0026-895X/01/6005-963–971$3.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 60:963–971, 2001

Vol. 60, No. 5
1107/942258
Printed in U.S.A.

Positive Allosteric Modulation of Native and Recombinant γ-Aminobutyric Acid$_B$ Receptors by 2,6-Di-*tert*-butyl-4-(3-hydroxy-2,2-dimethyl-propyl)-phenol (CGP7930) and its Aldehyde Analog CGP13501

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Received May 15, 2001; accepted July 20, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

The compounds CGP7930 [2,6-Di-*tert*-butyl-4-(3-hydroxy-2,2-dimethyl-propyl)-phenol] and its close analog CGP13501 were identified as positive modulators of γ-aminobutyric acid$_B$ (GABA$_B$) receptor function. They potentiate GABA-stimulated guanosine 5′-*O*-(3-[^{35}S]thiotriphosphate) (GTPγ[^{35}S]) binding to membranes from a GABA$_{B(1b/2)}$ expressing Chinese hamster ovary (CHO) cell line at low micromolar concentrations and are ineffective in the absence of GABA. The structurally related compounds propofol and malonoben are inactive. Similar effects of CGP7930 are seen in a GTPγ[^{35}S] binding assay using a native GABA$_B$ receptor preparation (rat brain membranes). Receptor selectivity is demonstrated because no modulation of glutamate-induced GTPγ[^{35}S] binding is seen in a CHO cell line expressing the metabotropic glutamate receptor subtype 2. Dose-response curves with GABA in the presence of different fixed concentrations of CGP7930 reveal an increase of both the potency and maximal efficacy of GABA at the GABA$_{B(1b/2)}$ heteromer. Radioligand binding studies show that CGP7930 increases the affinity of agonists but acts at a site different from the agonist binding site. Agonist affinity is not modulated by CGP7930 at homomeric GABA$_{B(1b)}$ receptors. In addition to GTPγ[^{35}S] binding, we show that CGP7930 also has modulatory effects in cellular assays such as GABA$_B$ receptor-mediated activation of inwardly rectifying potassium channels in *Xenopus laevis* oocytes and Ca^{2+} signaling in human embryonic kidney 293 cells. Furthermore, we show that CGP7930 enhances the inhibitory effect of L-baclofen on the oscillatory activity of cultured cortical neurons. This first demonstration of positive allosteric modulation at GABA$_B$ receptors may represent a novel means of therapeutic interference with the GABAergic system.

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system. It activates two classes of receptors: ionotropic, chloride-permeable GABA$_A$ receptors and metabotropic GABA$_B$ receptors. The structure and function of GABA$_B$ receptors have been reviewed extensively (Bettler et al., 1998; Marshall et al., 1999; Bowery and Enna, 2000; Couve et al., 2000; Jones et al., 2000; Kuriyama et al., 2000; Marshall, 2000). The GABA$_B$ receptor is a member of the "family 3" G-protein-coupled receptors (GPCRs) (reviewed in Couve et al., 2000), which also comprises metabotropic glutamate receptors (mGluRs), the calcium-sensing receptor, and a group of mammalian vomeronasal and candidate taste receptors (Hoon et al., 1999). Like the other members of this family, the GABA$_B$ receptor has a molecular structure that is characterized by its seven transmembrane-spanning domains and a large extracellular N-terminal ligand binding domain related to periplasmic bacterial amino acid binding proteins. GABA$_B$ receptors modulate the activity of inwardly rectifying potassium channels and high voltage-activated calcium channels. Furthermore, they also inhibit adenylate cyclase activity in native and recombinant systems. By these mechanisms, they act post- and presynaptically to inhibit neuronal excitability and neurotransmitter release, respectively. A thorough molecular investigation of GABA$_B$ receptors was initiated by the cloning of a first receptor protein GABA$_{B(1)}$, which exists in two N-terminal splice variants, 1a and 1b (Kaupmann et al., 1997). Unexpectedly, however, heterologous expression of GABA$_{B(1)}$ receptor protein has not made possible the measurement of robust func-

ABBREVIATIONS: GABA, γ-aminobutyric acid; GPCR, G-protein-coupled receptor; mGluR, metabotropic glutamate receptor; CHO, Chinese hamster ovary; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); SPA, scintillation proximity assay; CGP7930, 2,6-di-*tert*-butyl-4-(3-hydroxy-2,2-dimethyl-propyl)-phenol; APPA, 3-aminopropylphosphinic acid; HEK, human embryonic kidney; HBSS, Hanks' balanced salt solution; FLIPR, fluorescence imaging plate reader.

tional responses. This finding has remained unexplained until the discovery that the formation of heterodimeric assemblies between $GABA_{B(1)}$ and a novel $GABA_{B(2)}$ protein is a prerequisite to form functional $GABA_B$ receptors (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999).

Allosteric modulation of $GABA_B$ and some mGluR receptors by calcium has been described previously (Kubo et al., 1998; Saunders et al., 1998; Wise et al., 1999; Galvez et al., 2000a). The calcium sensing receptor is, in turn, allosterically activated by amino acids (Conigrave et al., 2000). Furthermore, noncompetitive inhibitors of "group I" mGluRs acting at a site distinct from the agonist binding site have also been found (for reviews, see Pin et al., 1999; Spooren et al., 2001). However, no allosteric modulation of $GABA_B$ receptor activity by low-molecular-weight organic compounds has been observed to date. This study describes two molecules with such effects, CGP13501 and CGP7930 (Fig. 1). Positive allosteric modulators act synergistically with an agonist, but have no intrinsic efficacy on their own. Thus, they act only when and where the endogenous agonist is present and thus have more physiological effects than pure agonists, which activate receptors independently of synaptic activity. Therefore, positive allosteric modulators are expected to have a better side effect profile than conventional agonists and thus are of considerable therapeutic interest.

Materials and Methods

Stable Transfection and Culture of CHO Cell Clones. Chinese hamster ovary K1 (CHO-K1) cells were stably transfected with $GABA_{B(1b)}$ and $GABA_{B(2)}$ cDNAs. Human $GABA_{B(1b)}$ [in pcDNA3.1, (Invitrogen, Carlsbad, CA)] and rat $GABA_{B(2)}$ [in pC1-neo (Promega, Madison, WI)] constructs were cotransfected (1:1 ratio of plasmids) using the Superfect transfection system from QIAGEN AG (Basel, Switzerland). Stably transfected cell clones were selected and cultured in Dulbecco's modified eagle medium (glutamine-free Dulbecco's modified Eagle's medium; Invitrogen) supplemented with 10% fetal calf serum, 20 μg/ml L-proline, 400 μg/ml L-glutamine, 1 mg/ml geneticin, 250 μg/ml zeocin. The cells were grown to 80 to 90% confluence in 14-cm cell culture dishes. For some specificity experiments, a CHO cell line stably expressing the mGluR2 metabotropic glutamate receptor (Flor et al., 1995) was also used.

