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Positive regulation of $GABA_B$ receptors dually coupled to cyclic AMP by the allosteric agent CGP7930

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Abstract

The ability of 2,6Di-*tert*-butyl-4-(-hydroxy-2,2-dimethyl-propyl)-phenol (CGP7930), a positive allosteric modulator of $GABA_B$ receptors, to regulate $GABA_B$ receptor-induced stimulation and inhibition of adenylyl cyclase activity in rat brain was investigated. In olfactory bulb granule cell layer and in frontal cortex, CGP7930 potentiated the stimulatory effects of (−)-baclofen and γ-aminobutyric acid (GABA) on basal and corticotropin-releasing hormone-stimulated adenylyl cyclase activities, respectively. In these stimulatory responses, CGP7930 enhanced both agonist potencies and maximal effects. When $GABA_B$ receptor-mediated inhibition of forskolin-stimulated adenylyl cyclase activity of frontal cortex was examined, CGP7930 increased the agonist potencies but failed to affect the maximal effect of (−)-baclofen and modestly increased that of GABA. Similar results were obtained for the inhibition of Ca^{2+}/calmodulin-stimulated adenylyl cyclase in striatum and cerebellum. Western blot analysis of each membrane preparation showed the presence of $GABA_{B2}$ receptor subunit, a putative site of action of CGP7930. These data indicate that CGP7930 positively modulates brain $GABA_B$ receptors coupled to either stimulation or inhibition of cyclic AMP signalling.

Keywords: $GABA_B$ receptor; cAMP; CGP7930; Allosteric modulation; Brain, rat

1. Introduction

Unlike other G protein-coupled receptors, the metabotropic $GABA_B$ receptor has been shown to operate as a heterodimer with the subunits, termed $GABA_{B1}$ and $GABA_{B2}$ (Bowery et al., 2002), subserving distinct signalling steps (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998; Kuner et al., 1999; Ng et al., 1999). Although both $GABA_{B1}$ and $GABA_{B2}$ contain a seven-transmembrane domain and a long extracellular chain at the N terminus, only $GABA_{B1}$ possesses the binding site for $GABA_B$ receptor agonists and antagonists (Kaupmann et al., 1998; Galvez et al., 1999). However, agonist-bound $GABA_{B1}$ appears to be unable to transduce the signal and requires the interaction with $GABA_{B2}$ for effective G protein activation (Galvez et al., 2001; Margeta-Mitrovic et al., 2001). $GABA_{B2}$ is also necessary for the $GABA_{B1}$ transportation to the plasma membrane (Couve et al., 1998; Margeta-Mitrovic et al., 2000) and its coexpression increases the binding affinity of GABA on $GABA_{B1}$ (Kaupmann et al., 1998; Galvez et al., 2001).

Recently, Urwyler et al. (2001) reported that the compound 2,6Di-*tert*-butyl-4-(-hydroxy-2,2-dimethyl-propyl)-phenol (CGP7930) and its aldehyde analog CGP13501 act as positive allosteric modulators of $GABA_B$ receptors. These compounds were found to potentiate γ-aminobutyric acid (GABA)-stimulated guanosine 5′-*O*-(3-[^{35}S]thiotriphosphate) ([^{35}S]GTPγS) binding mediated by either cloned or native $GABA_B$ receptors. Moreover, CGP7930 dose-dependently enhanced $GABA_B$ receptor-induced modulation of K^+ currents in *Xenopus laevis* oocytes (co-expressing the $GABA_B$ receptor subunits and the inwardly rectifying K^+ channel) and reduction of Ca^{2+} oscillation in primary cultures of cortical neurons (Urwyler et al., 2001). In each cell system examined, the positive allosteric modulators appeared inactive in the absence of GABA, but increased both the affinity and the efficacy of $GABA_B$ receptor agonists.

