GABA(_{1b/2c}))</td>
<td>(0.3) (\mu)M</td>
<td>2.55</td>
<td>121 (\pm) 3*</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>(1)</td>
<td>1.71</td>
<td>153 (\pm) 11*</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>(3) (\mu)M</td>
<td>1.13</td>
<td>187 (\pm) 22*</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.54</td>
<td>218 (\pm) 18*</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.45</td>
<td>217 (\pm) 14*</td>
<td>3</td>
</tr>
</tbody>
</table>

*\(p < 0.05\); **\(p < 0.01\) (ANOVA/Dunnett’s test; one-sample t test for maximal effects relative to GABA).

Experiments were conducted in the presence of 50 \(\mu\)M GDP, which was omitted in the experiments shown here in Table 3 and Fig. 5.) Furthermore, the relative proportion of high affinity sites was slightly, but consistently increased in all experiments in the presence of the modulator. If the percentages of high-affinity site values for all three agonists were pooled (assuming that high- and low-affinity receptor states are the same regardless of the agonist used), the means were 48 \(\pm\) 0.9% in the absence and 55 \(\pm\) 1.5% in the presence of 30 \(\mu\)M GS39783 (\(p < 0.01\), \(n = 12\) in each group).

**TABLE 2**

Effects of GS39783 on the potency and efficacy of GABA to stimulate \[^{35}S\]GTP\(\gamma\)S binding to membranes from CHO cells expressing recombinant GABA\(_b\) receptors

Concentration-response curves for GABA were measured in membranes from CHO cells expressing recombinant GABA\(_b\) receptors in the absence and in the presence of different fixed concentrations of GS39783. The data shown are means \(\pm\) S.E.M. from \(n\) independent experiments. A typical experiment with the data expressed in cpm values is shown in Fig. 4.

**Fig. 4.** Concentration-response curves for GABA in the \[^{35}S\]GTP\(\gamma\)S binding assay in the absence (■) and in the presence of GS39783 (○, 0.3 \(\mu\)M; ●, 1 \(\mu\)M; △, 3 \(\mu\)M; ●, 10 \(\mu\)M; □, 30 \(\mu\)M). GABA responses were measured at recombinant GABA\(_b\) receptors using membranes from stably transfected CHO cells as described under Materials and Methods. The data shown are means \(\pm\) S.E.M. from triplicate determinations in a typical experiment. See Table 2 for a summary of the results from several independent experiments.

**Fig. 5.** Displacement of \[^{3}H\]CGP62349 from native GABA\(_b\) receptors in rat cortical membranes by GABA (squares), l-baclofen (circles), and APPA (triangles) in the absence (open symbols) and in the presence (filled symbols) of 30 \(\mu\)M GS39783. The assay was performed as described under Materials and Methods. The results shown are from a single typical experiment; the data points represent means \(\pm\) S.E.M. from triplicate determinations. A summary of the relevant parameters from several such experiments is given in Table 3.

In these experiments, the control specific binding of 1 \(nM\) \[^{3}H\]CGP62349 was somewhat lower in the presence of 30 \(\mu\)M GS39783 than in its absence (not shown); however, this inhibition was obviously not of a competitive nature. In fact, saturation experiments with the antagonist radioligand \[^{3}H\]CGP62349 revealed a slight increase in the \(K_d\) value (0.54 nM in the absence and 0.92 nM in the presence of 30 \(\mu\)M GS39783), without any change in the total number of binding sites produced by 30 \(\mu\)M GS39783 (curves not shown). This change in affinity could be calculated to fully account for the lower control binding of the radioligand in the presence of the modulator and was taken into account for the calculation of the \(K_d\) values given in Table 3.

Thus, it follows that GS39783 does not bind to the orthosteric ligand binding site and does not act by changing the total number of available GABA\(_b\) receptors.