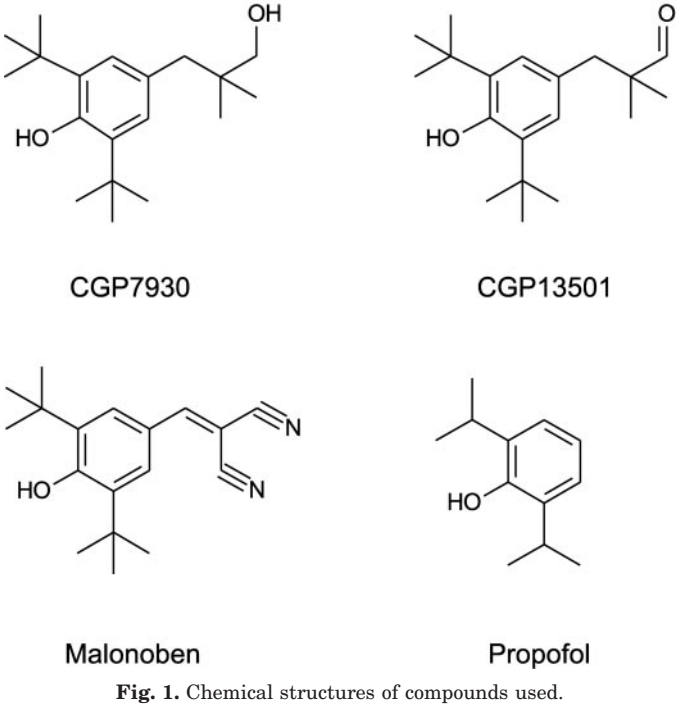

Fig. 1. Chemical structures of compounds used.

Preparation of Membranes from CHO Cells. The culture dishes were washed twice with ice-cold HEPES buffer, pH 7.4. Buffer was added and the cells were scraped off. Crude membranes from several dishes were collected in a 50-ml tube and centrifuged at 4°C for 20 min at 15,000 rpm in an SS34 rotor (Sorvall, Newton, CT). The pellet was resuspended in buffer and homogenized using a glass-glass homogenizer (10 strokes). Afterward, the suspension was centrifuged (18,000 rpm, 30 min, 4°C), and the pellet was resuspended in a small volume of buffer and homogenized again (20 strokes). Aliquots were frozen in liquid nitrogen and stored at −80°C. On the day of the experiment, the frozen membranes were thawed and then centrifuged for 10 min at 15,000 rpm and 4°C. The pellet was resuspended in 1 ml of ice-cold distilled water and incubated for 1 h on ice. After a further centrifugation as before, the final pellet was resuspended in the appropriate amount of assay buffer (see below).

Preparation of Rat Brain Membranes for Native Receptor Assays. Membranes from rat brain cortex were prepared as described in detail earlier (Olpe et al., 1990).

GTPγ[^{35}S] Assay. The composition of the assay mixtures [in a final volume of 250 μl in 96-well, clear-bottomed microtiter Isoplates (PerkinElmer Wallac, Turku, Finland)] was as follows: 50 mM Tris-HCl buffer, pH 7.7, 10 mM $MgCl_2$, 0.2 mM EGTA, 2 mM $CaCl_2$, 100 mM NaCl, 10 μM guanosine 5′-diphosphate (30 μM with rat cortical membranes; Sigma Chemical, Buchs, Switzerland), 50 μl of the membrane suspension described above (approximately 10–20 μg of protein), 1.5 mg of wheat germ agglutinin-coated SPA beads (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK), 0.3 nM [^{35}S]GTPγS (~1000 Ci/mmol, stabilized solution; Amersham Pharmacia Biotech), and the test compounds (agonists and/or modulators) at the appropriate concentrations. Nonspecific binding was measured in the presence of unlabeled GTPγS (Sigma) in excess (10 μM). The samples were incubated at room temperature for 60 min before the SPA beads were sedimented by centrifugation at 2600 rpm for 10 min. The plates were then counted in a Wallac 1450 MicroBeta liquid scintillation counter. 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, San Diego, CA) was used for all data calculations.

Radioligand Binding Experiments. The protocols for measuring the binding of the radioligands [^{3}H]CGP62349 (a competitive antagonist) and [^{3}H]APPA ([^{3}H]CGP27492, an agonist ligand) were based essentially on methods described previously (Olpe et al., 1990; Hall et al., 1995; Bittiger et al., 1996). The [^{3}H]CGP62349 binding assay was performed in the SPA format; in the [^{3}H]APPA binding assay, bound and free radioligand were separated by centrifugation. Saturation and displacement curves were analyzed by nonlinear curve fitting to the appropriate models and using Prism 3.0 software.

Measurement of Change in Intracellular Calcium Concentration by Fluorometry. For measurement of changes in intracellular calcium concentrations, HEK293 cells were transiently transfected with $GABA_{B(1b/2a)}$. All transfections included $G\alpha_{qo5}$ to couple $GABA_B$ receptors to phospholipase C (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). Twenty-four to seventy-two hours after transfection, cells were loaded for 45 min with 2 μM fluo-4 AM (Molecular Probes, Eugene, OR) in HBSS (Invitrogen) containing 50 μM probenecid (Sigma). Plates were washed twice in the incubation buffer (HBSS) and transferred to a fluorescence imaging plate reader (FLIPR; Molecular Devices, Sunnyvale, CA). Fluorescence was measured at room temperature for 3 min after the

addition of CGP7930 to check for agonistic effects of the compound. A second recording period of 3 min was initiated 10 min after the start of the first measurement. CGP7930 was present from the start, and 1 μM GABA in HBSS was added at 20 s after the start of the second reading. Relative fluorescence changes over baseline ($\Delta F/F$) were determined. Concentration-response curves were recorded with three to eight wells per concentration and experiment; the data were pooled and fitted using Igor Pro (Wavemetrics, Lake Oswego, OR) with a logistic equation using weighted nonlinear regression.

FLIPR Experiments on Neuronal Networks. Primary cultures of cortical neurons were prepared from embryonic day 16 to 18 Sprague-Dawley rats (Wang and Gruenstein, 1997). Dissociated cells were plated on poly-L-lysine coated plates and incubated at 37°C in 5% CO_2 for 7 to 10 days. About 15 min before experiments, the culture medium was removed and cells were loaded with 2 μM fluo-4 AM in HBSS supplemented with 10 mM HEPES, pH adjusted to 7.4. After loading, cells were washed twice in the incubation medium (HBSS without Mg^{2+}) and then transferred to the fluorescence reader. Fluorescence was measured at room temperature and at a sampling rate of 0.5 Hz. Drugs were dissolved in HBSS without Mg^{2+} and added to the cultures during recording. Oscillations were analyzed using IgorPro by peak detection and calculation of the ratio of peak frequencies before and after compound addition.

Oocyte Electrophysiology. Experiments were performed as described earlier (Lingenhoehl et al., 1999). Briefly, lobes of oocytes were removed surgically from anesthetized (1.2 g/l MS222) female *Xenopus laevis* frogs. Oocytes were separated and defolliculated and injected with 10 to 50 ng of rat $GABA_{B(1a)}$ (or $GABA_{B(1b)}$) together with $GABA_{B(2)}$ and rat Kir3.1, 3.2, and 3.4 coding mRNAs and incubated at 18°C for 3 to 8 days. Two-electrode voltage clamp recordings were done with electrodes filled with 3 M KCl. Oocytes were continuously perfused with normal frog Ringer solution (115 mM NaCl, 10 mM HEPES, 2.5 mM KCl, 1.8 mM $CaCl_2$, pH 7.2) or high-potassium Ringer solution (90 mM KCl, 27.5 mM NaCl, 10 mM HEPES, 1.8 mM $CaCl_2$, pH 7.2). Recordings were performed at a clamp potential of −70 mV. To test the positive modulatory activity of CGP7930, the compound was applied with ascending concentrations and a fixed GABA concentration.