Besides being coupled to K^+ and Ca^{2+} channels, $GABA_B$ receptors are known to stimulate and inhibit cyclic AMP formation in different brain regions (Enna and Karbon, 1984; Hill and Dolphin, 1984; Wojcik and Neff, 1984; Karbon and Enna, 1985; Watling and Bristow, 1986; Knight and Bowery,

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1996) and it has been proposed that these opposite responses may involve distinct pools of pre- and post-synaptic $GABA_B$ receptors (Hill, 1985). Moreover, the stimulatory and inhibitory responses have been shown to display different sensitivity to some specific $GABA_B$ receptor antagonists, suggesting the possible involvement of pharmacologically different receptor subtypes (Cunningham and Enna, 1996). We have recently demonstrated that the $GABA_B$ receptor-mediated dual regulation of cyclic AMP can be detected in membrane preparations of specific layers of the rat olfactory bulb and frontal cortex, and provided evidence that the stimulatory and inhibitory effects may result from the coupling to distinct Ca^{2+}-insensitive and Ca^{2+}-sensitive isoforms of adenylyl cyclase (Olianas and Onali, 1999; Onali and Olianas, 2001). Because of these unique properties, it was of interest to investigate whether the $GABA_B$ receptors coupled to cyclic AMP can be modulated by allosteric regulators and whether the stimulatory and inhibitory responses are differentially affected by these agents. With this goal, in the present study we examined the effects of CGP7930 on the stimulation and inhibition of adenylyl cyclase activity elicited by GABA and (−)-baclofen in membrane preparations of different rat brain areas.

2. Materials and methods

2.1. Materials

[α-^{32}P]ATP (30–40 Ci/mmol), [2,8-^{3}H]cyclic AMP (25 Ci/mmol) were obtained from New England Nuclear (Boston, MA, USA). CGP7930 was kindly provided by Dr. Stephan Urwyler (Novartis Pharma, Basel, Switzerland). The compound was dissolved in dimethyl sulfoxide to a concentration of 30 mM and subsequent dilutions were made in distilled water. Fresh solutions were prepared daily by diluting the stock solutions in the appropriate vehicle. The final concentration of dimethyl sulfoxide in the assay did not exceed 1%. Control samples received an equal concentration of vehicle. (−)-Baclofen was obtained from Tocris Cookson (Bristol, UK). Corticotropin-releasing hormone (CRH) (human, rat) was obtained from Neosystem (Strasbourg, France). GABA and the other reagents used in the assays were from Sigma (St. Louis, MO, USA).

2.2. Membrane preparation

Male Sprague–Dawley rats (200–300 g) were used. Experiments were performed according to the principles of laboratory animal care (Law on Animal Experiments in Italy, D.L. 116/92). The animals were killed by decapitation and their brain immediately immersed in an ice-cold phosphate-buffered saline. To prepare membranes from granule cell layer of the main olfactory bulb, each bulb was cut into 300-μm-thick coronal sections, which were kept in an ice-cold phosphate-buffered saline containing 2.5 mM $CaCl_2$. With the aid of a stereoscopic microscope equipped with a diascopic illuminator base, the granule cell layer was free-hand-dissected from each slice. The microdissected tissue layer from individual slices was pooled and homogenized by hand in an ice-cold buffer containing 10 mM HEPES–NaOH, 1 mM EGTA, 1 mM $MgCl_2$ and 1 mM dithiothreitol (pH 7.40) using a Teflon-glass tissue grinder. The homogenate was centrifuged at 27,000 × *g* for 20 min at 4 °C. The pellet was resuspended in the same buffer at a protein concentration of 0.8–1.0 mg/ml and used immediately for adenylyl cyclase assays. The frontal lobes were isolated from the rest of the brain by performing a cut in the coronal plane just anterior to the olfactory tubercles. The dorsal striatum was dissected from five to six coronal sections (300 μm thick) of the brain. The cerebellum was removed from the pons by using razor blade knives to cut the cerebellar peduncles. Each tissue was homogenized in 10–15 volumes of ice-cold homogenisation buffer, diluted 5-fold with the same medium and centrifuged at 27,000 × *g* for 20 min at 4 °C. The pellet was resuspended and centrifuged as above. The final pellet was resuspended in the same buffer to a protein concentration of 1.0–1.5 mg/ml and used immediately for the adenylyl cyclase assay.