The high agonist-affinity state of the GABA\(_b\) receptor can be directly labeled by the agonist radioligand \[^{3}H\]APPA. We made use of this possibility to determine the effects of GS39783 on the rates of association and dissociation of this agonist at native GABA\(_b\) receptors (Fig. 6). In association experiments, as expected, the level of binding once equilibrium was reached was higher in the presence of the modulator than in its absence (top, inset), due to the increased affinity for APPA. However, somewhat unexpectedly, the rate of association was slower in the presence of 30 \(\mu\)M GS39783. This effect was overcompensated by an even greater decrease in the rate of dissociation produced by the modulator, the net effect thus resulting in an increased binding affinity of \[^{3}H\]APPA, as is reflected in the \(K_d\) values, which can be calculated from the different rate constants given in Table 4.
Effects of GS39783 on the affinities of agonists for native GABA<sub>B<sub> receptors from rat cortical membranes in radioligand binding assays

The binding of [3H]CGP22349 to membranes from rat brain cortex was measured as described under Materials and Methods. Inhibition curves with the three agonists shown as displacers were constructed as illustrated in Fig. 5. Best curve fits were obtained with a two-site model in all cases. The results shown are means ± S.E.M. from four independent experiments.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>pK&lt;sub&gt;i&lt;/sub&gt; Values Control</th>
<th>pK&lt;sub&gt;i&lt;/sub&gt; Values with 30 μM GS39783</th>
<th>% High-Affinity Sites Control</th>
<th>% High-Affinity Sites with 30 μM GS39783</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High/Low Affinity</td>
<td>High/Low Affinity</td>
<td>% High-Affinity</td>
<td>% High-Affinity</td>
</tr>
<tr>
<td>GABA</td>
<td>7.43 ± 0.04</td>
<td>7.68 ± 0.07***</td>
<td>47 ± 1.6</td>
<td>56 ± 3.3*</td>
</tr>
<tr>
<td>l-Baclofen</td>
<td>7.15 ± 0.04</td>
<td>7.49 ± 0.10**</td>
<td>50 ± 1.9</td>
<td>56 ± 3.4</td>
</tr>
<tr>
<td>APPA</td>
<td>8.24 ± 0.07</td>
<td>8.56 ± 0.08**</td>
<td>48 ± 1.0</td>
<td>53 ± 1.0*</td>
</tr>
</tbody>
</table>

*p < 0.05; **p < 0.01 compared with the corresponding control values (t test).

GS39783 Augments GABA<sub>B<sub> Receptor-Mediated Calcium Signaling in a Cellular Assay. In HEK293 cells transiently transfected with human GABA<sub>B<sub>(1a/2) or GABA<sub>B<sub>(1b/2) and cotransfected with G<sub>q</sub>α1c to couple GABA<sub>B<sub> receptors to PLC, GABA elicited a transient calcium signal (Fig. 7, inset). Thus, as observed previously with CGP7930 in Xenopus oocytes (Urwyler et al., 2001), this effect of GS39783 was not dependent on the GABA<sub>B<sub>(1) receptor isoform. At a low (0.3 μM) and at a higher (3 μM) concentration of GABA, the signal was potentiated in a concentration-dependent manner by GS39783 (Fig. 7). Like in the [35S]GTPγS assay, the signal measured in the presence of GS39783 clearly exceeded the maximal effect produced by GABA on its own. Table 5 gives the corresponding EC<sub>50</sub> values and maximal effects of GS39783 under the different conditions used. GS39783, when applied in the absence of GABA, did not produce any calcium signal on its own (Fig. 7).

GS39783 Enhances the Effects of GABA on Inwardly Rectifying Potassium Channels in Xenopus Oocytes. The effects of GS39783 on the regulation of inwardly rectifying potassium channels via GABA<sub>B<sub> receptors in Xenopus oocytes is shown in Fig. 8. Exposure of the oocytes to a high potassium (90 mM) Ringer solution elicited an inward current that was reversibly amplified in the presence of GABA. The effect of a low concentration (0.3 μM, ~EC<sub>20</sub> value) of GABA was increased in the presence of GS39783 (Fig. 8, top). Upon adding 10 μM GS39783 to ascending concentrations of GABA, a left-shift in the GABA concentration-response curve (EC<sub>50</sub> = 1 μM (95% confidence interval: 0.58–1.74 μM), compared with 1.63 μM (95% confidence interval: 1.34–1.98 μM) for the control curve) and an increase in the maximal GABA response to 127 ± 5 (S.E.M.) percentage of the control value were observed (Fig. 8, bottom). No intrinsic agonistic activity of GS39783 was seen in oocytes (data not shown).