Chemicals. CGP7930 and CGP13501 were synthesized in house. Propofol and malonoben were from Tocris Cookson Ltd. (Bristol, UK). Stock solutions of these compounds were prepared in dimethyl sulfoxide and subsequently diluted in the respective assay buffers. The final concentrations of dimethyl sulfoxide in the various assays usually did not exceed 0.3% and did not interfere with the measured parameters. [^{3}H]APPA ([^{3}H]CGP27429, 50 Ci/mMol) and [^{3}H]CGP62349 (85 Ci/mMol) were obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO).

Results

CGP7930 and CGP13501 Positively Modulate Recombinant and Native $GABA_B$ Receptor Activity in a GTPγ[^{35}S] Binding Assay. The stimulation of GTPγ[^{35}S] binding is a widely used functional assay for GPCRs. GABA stimulated GTPγ[^{35}S] binding in membranes from CHO cells stably expressing $GABA_{B(1b/2)}$. The maximal stimulation obtained with a saturating concentration (100 μM) corresponded to a 2- to 3-fold of the basal activity measured in the absence of an agonist (Fig. 2). This effect of GABA was mediated via $GABA_B$ receptors, because it was blocked by the competitive $GABA_B$ receptor antagonist CGP56999A (Fig. 2C) and it was not observed in membranes from CHO cells that had not been transfected with $GABA_B$ receptor cDNA (not shown). The compounds CGP7930 and its aldehyde analog CGP13501 (Fig. 1) were found to substantially increase the effects of different GABA concentrations (Fig. 2, A and B). Similarly, CGP7930 increased the agonistic effect of L-baclofen (not shown). The compounds propofol and malonoben (Fig. 1), which are closely related chemically, had no such effects (Fig. 2, A and B). Propofol (2.5 μM and 25 μM) also did not antagonize the effects of CGP7930 (2.5 μM and 25 μM, not shown). CGP7930 and CGP13501 produced little or no stimulation of GTPγ[^{35}S] binding in the absence of GABA or when the effect of GABA was blocked by a competitive antagonist (Fig. 2C). They also did not potentiate glutamate-induced GTPγ[^{35}S] binding in membranes from CHO cells expressing the mGluR2 metabotropic glutamate receptor (Fig. 3).

To characterize the positive modulatory effects in more detail, further experiments were performed with the more active compound CGP7930. To evaluate whether CGP7930 also acts on native $GABA_B$ receptors, GTPγ[^{35}S] binding studies on membranes from rat brain cortex were performed. The addition of GABA to this preparation stimulated GTPγ[^{35}S] binding; the stimulation could be inhibited by well-established $GABA_B$ receptor antagonists (data not shown). The effect of GABA was again potentiated by CGP7930 (Fig. 4, bottom). Concentration-response curves, established with native or recombinant receptor preparations at fixed concentrations of GABA (1 μM and 20 μM), revealed EC_{50} values for CGP7930 in the low micromolar range (Fig. 4, Table 1). The EC_{50} value for CGP7930 was similar for recombinant and native receptors (Table 1).

$GABA_B$ Receptor Modulation Is Mediated via an Increase in Both Agonist Affinity and Efficacy. Concentration-response curves for GABA at different fixed concentrations of CGP7930 revealed a dual mechanism of recombinant $GABA_B$ receptor modulation, involving an increase of agonist potency as well as of maximal efficacy (Fig. 5, Table 2).

An increase of agonist affinity induced by CGP7930 also became apparent in radioligand binding assays. In saturation experiments with the agonist radioligand [^{3}H]APPA, labeling native $GABA_B$ receptors in rat cortical membranes, 30 μM CGP7930 produced an increase in affinity, without a change in the B_{max} value (Fig. 6, top; Table 3). The displacement of [^{3}H]CGP62349 (a competitive antagonist) from recombinant $GABA_B$ receptors by GABA revealed a more complex situation (Fig. 6, bottom). When membranes from cells expressing the $GABA_{B(1b/2)}$ heterodimer were used, the displacement curves were biphasic, with a minor high-affinity component and a major low-affinity component (Fig. 6, bottom; Table 4). The modulator CGP7930 increased the affinity of GABA for the minor component, whereas the remaining part of the displacement curve was unchanged in the presence or absence of this compound. The biphasicity of these curves was presumably caused by the presence of overexpressed monomeric $GABA_{B(1b)}$ subunits in addition to $GABA_{B(1b/2)}$ heterodimers (see the discussion section). In fact, in membranes from cells expressing the $GABA_{B(1b)}$ subunit alone, only a single component with low affinity for GABA was detected, which was not influenced by CGP7930. The inset in the bottom panel of Fig. 6 also shows that CGP7930 did not displace the competitive antagonist radioligand directly from the agonist recognition site on the $GABA_B$ receptor.

Positive Modulation of $GABA_B$ Receptors by CGP7930 Does not Differentiate $GABA_{B(1)}$ Splice Vari-