2.3. Adenylyl cyclase assay

The enzyme activity was assayed in a reaction mixture (final volume, 100 μl) containing 50 mM HEPES–NaOH (pH 7.40), 2.3 mM $MgCl_2$, 0.5 mM [^{3}H]cyclic AMP (80 cpm/nmol), 0.3 mM EGTA, 1.3 mM dithiothreitol, 1 mM 3-isobutyl-1-methylxanthine, 5 mM phosphocreatine, 50 u/ml of creatine kinase, 50 μg of bovine serum albumin, 10 μg of bacitracin, 10 kallikrein inhibitor units (KIU) of aprotinin, and 20–30 μg of membrane protein. The concentrations of [α-^{32}P]ATP and GTP were 0.2 and 0.1 mM in the assay of the enzyme activity of granule cell layer and 0.1 mM and 10 μM when the regulation of enzyme activity was studied in frontal cortex, striatum and cerebellum. When included in the reaction mixture, the concentrations of CRH, forskolin, free Ca^{2+}, and calmodulin were 0.5, 10, 1 and 2 μM, respectively. The reaction was started by adding the tissue preparations and carried out at 30 °C for 10 min. The reaction was stopped by adding 200 μl of a solution containing 2% (w/v) sodium dodecyl sulfate, 45 mM ATP, 1.3 mM cyclic AMP (pH 7.5). Cyclic AMP was isolated by sequential chromatography on Dowex and alumina columns as described by Salomon et al. (1974). The recovery of [^{32}P]cyclic AMP from each sample was calculated on the basis of the recovery of [^{3}H]cyclic AMP. Assays were carried out in duplicate.

Protein content was determined by the method of Bradford (1976), using bovine serum albumin as a standard.

2.4. Western blot analysis of $GABA_{B2}$

Freshly dissected brain areas were homogenized with a Teflon-glass tissue grinder in an ice-cold buffer containing

10 mM HEPES–NaOH (pH 7.4), 1 mM EDTA and a protease inhibitor mixture (300 KIU/ml of aprotinin, 10 μg/ml of leupeptin, 10 μg/ml of soybean trypsin inhibitor, 3 μM pepstatin and 1 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 27,000 × *g* for 20 min at 4 °C and the pellet was resuspended in 1% sodium dodecyl sulphate (SDS), 0.5% sodium deoxycholate, 0.2 mM dithiothreitol, 1 mM EDTA and the protease inhibitor mixture. The samples were heated at 100 °C for 5 min, cooled, briefly sonicated and mixed with a 5 × solution of sample buffer (300 mM Tris–HCl, 10% SDS, 40% glycerol, 10% β-mercaptoethanol and 0.008% bromophenol blue, pH 6.8), heated at 90 °C for 2 min and subjected to SDS-polyacrilamide gel electrophoresis. The proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bradford, MA, USA) according to the method of Towbin et al. (1979). The efficiency of the transfer was controlled by staining the gel with Coomassie Brilliant Blue and by following the transfer of prestained protein standards (Amersham, Buckinghamshire, UK). Nonspecific binding sites were blocked by incubation in 20 mM Tris–HCl, 137 mM NaCl and 0.05% Tween-20 (pH 7.6) (TBS-T buffer) containing 5% bovine serum albumin overnight at 4 °C. After washing with TBS-T buffer, the membranes were incubated for 1 h at room temperature with an affinity-purified antibody (1 μg/ml in TBS-T containing 0.1% bovine serum albumin) raised in rabbits against a 24-amino acid peptide located at the C-terminus of rat $GABA_{B2}$ (Alpha Diagnostic International, San Antonio, TX, USA). When indicated, the antibody was blocked by incubation with a 5-fold excess of the corresponding peptide for 1 h at room temperature. The membranes were then washed with TBS-T and incubated with a horseradish peroxidase-conjugated second antibody (1:5000; Santa Cruz Biotecnology, Santa Cruz, CA, USA) for 45 min at room temperature. Immunoreactive bands were detected by using an enhanced chemiluminescence system (ECL Plus) and ECL Hyperfilm (Amersham). Preabsorption of the $GABA_{B2}$ antibody with the respective blocking peptide was performed by incubating the primary antibody in the presence of a 5-fold higher concentration of the blocking peptide for 1 h at room temperature.

2.5. Statistical analysis

Results are given as mean ± S.E. Concentration response curves were analysed by a least squares curve-fitting computer program (Graph Pad Prism, San Diego, CA, USA), which yielded concentrations producing half-maximal effects (EC_{50} values) and maximal responses. For statistical analysis, EC_{50} values were converted to the negative logarithmic form (pEC_{50}), as these values are log-normally distributed (Fleming et al., 1972). Statistical significance of the difference between means was determined by Student's *t*-test.