GS39783, Like l-Baclofen, Reduces Paired-Pulse Inhibition in Hippocampal Slices. In a hippocampal slice preparation, the application of two consecutive stimuli to afferent pathways results in inhibition of the second population response. In our experiments, addition of l-baclofen (1 μM) completely suppressed paired pulse inhibition (U<sub>test</sub>/U<sub>cond</sub> = 0.36 ± 0.08 for controls and 1.07 ± 0.09 in the presence of baclofen; mean ± S.E.M., n = 8, p < 0.01; recordings not shown). This effect of l-baclofen was reversed in the presence of the competitive antagonist CGP55845A (3 μM; data not shown). A similar reduction of paired pulse inhibition was produced by 10 μM GS39783 (Fig. 9); in fact, the ratio U<sub>test</sub>/U<sub>cond</sub> was 0.36 ± 0.03 in the absence and 0.90 ± 0.1 in the presence of 10 μM GS39783 (n = 4; p = 0.01). This effect was also antagonized by 3 μM CGP55845A (Fig. 9).
Effects of GS39783 on kinetic rate constants of the binding of $[^3H]$APPA to the high-affinity state of native GABA$_B$ receptors from rat cortical membranes

The association and dissociation of the binding of $[^3H]$APPA (3 nM) to membranes from rat brain cortex was measured as described under Materials and Methods. The results shown are means $\pm$ S.E.M. from three independent experiments. Typical data from a representative experiment are shown in Fig. 6.

<table>
<thead>
<tr>
<th></th>
<th>Without GS39783 (Control)</th>
<th>With 30 $\mu$M GS39783</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Association</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Observed rate constant ($k_{on}$) (min$^{-1}$)</td>
<td>0.972 $\pm$ 0.12</td>
<td>0.434 $\pm$ 0.09**</td>
</tr>
<tr>
<td>Half life (min)</td>
<td>0.73 $\pm$ 0.08</td>
<td>1.73 $\pm$ 0.33*</td>
</tr>
<tr>
<td>Corrected rate constant ($k_c$) (nM$^{-1}$ min$^{-1}$)</td>
<td>0.187</td>
<td>0.109</td>
</tr>
<tr>
<td><strong>Dissociation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rate constant ($k_{-1}$) (min$^{-1}$)</td>
<td>0.41 $\pm$ 0.03</td>
<td>0.108 $\pm$ 0.003**</td>
</tr>
<tr>
<td>Half life (min)</td>
<td>1.70 $\pm$ 0.10</td>
<td>6.44 $\pm$ 0.16**</td>
</tr>
<tr>
<td>Calculated $k_{eq}$ ($k_{-1}/k_{1}$) (nM)</td>
<td>2.2</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*p = 0.05; **p < 0.01, paired t test.

Discussion

Only recently, the first allosteric enhancers of GABA$_B$ receptor function, CGP7930 and CGP13501, have been described (Urwyler et al., 2001). Three arylalkylamine derivatives have meanwhile also been reported to enhance GABA$_B$ receptor-mediated responses in rat cortical slices (Kerr et al., 2002). However, in that report molecular mechanisms of action were not addressed. Two of these compounds were found to be totally inactive in our biochemical assays (S. Urwyler, T. Gjoni, K. Kaufmann, M. F. Pozza, and J. Mosbach, manuscript in preparation). In the present study, we introduce for the first time a novel structure, GS39783 (Fig. 1), with the pharmacological characteristics of an allosteric GABA$_B$ receptor modulator. Some analogs of this compound (Fig. 1) have also been found to amplify GABA$_B$ receptor-mediated responses; however, GS39783 was the most efficacious modulator in this series (Fig. 2).