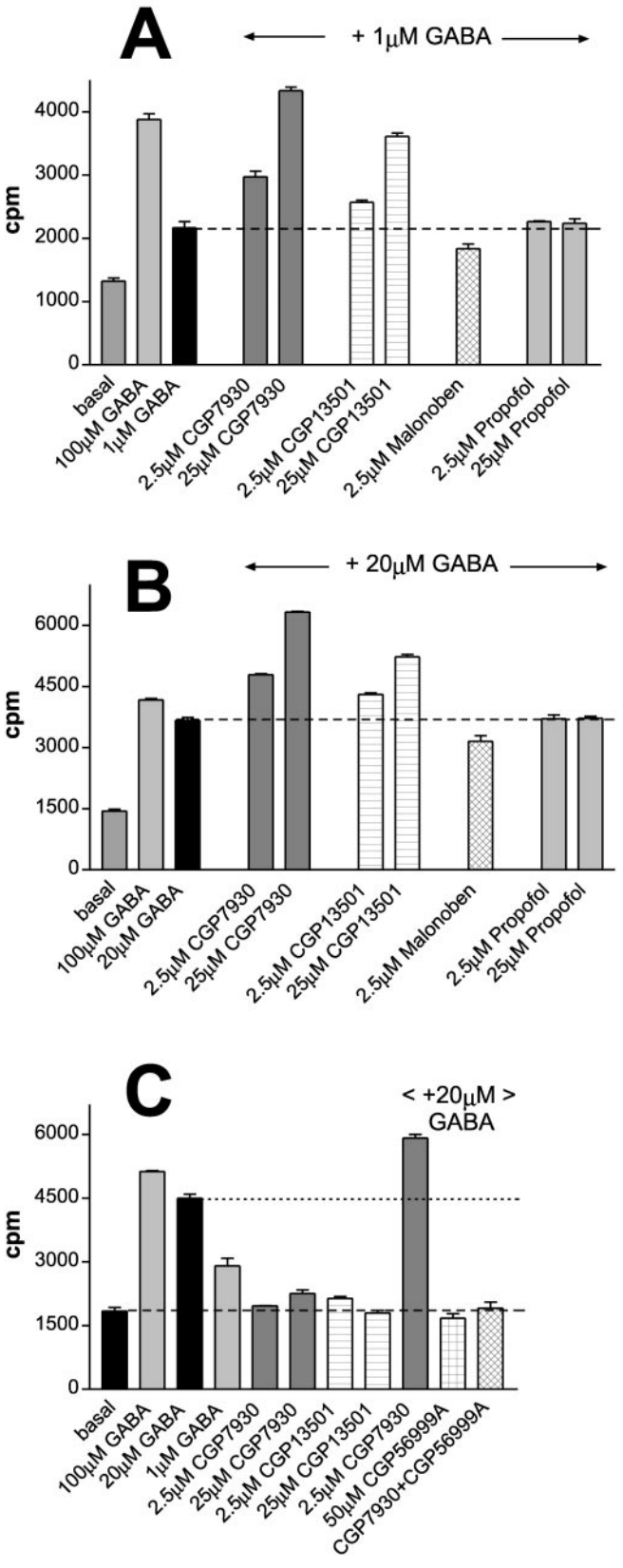

Fig. 2. Potentiation of the GABA-induced stimulation of GTPγ[^{35}S] binding via $GABA_{B(1b/2)}$ expressed in CHO-K1 cells. The effects of the four compounds shown in Fig. 1 on GTPγ[^{35}S] binding to membranes from stably transfected CHO cells were measured in the presence of a low (1 μM, A) or a high (20 μM, B) concentration of GABA, and in the absence of GABA or in the presence of a competitive antagonist (CGP56999A, C), as described under *Materials and Methods*. The respective control levels are shown by black columns and broken lines. Basal levels and maximally active GABA (100 μM) effects, as well as the effect of a low (1 μM) concentration of GABA in C, are also shown for reference. The data shown are from a typical experiment, performed in quadruplicate, and expressed as mean cpm values with S.E.M. At 25 μM, malonoben produced a strong inhibition of GTPγ[^{35}S] binding (not shown), which was, however, probably caused by color-quenching effects.

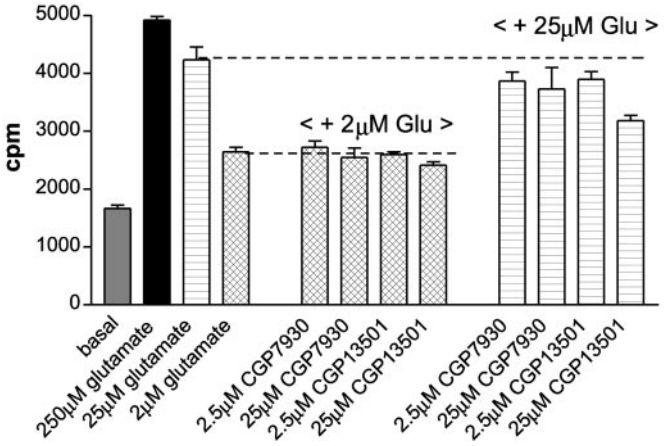

Fig. 3. Lack of effect of CGP7930 and CGP13501 on L-glutamate-induced stimulation of GTPγ[^{35}S] binding in membranes from CHO cells stably expressing the human mGluR2 receptor. The GTPγ[^{35}S] assay was performed as described under *Materials and Methods*. The data shown are from a typical experiment, performed in quadruplicate, and expressed as mean cpm values with S.E.M. GABA, alone or in combination with CGP7930, did not stimulate GTPγ[^{35}S] binding in these cells (not shown).

ants 1a and 1b. The effects of CGP7930 on the regulation of inwardly rectifying potassium channels via $GABA_B$ receptors in *Xenopus laevis* oocytes are shown in Fig. 7. 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) of GABA was increased in the presence of CGP7930; the current traces obtained during the preincubation with CGP7930 clearly show that this compound had no effect on its own. The positive modulation produced by CGP7930 was observed with both $GABA_B$ receptor subunit combinations, $GABA_{B(1a/2)}$ and $GABA_{B(1b/2)}$. The effect of CGP7930 was reversible, because upon washout, the peak size was near control levels after about 15 min (data not shown). The EC_{50} value of CGP7930 in this assay was approximately 1 μM, similar to the values obtained in GTPγ[^{35}S] experiments with recombinant receptors.

Effects of CGP7930 on $GABA_B$ Receptors in Intact HEK293 Cells. HEK293 cells were transiently transfected with $GABA_{B(1/2)}$ and the $G\alpha_{qo5}$ G-protein subunit. CGP7930 concentration-dependently increased a transient Ca^{2+} signal induced by 1 μM GABA (Fig. 8). The pEC_{50} value for CGP7930 in this assay was 5 ± 0.04. CGP7930, up to 30 μM, added during the preincubation phase, elicited no calcium signal on its own.

CGP7930 Reduces Calcium Oscillations in Rat Cortical Neuron Primary Cultures. Dissociated rat cortical neurons in primary culture form synaptically connected networks. Removal of Mg^{2+} from the incubation medium elicits synchronized calcium oscillations in these neurons (Fig. 9A; Wang and Gruenstein, 1997). The $GABA_B$ receptor agonist L-baclofen (3 μM) reduced the firing frequency in this neuronal network (Fig. 9B), an effect that was reversed by the competitive antagonist CGP54626A (Fig. 9C). At a low con-

centration (0.3 μM), at which it had no effect on its own, CGP7930 increased the effect of 3 μM L-baclofen (Fig. 9, D and E).

Discussion

This study describes for the first time the identification of low-molecular-weight organic compounds that act as positive allosteric modulators at $GABA_B$ receptors in a native environment (rat brain membranes, neuronal cultures) or in recombinant expression systems (stably or transiently transfected mammalian cell lines, *X. laevis* oocytes).

The compound CGP7930, structurally close to the general anesthetic propofol, and its aldehyde analog CGP13501 potentiated GABA-induced signals in a functional receptor test (GTPγ[^{35}S] binding), using membranes from CHO cells stably expressing the $GABA_B$ receptor (Fig. 2). The findings that these signals exceeded the response elicited by a maximally active concentration of GABA alone and that these two compounds did not stimulate GTPγ[^{35}S] binding in the absence of GABA to any relevant extent clearly show that they acted as positive modulators, without intrinsic agonistic activity. In GTPγ[^{35}S] experiments, but not in the other assays, a very marginal effect was seen with the modulators alone (Fig. 2C) that might, however, be due to a small constitutive activity of a part of the receptor population. The modulatory effects of CGP7930 and CGP13501 were $GABA_B$ receptor selective because they were not observed in the same host cells (CHO-K1) expressing mGluR2, which couples to the same G-proteins (G_o/G_i) as the $GABA_B$ receptor (Fig. 3). This finding also strongly suggests that the modulators affect the $GABA_B$ receptor itself, rather than the G-protein or the membrane. Moreover, chemical specificity of these effects is also indicated by the fact that the two structurally related compounds malonoben and propofol were without effect in this assay. This is interesting insofar as propofol acts as a general anesthetic by a mechanism that involves positive modulation at the ionotropic $GABA_A$ receptor (Hales and Lambert, 1991). Propofol differs from the two active compounds in that it has two isopropyl- instead of *t*-butyl substituents in positions 2 and 6 and lacks a further side chain in position 4. This side chain apparently has to fulfill relatively stringent structural requirements, because a rather small difference between CGP7930 and CGP13501 (an alcohol instead of an aldehyde function in the terminal position of the side chain) conferred a more pronounced modulatory activity to the former compound. Also, malonoben with its more different side chain was completely inactive in our experiments.