3. Results

3.1. Effects of CGP7930 on $GABA_B$ receptors stimulating basal adenylyl cyclase activity in granule cell layer of olfactory bulb

As previously reported (Olianas and Onali, 1999), in membranes of granule cell layer (−)-baclofen caused a concentration-dependent stimulation of basal adenylyl cyclase activity with a pEC_{50} value of 4.40 ± 0.04 (Fig. 1A). CGP7930, added at 30 and 100 μM, failed to affect basal cyclase activity per se, but increased the potency of (−)-baclofen by 2.2- and 3.0-fold, respectively (pEC_{50} values were 4.75 ± 0.05, $P < 0.01$, and 4.87 ± 0.09, $P < 0.01$, respectively). CGP7930 enhanced the maximal stimulatory effect of the $GABA_B$ receptor agonist by

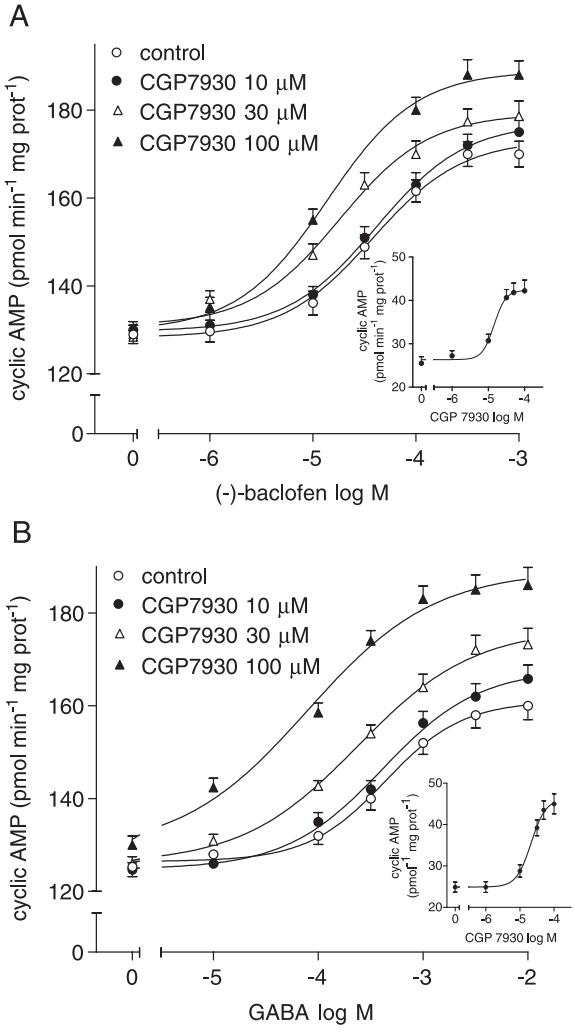

Fig. 1. Effects of CGP7930 on (−)-baclofen (A) and GABA (B) stimulation of basal adenylyl cyclase activity in membranes of granule cell layer of the rat olfactory bulb. Data are the mean ± S.E. of three experiments. Insets: Concentration–response curves of CGP7930 in potentiating cyclic AMP formation stimulated by 50 μM (−)-baclofen (A) and 500 μM GABA (B). Data are the mean ± S.E. of three experiments.

18 ± 2% ($P < 0.05$) and 32 ± 5% ($P < 0.01$) at 30 and 100 μM, respectively. At a lower concentration (10 μM), CGP7930 had no significant effect on the (−)-baclofen stimulation. Concentration–response curves of CGP7930 in the presence of a fixed concentration of (−)-baclofen (50 μM) showed that the allosteric modulator potentiated the (−)-baclofen effect with a pEC_{50} value of 4.84 ± 0.10 (Fig. 1A, inset).

In the same membrane preparation, CGP7930 significantly enhanced the maximal stimulation of cyclic AMP formation elicited by GABA by 28.5 ± 7.0% ($P < 0.05$) and 48.8 ± 8% ($P < 0.01$) at the concentrations of 30 and 100 μM, respectively (Fig. 1B). The same CGP7930 concentrations increased the potency of GABA by 2.0- and 4.7-fold (pEC_{50} values were: control, 3.34 ± 0.05; CGP7930 30 μM, 3.65 ± 0.07, $P < 0.05$; 100 μM, 4.02 ± 0.09, $P < 0.01$). When the effect of increasing concentrations of CGP7930 on the stimulation by a fixed concentration of GABA (500 μM) was investigated, it was found that the compound enhanced the agonist effect with a pEC_{50} of 4.67 ± 0.08 (Fig. 1B, inset).