The actions of GS39783 are very similar to those of the previously described compound CGP7930 (Urwyler et al., 2001) in $[^{35}]$GTP$\gamma$S binding and cellular assays (intracellular calcium release, effects on Kir3 channels in Xenopus oocytes). In these experiments, GS39783 had no effect without the concomitant activation of the orthosteric ligand binding site by an agonist, but potentiated the effects of agonists, and the maximal effects of GABA in the presence of the modulator exceeded those of GABA at a saturating concentration alone (Figs. 3, 4, 7, and 8). However, although the potencies of the two compounds (in terms of EC$_{50}$ values; Tables 1 and 5) are very similar, GS39783 seems to be more efficacious than CGP7930 in enhancing GABA$_B$ receptor-mediated responses. In fact, the maximal stimulation of $[^{35}]$GTP$\gamma$S binding by GABA was increased to about 200% of control at maximally active concentrations of GS39783 (Table 2), whereas with CGP7930 only about a 40% increase was obtained (Urwyler et al., 2001). On the other hand, at the highest concentration tested, GS39783 increased the potency of GABA by an 8-fold, whereas a maximally 6-fold increase was obtained previously with CGP7930. Thus, like CGP7930, GS39783 acts through a dual mechanism, by enhancing at the same time the affinity and the maximal efficacy of GABA. This mode of action seems unusual compared with that of previous examples of allosteric modulators at other GPCRs, which affect only agonist potencies but not the maximal

Table 5: Characteristics of the amplification of a GABA$_B$ receptor-mediated transient calcium signal in HEK293 cells by GS39783

<table>
<thead>
<tr>
<th>Recombinant Receptor</th>
<th>[GABA]</th>
<th>GS39783 $\mu$M</th>
<th>EC$_{50}$ $\mu$M</th>
<th>Maximal Effect</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td></td>
<td>$\log M$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GABA$_{B1a/2}\alpha$C</td>
<td>3</td>
<td>5.49 $\pm$ 0.19</td>
<td>6.3</td>
<td>130 $\pm$ 41</td>
<td>5</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td>$\log M$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GABA$_{B1b/2}\alpha$C</td>
<td>3</td>
<td>5.63 $\pm$ 0.52</td>
<td>2.4</td>
<td>213 $\pm$ 20</td>
<td>4</td>
</tr>
</tbody>
</table>

FLIPR experiments on HEK293 cells transiently transfected with human GABA$_{B1a/2}\alpha$C or GABA$_{B1b/2}\alpha$C and cotransfected with Go$_{s}\alphaq$ to couple the receptors to the PLC pathway were performed as described under Materials and Methods. Concentration-response curves for GS39783 were constructed at two different GABA concentrations as illustrated in Fig. 7 for GABA$_{B1a/2}\alpha$C. The data shown are means $\pm$ S.E.M. from n independent experiments.
responses. However, recently at least two other examples of allosteric enhancers have become known, which increase the affinities as well as the intrinsic efficacies of agonists at glutamate metabotropic glutamate receptor 1 (Knoflach et al., 2001) and adenosine A3 receptors (Gao et al., 2002). This mechanism of allosteric modulation is accounted for by a recently developed theoretical model (Hall, 2000).

The allosteric effects of GS39783 on native and recombinant GABA<sub>B</sub> receptors were further corroborated in radioligand binding experiments. The effects of the previously described GABA<sub>B</sub> receptor modulator CGP7930 on the displacement of [³H]CGP62349 by GABA in recombinant receptor preparations were found to be complex due to the lack of sensitivity of the overexpressed GABA<sub>B(1)</sub> monomer to the allosteric effects of this compound (Urwyler et al., 2001). For this reason, we have chosen to use native receptors for this type of experiment in this study. Agonist displacement curves were biphasic (Fig. 5), presumably due to the presence of GABA<sub>B</sub> receptors coupled to and uncoupled from their G proteins. In fact, it is well known that GABA<sub>B</sub> receptors, like most GPCRs, have different agonist affinities in these two different states (Hill et al., 1984; Parmentier et al., 2002). Interestingly, the affinities of both receptor forms for agonists were significantly enhanced by GS39783 (Table 3; Fig. 5). Moreover, the relative proportion of the high-affinity state was increased to some extent by GS39783, strongly suggesting that the coupling of the receptor to its G proteins was also promoted by this compound. This mechanism might well contribute to the finding that the maximal efficacy of GABA<sub>B</sub> receptor activation was enhanced by the positive modulator.