Concentration-response curves established with CGP7930 in the presence of fixed GABA concentrations revealed micromolar potencies (EC_{50} values) at recombinant and native $GABA_B$ receptors (Fig. 4). On the other hand, when concentration-response curves were measured for GABA at different fixed concentrations of CGP7930 (Fig. 5), it became evident that the modulator simultaneously increased the potency and the maximal activity of GABA. Such dual effects are unusual for allosteric enhancers at GPCRs and ionotropic receptors. For example, benzodiazepines modulate $GABA_A$ receptors by enhancing GABA responses only at subsaturating, not at maximally active, GABA concentrations (Choi et al., 1981). Similarly, brucine and some analogs thereof act as allosteric muscarinic receptor modulators by increasing agonist potency in radioligand binding and functional assays without affecting the maximal response (e.g., in GTPγ[^{35}S] experiments) (Lazareno et al., 1998; Birdsall et al., 1999). Whereas these effects can be described by a ternary allosteric

TABLE 1

Characteristics of the potentiation of the effect of GABA on GTPγ[^{35}S] binding by CGP7930

Concentration-response curves for CGP7930 were measured with recombinant or native receptor preparations in the presence of a low (1 μM) and a higher (20 μM) concentration of GABA, as shown in Fig. 4 and as described under *Materials and Methods*. The data shown are means ± S.E.M. from *n* independent experiments.

Receptor Source	GABA	CGP7930 EC_{50}	Hill coefficient	*n*
	μM	*μM*		
Native (rat cortex membranes)	1	5.37 ± 1.02	1.2 ± 0.15	6
	20	3.65 ± 0.70	1.5 ± 0.11	6
Recombinant (expressed in CHO cells)	1	4.60 ± 0.83	1.00 ± 0.08	3
	20	1.87 ± 0.10	1.29 ± 0.12	3

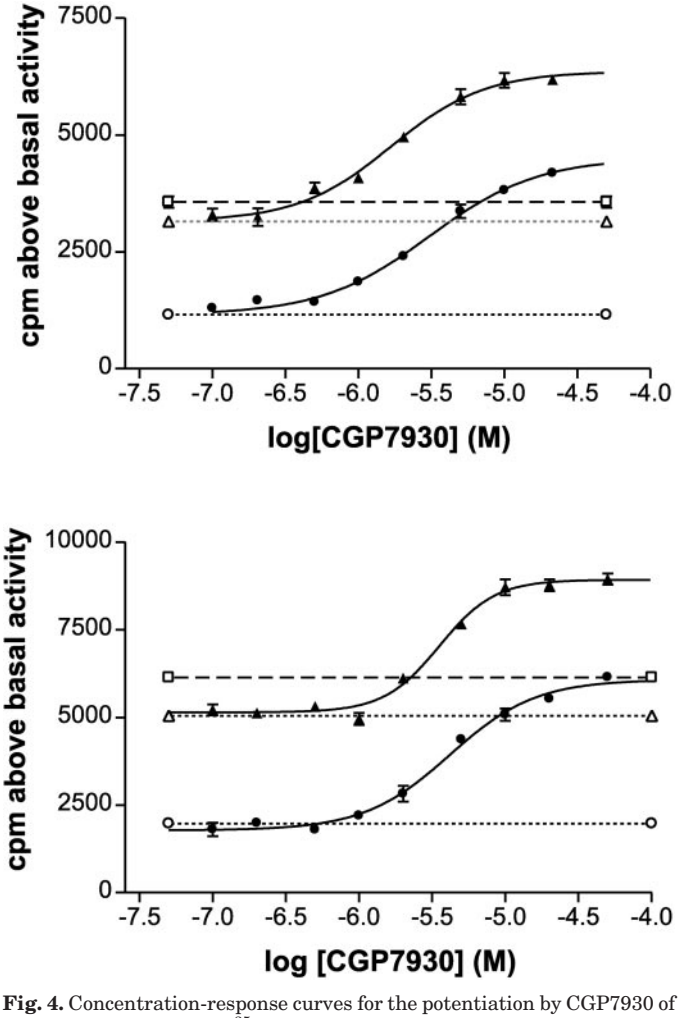

Fig. 4. Concentration-response curves for the potentiation by CGP7930 of GABA-stimulated GTPγ[^{35}S] binding to membranes from CHO cells stably expressing $GABA_B$ receptors (top) or to rat cortical membranes (bottom). The potentiating effect of CGP7930 was measured at two different fixed concentrations of GABA, 1 μM (●) and 20 μM (▲); the corresponding control levels, measured in the presence of GABA alone, are indicated by horizontal lines (○, 1 μM GABA; △, 20 μM GABA). The upper broken line (□) represents the level of maximal stimulation obtained by a saturating concentration (100 μM) of GABA alone. Basal activities (above nonspecific binding) in the representative experiments shown here were 1600 cpm for the recombinant and 7500 cpm for the native receptor preparation. The data are means ± S.E.M. from triplicate determinations.

model in which both the primary and allosteric ligands simultaneously bind to the receptor and reciprocally modulate their respective affinities, the situation with our $GABA_B$ receptor modulators is obviously more complex. The recently described extension of the two-state model of receptor activation (Hall, 2000) accounts for the allosteric effects of compounds that, like CGP7930, affect not only the affinity but also the intrinsic efficacy of agonists. On the other hand, the interpretation that the augmentation of the maximal stimulation obtained in our GTPγ[^{35}S] experiments reflects an increase in receptor number can be ruled out by the finding that the B_{max} value in our [^{3}H]APPA binding experiments remained unchanged in the presence of CGP7930. Also, the effects found with CGP7930 are clearly different from those described for saponin, which increases not only the maximal level of stimulation (presumably via a nonreceptor mechanism) but, unlike CGP7930, also the baseline values in GTPγ[^{35}S] assays (Cohen et al., 1996).

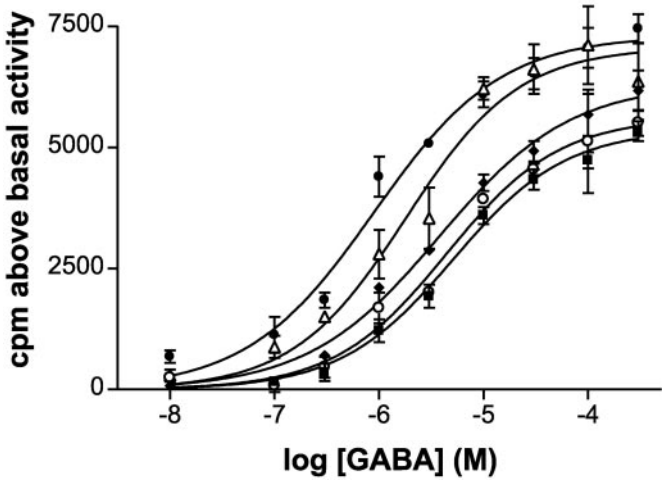

Fig. 5. Concentration-response curves for GABA in the GTPγ[^{35}S] binding assay in the absence (■) and in the presence of CGP7930 (○, 1 μM; ♦, 3 μM; △, 10 μM; ●, 30 μM). GABA responses were measured using a recombinant $GABA_B$ receptor preparation as described under *Materials and Methods*. The parameters describing the different curves are given in Table 2. Basal activity (above nonspecific binding) in the typical experiment shown here was 2065 cpm.

TABLE 2

Effects of CGP7930 on the potency and efficacy of GABA to stimulate GTPγ[^{35}S] binding in 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 the positive modulator CGP7930. Because the maximal effect of GABA alone differed between experiments, the data were normalized either to the maximal effect of GABA under control conditions or to basal activity. The data shown are means ± S.E.M. from *n* independent experiments. A typical experiment with the data expressed in cpm values is shown in Fig. 5.