3.2. Effects of CGP7930 on $GABA_B$ receptor-induced potentiation of CRH-stimulated adenylyl cyclase activity in frontal cortex

In membranes of frontal cortex activation of $GABA_B$ receptors markedly potentiates the stimulation of the enzyme produced by CRH (Onali and Olianas, 2001). As shown in Fig. 2A, CGP7930 enhanced the potentiation of CRH-stimulated enzyme activity elicited by either (−)-baclofen or GABA. Thus, the addition of 10, 30 and 100 μM CGP7930 increased the potency of (−)-baclofen ($pEC_{50} = 3.75 \pm 0.03$) by 1.1-fold ($pEC_{50} = 3.79 \pm 0.04$, $P > 0.05$), 1.7-fold ($pEC_{50} = 3.98 \pm 0.04$, $P < 0.05$) and 6.6-fold ($pEC_{50} = 4.57 \pm 0.08$, $P < 0.001$), respectively. At these concentrations, the compound enhanced the maximal effect of the agonist by 4.5 ± 2.0 ($P > 0.05$), 29 ± 6 ($P < 0.01$) and 67 ± 9% ($P < 0.001$), respectively. The GABA-induced potentiation of CRH-stimulated cyclic AMP formation was similarly enhanced by CGP7930 (Fig. 2B). At the concentration of 10 μM, the allosteric modulator caused an insignificant increase of the GABA effect, but at 30 and 100 μM, CGP7930 increased the agonist potency by 2.3- and 5.7-fold, respectively (pEC_{50} values were: control, 3.01 ± 0.03; CGP7930 30 μM, 3.37 ± 0.05, $P < 0.01$; 100 μM, 3.76 ± 0.08, $P < 0.001$) and enhanced the maximal effect by 49 ± 7% ($P < 0.001$) and 97 ± 8% ($P < 0.001$), respectively. Concentration–response curves of CGP7930 (from 1 to 100 μM) performed in the presence of a fixed concentration of either (−)-baclofen (150 μM) or GABA (1 mM) yielded pEC_{50} values of 4.61 ± 0.12 and 4.47 ± 0.08, respectively (results not shown). At each concentration tested, CGP7930 did not significantly affect the stimulation of adenylyl cyclase activity elicited by CRH.

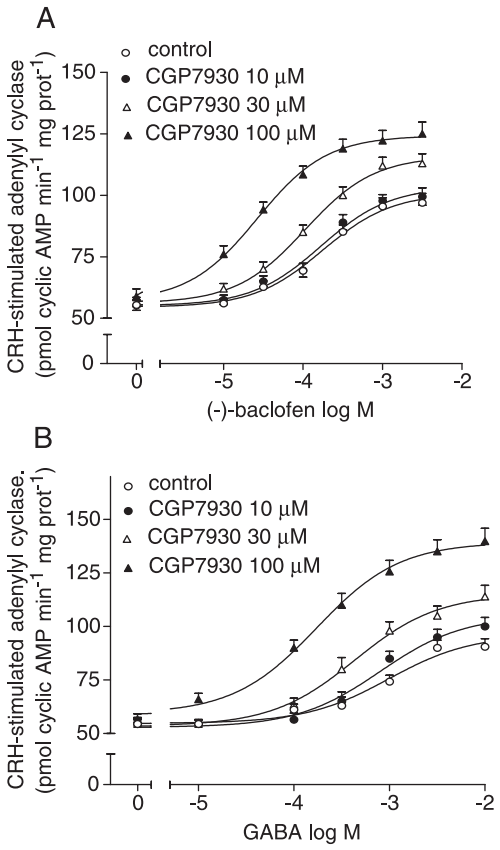

Fig. 2. Effects of CGP7930 on (−)-baclofen (A) and GABA (B) potentiation of CRH-stimulated adenylyl cyclase activity in membranes of rat frontal cortex. Data are the mean ± S.E. of three experiments.

3.3. Effects of CGP7930 on $GABA_B$ receptor-induced inhibition of forskolin- and Ca^{2+}/calmodulin-stimulated adenylyl cyclase activities