A reliable and widely used method to demonstrate and quantify allosteric effects is kinetic (nonequilibrium) radioligand binding experiments (Christopoulos and Kenakin, 2002). The rate constants of association and dissociation of an orthosteric ligand are very sensitive to changes in receptor conformation induced by an allosteric ligand. In particular, whereas the rate of association can also be decreased by conventional competitive agents, the dissociation is a zero-order reaction and is most indicative of such a conformational change induced by another agent acting at a distant site. It is the balance of the changes in kinetic association and/or dissociation rate constants that makes up the effects of allosteric modulators on orthosteric ligand affinity at equilibrium. Thus, a positive modulator could enhance agonist affinity by enhancing the rate of association or reducing the rate of dissociation of an agonist, or both. In our experiments, how-

Fig. 8. Positive modulation of the effects of GABA on inwardly rectifying potassium channels in X. laevis oocytes by GS39783. Top, typical current record of an oocyte expressing GABA<sub>B(1/2)</sub> receptors in the presence of high potassium (90 mM) Ringer solution. Control responses to GABA are shown at two different concentrations of GABA (0.3 μM (EC<sub>20</sub>) and 5.4 μM (EC<sub>90</sub>), black columns). In the presence of 3 μM GS39783 (gray column), the response to 0.3 μM GABA is enhanced. Bottom, concentration-response curves for GABA in the absence (■) and in the presence of 10 μM GS39783 (▲). GS39783 caused a left shift of the GABA concentration-response curve and an increase in the maximal response amplitude. All values were normalized to a 100 μM GABA response (Io) that was measured on every oocyte. The values shown are means ± S.E.M. from at least three different oocytes.

Fig. 9. Effect of the GABA<sub>B</sub> receptor modulator GS39783 on synaptic inhibition of extracellularly recorded pyramidal cell population spikes and its reversal by the GABA<sub>B</sub> receptor antagonist CGP55845A. The traces show typical records of pairs of evoked conditioning (U<sub>cond</sub>) and test (U<sub>test</sub>) potentials at an interstimulus interval of 20 ms. Shown are control responses in artificial cerebrospinal fluid (top trace) and responses under the influence of 10 μM GS39783 alone (middle trace) or in combination with 3 μM CGP55845A (bottom trace). The stimulus strength was identical for all records. Asterisks mark the time of stimulation. The calibration pulse has, as indicated, an amplitude of 5 mV and a duration of 10 ms.
ever, GS39783 reduced the rate of association of [3H]APP antagonists, but this effect was overcompensated by an even greater reduction in its rate of dissociation, resulting in a net increase of affinity for this agonist radioligand. It is of interest in this context that, although an increase of association rate could theoretically be the underlying mechanism of action of a positive allosteric modulator, to date no such mechanism seems to have been conclusively shown for any GPCR (Holzgrabe and Mohr, 1998; Christopoulos and Kenakin, 2002).

In a hippocampal slice preparation, the application of two consecutive stimuli to different pathways results in inhibition of the second population response, due to the activation of local inhibitory GABAergic interneurons (Alger and Nicoll, 1982). Therefore, paired-pulse inhibition provides a reliable measure of GABA receptor-mediated synaptic inhibition. Activation of presynaptic GABA_B receptors leads to a suppression of this paired pulse synaptic inhibition, most likely through inhibition of GABA release from interneurons (Deisz and Prince, 1989; Thompson and Gaehwiler, 1989). Thus, in our experiments, 1 µM l-baclofen completely suppressed paired pulse inhibition; upon coapplication of the competitive GABA_B receptor antagonist CGP55845A (Davies et al., 1993), this effect of baclofen was completely reversed (data not shown). The positive modulator GS39783, on its own, had an effect similar to that of the agonist baclofen (Fig. 9). However, because our other assays have shown that the compound does not directly activate the GABA_B receptor on its own, its suppression of paired pulse inhibition is most likely due to modulation of the effects of endogenous GABA. The finding that the competitive antagonist CGP55845A reversed the effect of GS39783 is in line with this interpretation. In fact, a competitive antagonist would not be expected to inhibit GABA_B receptor activation by a compound acting at a different site than GABA, if any agonistic activity by GS39783 were to be present.

In conclusion, GS39783 has been shown to act as a positive allosteric modulator at native and recombinant GABA_B receptors in different in vitro assay systems. A key question that remains to be answered is certainly that of whether the two structurally different positive modulators CGP7930 and GS39783 exert their effects through the same or through distinct allosteric sites on the GABA_B receptor. Parmentier et al. (2002) have recently suggested that allosteric modulators of family 3 GPCRs act through their seven transmembrane domains; however, in the case of the GABA_B receptor this hypothesis awaits experimental confirmation.

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