CGP7930	EC_{50} GABA	Maximal effect relative to		*n*
		GABA Control	Basal Activity	
μM	μM	%		
0 (control)	4.9 ± 0.04	100	301 ± 29	3
1	3.8 ± 0.43	109 ± 6	328 ± 27	3
3	2.9 ± 0.65*	128 ± 8	377 ± 20	3
10	1.6 ± 0.11**	132 ± 17	394 ± 50*	3
30	0.8 ± 0.14**	143 ± 14*	427 ± 33**	3

Asterisks indicate significant differences from the control group (*, $p < 0.05$; **, $p < 0.01$, analysis of variance followed by Dunnett's test).

An increase of agonist potency would presumably be related to a concurrent increase in affinity, which should be detectable in radioligand binding assays. When we displaced the antagonist radioligand [^{3}H]CGP62349 with GABA, biphasic inhibition curves were obtained in membranes from CHO cells expressing $GABA_{B(1b/2)}$ heterodimers (Fig. 6, Table 4). Only the minor high-affinity component was influenced by CGP7930. The two phases of the displacement curves could well be caused by the presence of both receptors coupled to and uncoupled from G-proteins, as is known for other GPCRs. On the other hand, they could also indicate that in the stably transfected CHO $GABA_{B(1b/2)}$ cell line used,

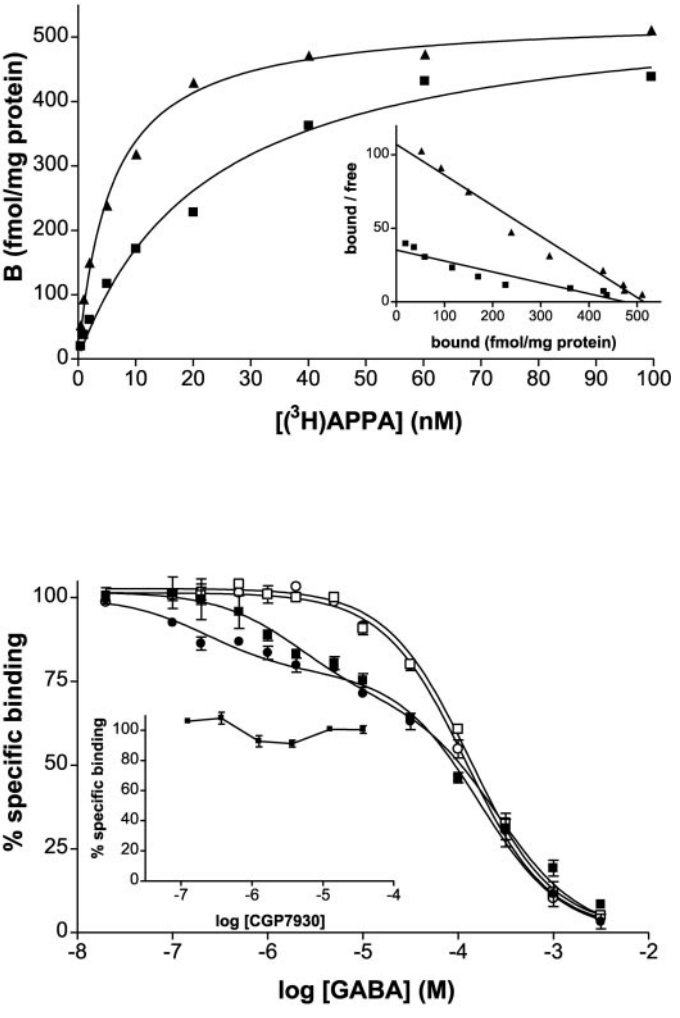

Fig. 6. Top, effect of CGP7930 on the binding of the agonistic radioligand [^{3}H]APPA to native $GABA_B$ receptors in rat cortical membranes. Saturation curves with [^{3}H]APPA were established in the absence (■) and in the presence (▲) of 30 μM CGP7930. The inset shows the corresponding Scatchard plot. The Scatchard plot is shown for display only; the relevant binding parameters were obtained through nonlinear regression to the actual saturation curve. The means of the K_D and B_{max} values from three such experiments are given in Table 3. Bottom, effect of CGP7930 on the displacement of the antagonist radioligand [^{3}H]CGP62349 by GABA in recombinant receptor preparations (filled symbols, $GABA_{B(1b/2)}$ heterodimers; open symbols, $GABA_{B(1b)}$ monomers). The binding of [^{3}H]CGP62349 to membranes from stably transfected CHO cells was measured in a scintillation proximity assay using wheat germ agglutinin-coated SPA beads. Displacement curves with different concentrations of GABA were established in the absence (squares) and in the presence (circles) of 30 μM CGP7930. Inset: Lack of a direct inhibition of [^{3}H]CGP62349 binding by CGP7930 in the concentration range in which the compound acts as a positive modulator.

the $GABA_{B(1)}$ subunit is strongly overexpressed and exists to a large extent in a monomeric form. In fact, the IC_{50} value for the predominant low-affinity component was similar to that found in experiments using cell membranes containing the $GABA_{B(1b)}$ subunit only, which was also not modulated by CGP7930. It is known that GABA and competitive antagonists bind to the $GABA_{B(1)}$ subunit and that agonist affinities are higher in $GABA_{B(1/2)}$ heterodimers compared with $GABA_{B(1)}$ monomers (Kaupmann et al., 1997, 1998; White et al., 1998). On the other hand, saturation curves with native receptors using the agonist [^{3}H]APPA, which preferentially detects the high affinity agonist site on the heteromer $GABA_{B(1/2)}$, revealed a clear increase in ligand affinity induced by the modulator (Fig. 6). It seems therefore that CGP7930 can exert its modulatory action only in $GABA_{B(1/2)}$ heterodimeric receptor assemblies, not in the $GABA_{B(1)}$ subunit alone, implying that CGP7930 either acts via $GABA_{B(2)}$ (by binding to this subunit) or at least needs the presence of $GABA_{B(2)}$ to be able to exert its effect.

Positive allosteric modulation of $GABA_B$ receptor activity was not only demonstrated in membrane preparations, but also in more complex cellular assay systems. In *X. laevis* oocytes injected with mRNA for the $GABA_B$ receptor and for inwardly rectifying (Kir 3) potassium channels, CGP7930 potentiated the effect of GABA on potassium currents (Fig. 7). These experiments were carried out with both the $GABA_{B(1a/2)}$ and $GABA_{B(1b/2)}$ subunit combinations, and similar effects were seen in both cases. Thus, the modulatory effect of CGP7930 was independent of the splice variant of the $GABA_{B(1)}$ subunit of the $GABA_B$ receptor. In transiently transfected cell lines, $GABA_B$ receptors induce a calcium signal when they are coexpressed with an appropriate chi-

TABLE 3

Effects of CGP7930 on the binding of [^{3}H]APPA to native $GABA_B$ receptors in rat cortical membranes

The binding of [^{3}H]APPA ([^{3}H]CGP27492) to rat cortical membranes was measured in the absence and in the presence of CGP7930 as described under *Materials and Methods*. The data shown are means ± S.E.M. from three independent experiments.

CGP7930	K_D	B_{max}	*n*
μM	*nM*	*fmol/mg of protein*	
0 (control)	23.4 ± 0.5	581 ± 23	3
30	8.5 ± 1.6*	624 ± 46	3

*, significantly different from the control value ($p < 0.01$, *t* test).