In membranes of rat frontal cortex, the adenylyl cyclase activity stimulated by forskolin (10 μM) was inhibited by (−)-baclofen in a concentration-dependent manner (Fig. 3A). The addition of CGP7930 (10, 30 and 100 μM) caused a progressive increase in the potency of (−)-baclofen (pEC_{50} values were: control 4.09 ± 0.04; CGP7930 10 μM, 4.25 ± 0.05, $P > 0.05$; 30 μM, 4.39 ± 0.04, $P < 0.05$; 100 μM, 4.95 ± 0.06, $P < 0.01$). The maximal inhibitory effect of (−)-baclofen, however, was not significantly enhanced by any CGP7930 concentration tested. When the inhibitory effect of GABA was investigated (Fig. 3B), CGP7930, at the concentrations of 10, 30 and 100 μM, increased the potency of GABA ($pEC_{50} = 3.07 \pm 0.03$) by 1.2-fold ($pEC_{50} = 3.15 \pm 0.04$, $P > 0.05$), 1.7-fold ($pEC_{50} = 3.29 \pm 0.04$, $P < 0.05$) and 6.5-fold ($pEC_{50} = 3.85 \pm 0.07$, $P < 0.001$), respectively. The maximal effect was enhanced only by the highest concentration of CGP7930 (17.5 ± 2.5%, $P < 0.05$). In a separate set of experiments, it was found that the inhibitory effects elicited by a submaximal concentration of either (−)-baclofen (100 μM) or GABA (0.8 mM) were potentiated by CGP7930 (tested at concentrations ranging

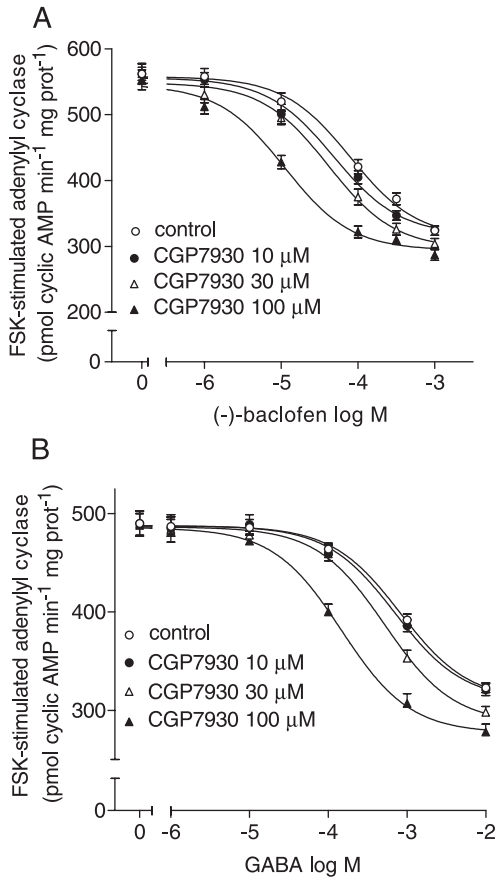

Fig. 3. Effects of CGP7930 on (−)-baclofen (A) and GABA (B) inhibition of forskolin-stimulated adenylyl cyclase activity in membranes of rat frontal cortex. Data are the mean ± S.E. of four experiments.

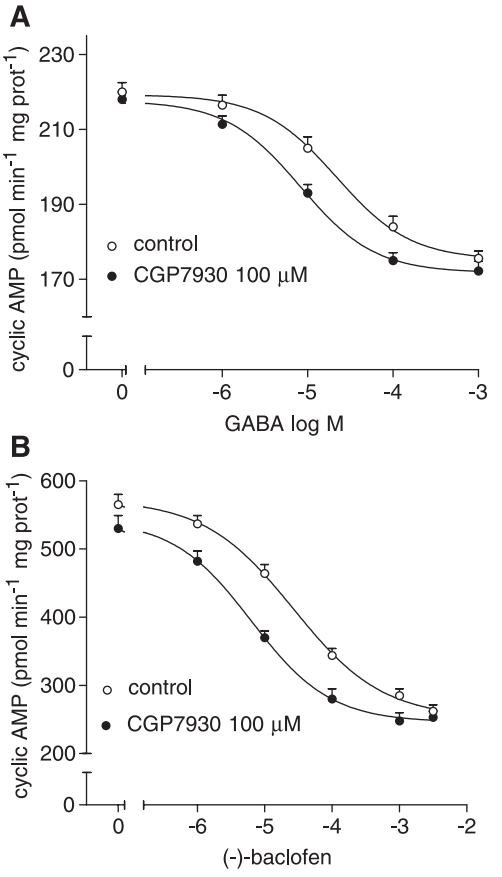

Fig. 4. Effects of CGP7930 on the inhibition of Ca^{2+}/calmodulin-stimulated adenylyl cyclase activity by GABA in rat striatal membranes (A) and by (−)-baclofen in rat cerebellar membranes (B). Data are the mean ± S.E of three experiments.

from 1 to 100 μM) with pEC_{50} values of 4.55 ± 0.08 and 4.43 ± 0.10, respectively (results not shown).

In dorsal striatum, GABA maximally inhibited the enzyme activity stimulated by Ca^{2+}/calmodulin by 20.2 ± 2.0% ($P < 0.05$) with a pEC_{50} value of 4.66 ± 0.02 (Fig. 4A). The addition of 100 μM CGP7930 increased the potency of GABA by 2.6-fold ($pEC_{50} = 5.08 \pm 0.03$, $P < 0.05$) without changing the maximal effect. In rat cerebellar membranes, (−)-baclofen inhibited the Ca^{2+}/calmodulin-stimulated enzyme activity by 55 ± 5% with a pEC_{50} of 4.57 ± 0.03 (Fig. 4B). Also in this case, CGP7930 (100 μM) enhanced the potency of the agonist ($pEC_{50} = 5.20 \pm 0.05$, $P < 0.01$), with no significant change of the maximal inhibitory effect.

3.4. Western blot analysis of $GABA_{B2}$ expression

As shown in Fig. 5, immunoblots analysis of granule cell layer and striatal membranes using a $GABA_{B2}$ specific antibody detected an immunoreactive band of ~ 110 kDa which either disappeared or faded when the antibody was preincubated with the blocking peptide. An immunoreactive band with the same electrophoretic mobility and staining sensitivity was detected in frontal cortex and cerebellum. In each tissue preparations, two additional bands of ~ 90 and 160 kDa, respectively, were detected, but their labelling was not affected by the blocking peptide, indicating that the immunoreactivity was nonspecific.

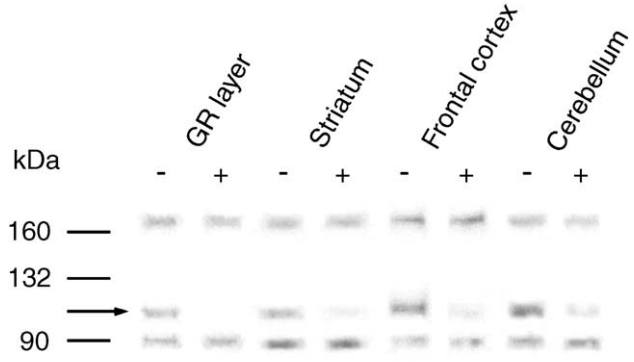

Fig. 5. Immunoblot analysis of $GABA_{B2}$ in membranes of granule cell (GR) layer, dorsal striatum (striatum), frontal cortex and cerebellum. The symbols (−) and (+) indicate that the primary antibody was preincubated without and with the blocking peptide, respectively. The arrow indicates the immunoreactive band corresponding to $GABA_{B2}$. The positions of molecular mass standards are shown on the left. The results are representative of three experiments.

4. Discussion

The data presented in this study show that CGP7930, an allosteric modulator of the $GABA_B$ receptor, positively regulates $GABA_B$ receptor-induced stimulation and inhibition of cyclic AMP formation in membranes obtained from specific rat brain areas. The stimulatory effect on adenylyl cyclase activity was studied in membrane preparations of granule cell layer of the main olfactory bulb and frontal cortex, where previous studies have shown that $GABA_B$ receptor activation enhanced basal and CRH-stimulated enzyme activities, respectively, likely through the stimulatory action of βγ subunits of the G proteins G_i/G_o on type II/IV adenylyl cyclases (Olianas and Onali, 1999; Onali and Olianas, 2001). In both assay systems, CGP7930 elicited a concentration-dependent potentiation of the stimulatory effects induced by either (−)-baclofen or GABA, without displaying intrinsic agonist activity. As previously observed for other $GABA_B$ receptor-mediated responses (Urwyler et al., 2001), the CGP7930 potentiation consisted in an increase of both agonist potencies and maximal effects. In both granule cell layer and frontal cortex membranes, CGP7930 enhanced the stimulatory effect of (−)-baclofen and GABA on cyclase activity with pEC_{50} values (4.47–4.79), which were close to those previously reported for the potentiation of GABA-induced stimulation of intracellular Ca^{2+} accumulation and [^{35}S]GTPgS binding in cell lines transfected with the cloned $GABA_B$ receptor (pEC_{50} = 5.00 and 5.33, respectively; Urwyler et al., 2001). Moreover, the magnitude of the changes induced by CGP7930 on agonist concentration–response curves were generally comparable to those previously obtained with the cloned receptor. For instance, the 4.7- to 5.7-fold increase in the potency and the 49–97% enhancement of the maximal response of GABA induced by 100 μM CGP7930 in granule cell layer and frontal cortex membranes, respectively, were close to the 6.1-fold shift in the agonist potency and to the 43% increase in the agonist efficacy observed in Chinese hamster ovary cells expressing the recombinant $GABA_B$ (Urwyler et al., 2001).