TABLE 4

Effect of CGP7930 on the displacement of [^{3}H]CGP62349 by GABA from recombinant heterodimeric $GABA_{B(1b/2)}$ and monomeric $GABA_{B(1b)}$ receptors

The binding of the antagonist radioligand [^{3}H]CGP62349 to membranes from stably transfected CHO cells was measured in a scintillation proximity assay using WGA coated SPA beads. The displacement of [^{3}H]CGP62349 from heterodimeric receptors by GABA was significantly better fitted by a two-site model, whereas for the $GABA_{B(1b)}$ monomer, the data were adequately fitted by a one-site model (F-test, GraphPad Prism software). The data shown are means ± S.E.M. from *n* independent experiments.

$GABA_B$ Receptor Type	CGP7930	IC_{50} GABA	High-Affinity Component	*n*
	μM		%	
Dimer (1b/2)	0 (control)	5.2 ± 1.3 356 ± 66	34 ± 3.1	4
	30	1.9 ± 1.0 (*) 291 ± 91	27 ± 4.2	4
Monomer (1b)	0 (control)	121 ± 6		3
	30	111 ± 5		3

*, $p = 0.06$ versus the corresponding control value.

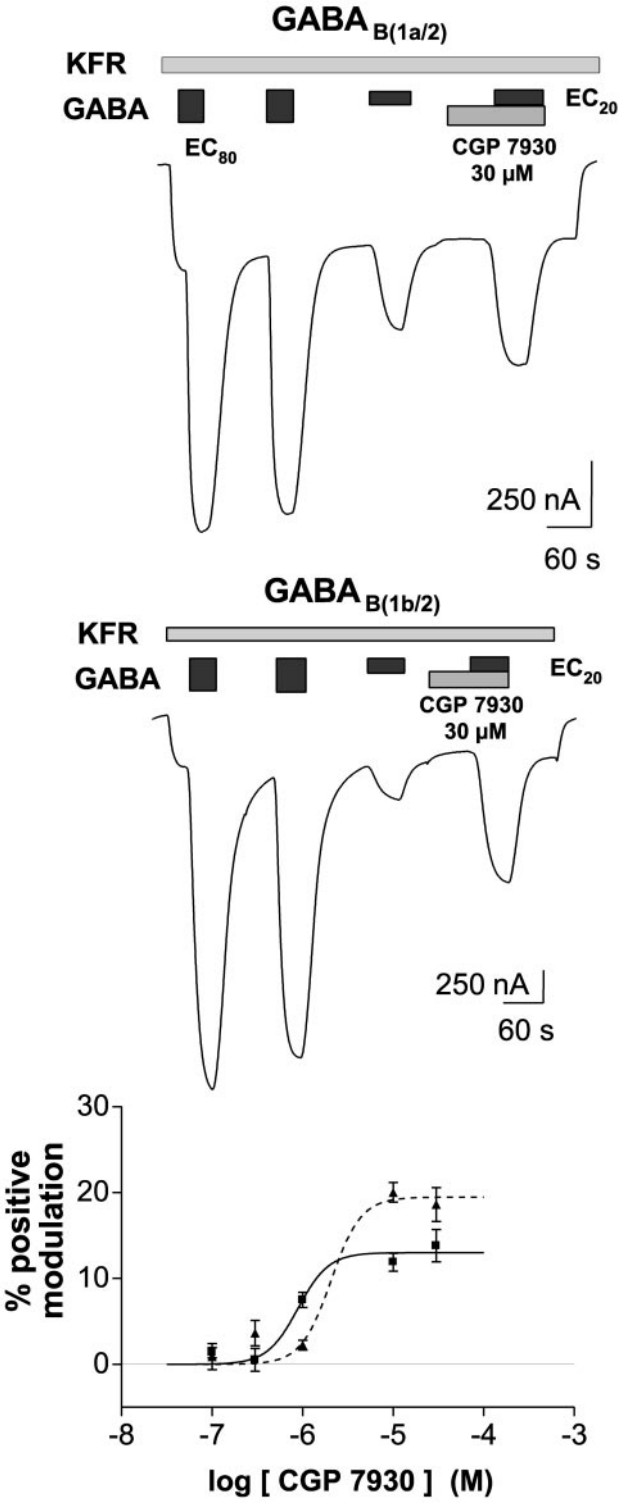

Fig. 7. Positive modulation of the effects of GABA on inwardly rectifying potassium channels in *X. laevis* oocytes by CGP7930. Top, current record of an oocyte expressing $GABA_{B(1a/2)}$ receptors in the presence of high-potassium (90 mM) Ringer solution (KFR, light gray bar). Control responses to GABA are shown at two different concentrations of GABA (0.3 μM [~EC_{20}] and 5.4 μM [~EC_{80}], black bars). In the presence of 30 μM CGP7930 (dark gray bar), the response to 0.3 μM GABA is enhanced. Middle, a similar trace with $GABA_{B(1b/2)}$ (GABA EC_{20}, 0.4 μM, EC_{80}, 2.5 μM). Bottom, concentration-response curves for CGP7930 (squares, $GABA_{B(1a/2)}$; triangles, $GABA_{B(1b/2)}$). In these curves, the positive modulation is expressed as increase above the control level in percentage of the maximal response of GABA alone. The values shown are means ± S.E.M. from at least three different oocytes.

meric G-protein, enabling them to couple to the phospholipase C pathway (Bräuner-Osborne and Krogsgaard-Larsen, 1999; Franek et al., 1999; Pagano et al., 2001; Wood et al., 2000). The increase of intracellular calcium concentrations elicited by the addition of GABA to HEK293 cells transiently transfected with $GABA_B$ receptors and the chimeric G-protein $G\alpha_{qo5}$ was again potentiated by CGP7930 in a concentration-dependent fashion (Fig. 8), whereas CGP7930 on its own did not produce an increase of intracellular calcium.

At the next level of complexity, the modulatory effects of CGP7930 were confirmed in a test system representing a neuronal network. Dissociated rat cortical neurons in primary culture produce synchronized calcium oscillations in low extracellular Mg^{2+} (Wang and Gruenstein, 1997; Fig. 9), resulting from the interplay of spontaneous depolarizations of inhibitory and excitatory neurons. The $GABA_B$ receptor agonist L-baclofen reduced the frequency of these oscillations, an effect that was again potentiated by CGP7930 (Fig. 9).

It is well known that $GABA_B$ receptors are positively modulated by calcium ions in an allosteric fashion (Wise et al., 1999; Galvez et al., 2000a). However, the following findings show that this action of calcium is of a different nature and occurs via another site than the modulation by the compounds described in this study: in GTPγ[^{35}S] stimulation experiments, Ca^{2+} increases the affinity of GABA without influencing its maximal effect (Wise et al., 1999; Galvez et al., 2000a). CGP7930 positively modulates agonism produced by GABA and L-baclofen, whereas Ca^{2+} enhances only the potency of GABA, not that of baclofen (Galvez et al., 2000a). Also, in contrast to CGP7930, calcium enhances the affinity of GABA as a displacer of an antagonist radioligand in membranes from CHO cells expressing the $GABA_{B(1)}$ subunit only (Galvez et al., 2000a). Furthermore, our experiments were conducted in the presence of a saturating concentration of calcium; therefore, the effects observed with CGP7930 and CGP13501 were additive with those of calcium ions.

In summary, we have shown that CGP7930 and CGP13501 act as positive allosteric modulators of $GABA_B$ receptor function. The allosteric nature of the effects of these compounds is supported by three main findings: first, they have no relevant agonistic effect when applied without GABA; second, the maximal stimulation of GTPγ[^{35}S] binding in the presence of these compounds exceeds the effect of a saturating concentration of GABA alone (i.e., the modulators act *synergistically* with GABA); finally, the compound CGP7930 does not bind to

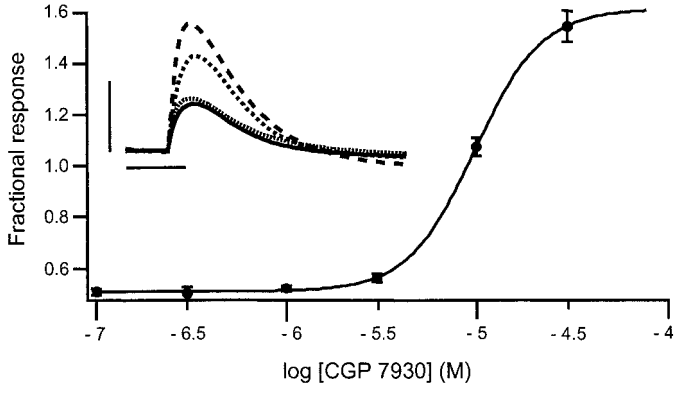

Fig. 8. Effects of CGP7930 on a calcium signal induced by GABA via recombinant $GABA_B$ receptors ($GABA_{B(1b/2a)}$) transiently coexpressed with $G\alpha_{qo5}$ in HEK293 cells. The concentration-response curve shown is expressed as relative fluorescence increase (ΔF/F), normalized to the values obtained with a saturating concentration of GABA (100 μM). The data shown are means ± S.E.M. from three experiments, each performed with eight wells per concentration. Inset, typical traces (raw fluorescence counts) from representative wells pretreated with no (control, solid line), 3 μM (small dotted line), 10 μM (medium dotted line), and 30 μM (large dotted line) CGP7930. GABA (1 μM) was added at t = 20 s. The calibration bars represent 30 s (horizontal) and 5000 FLIPR counts (vertical).

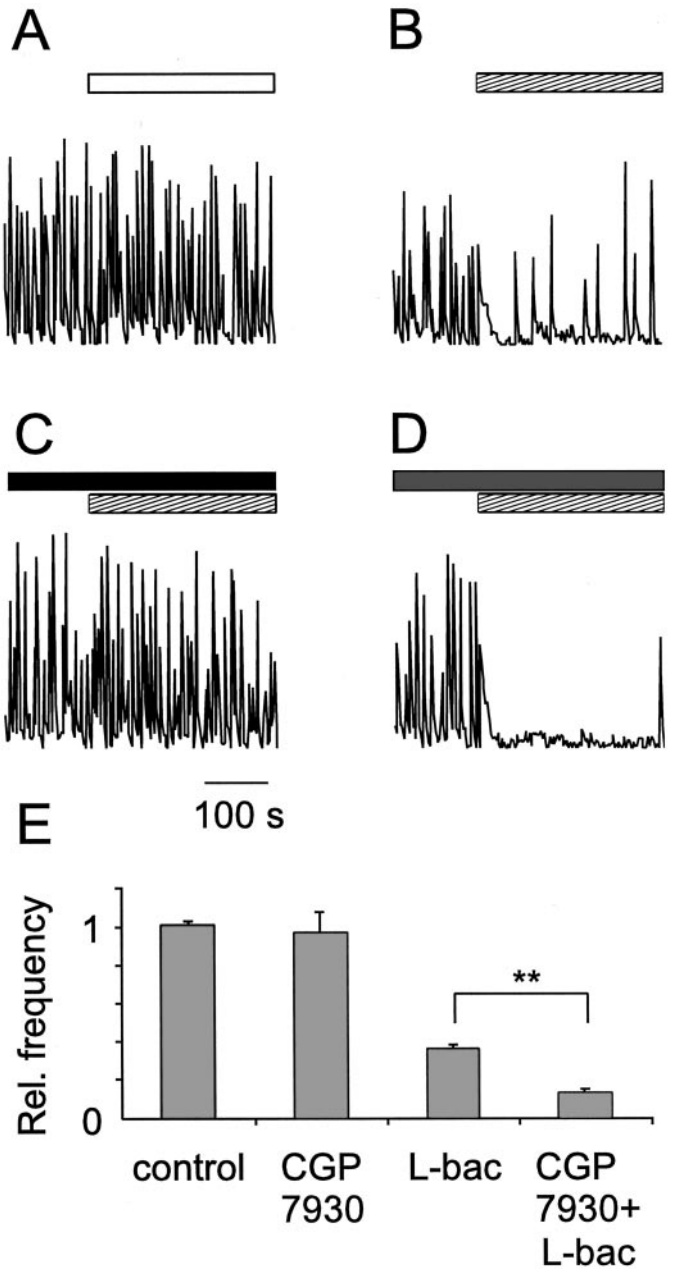

Fig. 9. Network oscillations in neuronal cultures. Calcium fluctuations in primary cultures from cortical neurons were monitored in a FLIPR using fluo-4 as described under *Materials and Methods*. A, calcium oscillations under control conditions (open bar, buffer application). B, the $GABA_B$ receptor agonist L-baclofen (3 μM, hatched bar) reduces oscillation frequency without decreasing the peak amplitudes. C, the effect of L-baclofen (3 μM, hatched bar) is completely blocked by 1 μM CGP54626A (black bar), a specific $GABA_B$ receptor antagonist. D, CGP7930 (gray bar) potentiates the effect of L-baclofen (3 μM, hatched bar) to reduce oscillations at a concentration (0.3 μM) at which it has no effect on its own (left part of the traces, before the addition of 3 μM L-baclofen). E, bar graph showing average effects of 0.3 μM CGP7930, 3 μM L-baclofen (L-bac), and both together on relative oscillation frequencies. The data shown are means ± S.E.M. of at least four wells. $^{**}p < 0.01$ (Student's *t* test).

the agonist recognition site of the $GABA_B$ receptor. As discussed above, our radioligand binding studies show that the presence of the $GABA_{B(2)}$ subunit is necessary for the positive modulation. At present, it is unclear whether CGP7930 binds to $GABA_{B(1)}$ or $GABA_{B(2)}$ or even acts at the interface between the two subunits. All agonist and antagonist ligands known so far bind to the $GABA_{B(1)}$ subunit (Kaupmann et al., 1997; Malitschek et al., 1999; Galvez et al., 2000b). On the other hand, not only is $GABA_{B(2)}$ responsible for the targeting of the $GABA_B$ receptor to the cell surface (Pagano et al., 2001), its extracellular domain also enhances agonist affinity at $GABA_{B(1)}$ and is necessary for agonist activation of the receptor (Galvez et al., 2001). Thus, it seems that $GABA_{B(1)}$ serves as the orthosteric ligand binding subunit and $GABA_{B(2)}$ as an allosteric subunit, through which positive modulators might well act. Alternatively, it is also conceivable that such modulators might bind to the transmembrane domain of one or both $GABA_B$ receptor subunits, as has been demonstrated for noncompetitive mGluR antagonists (Pagano et al., 2000). To address this question, studies with site-directed mutagenesis on individual $GABA_B$ receptor subunits will be needed.

Acknowledgments

We thank Dr. R. Kuhn for critically reading the manuscript, Dr. P. Flor for providing cells expressing mGluR2, and R. Brom, M. Erb, C. Lampert, M. Horvath, D. Ristig, and V. Schuler for their excellent technical assistance.

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