When the $GABA_B$-mediated inhibition of adenylyl cyclase was investigated, CGP7930 was found to modulate the response to agonists in a manner different from that observed for the cyclase stimulation. In fact, the allosteric modulator increased the agonist potency with little or no change in the apparent agonist efficacy. For instance, in frontal cortex membranes the exposure to 100 μM CGP7930 enhanced the potency of (−)-baclofen in inhibiting the forskolin-stimulated adenylyl cyclase activity by 7.2-fold, with no change of the maximal inhibition. When GABA was used as an agonist, there was a 5.6-fold increase in potency with little enhancement of the maximal effect. Similar results were obtained by examining the effect of CGP7930 on GABA- and (−)-baclofen-induced inhibition of Ca^{2+}/calmodulin-stimulated adenylyl cyclase activity in dorsal striatum and cerebellum, respectively. This indicated that the effect of the allosteric modulator occurred independently of the brain region examined and the type of adenylyl cyclase stimulator used.

Previous studies have shown that in rat cortical slices some $GABA_B$ receptor antagonists discriminated between $GABA_B$ receptor-induced stimulation and inhibition of cyclic AMP, suggesting the possible involvement of pharmacologically distinct subclasses of $GABA_B$ receptors (Cunningham and Enna, 1996). However, studies using heterologous systems expressing the cloned splice variants of $GABA_{B1}$, $GABA_{B1(a)}$ and $GABA_{B1(b)}$, in association with $GABA_{B2}$, have failed to demonstrate the pharmacological differences between $GABA_{B1(a)/B2}$ and $GABA_{B1(b)B2}$ (Kaupmann et al., 1998; Green et al., 2000). Moreover, when expressed in *X. laevis* oocytes, the two receptor isoforms have been found to display a similar sensitivity to the allosteric action of CGP7930 (Urwyler et al., 2001). Thus, the current information on the identified isoforms does not support the possibility that the differential modulation by CGP7930 of $GABA_B$ receptors coupled to cyclic AMP derives from an action on distinct receptor subtypes. On the other hand, differences in post-receptor events may influence the ability of allosteric modulators to enhance the agonist efficacy (Hall, 2000). Thus, in the case of the inhibitory response agonists may maximally activate the signalling cascade (for example, the G protein G_i mediating cyclase inhibition) so that further amplification is not possible. On the other hand, in the case of adenylyl cyclase stimulation full activation of the transduction pathway may be limited by the fact that this response likely involves a larger pool of G proteins (both G_o and G_i) as a source of βγ subunits, so that enhanced receptor activation by CGP7930 can be followed by an enhanced maximal response. Thus, differences in the efficacy of the transduction machinery may well explain the different sensitivity to CGP7930 of the two functional responses.

In radioligand binding studies, CGP7930 has been shown to increase GABA affinity to heterodimeric but not to homomeric $GABA_{B1}$ receptors, indicating that $GABA_{B2}$ was required for the binding and/or the action of the allosteric modulator (Urwyler et al., 2001). In situ hybridisation studies examining the distribution of $GABA_{B1}$ and $GABA_{B2}$ in the various rat brain areas, have demonstrated that the olfactory bulb expresses the mRNA encoding $GABA_{B1}$ but lacks the mRNA for $GABA_{B2}$ (Kaupmann et al., 1998; Kuner et al., 1999). As our data indicated that CGP7930 was effective in modulating the $GABA_B$ receptor function in granule cell layer, it was important to investigate whether $GABA_{B2}$ could be detected in this brain region at the protein level. Immunoblot analysis using a specific affinity-purified antibody revealed the presence in membranes of granule cell layer and the other brain regions investigated of an immunoreactive band with an apparent molecular mass (110 kDa) corresponding to that reported for the $GABA_{B2}$ protein (110–120 kDa) (Kaupmann et al., 1998; White et al.,

1998). The presence of immunoreactivity in a brain area lacking the mRNA signal suggests that in granule cell layer $GABA_{B2}$ is predominantly expressed in nerve terminals of neurons projecting to the olfactory bulb. Importantly, the detection of $GABA_{B2}$ immunoreactivity in granule cell layer is consistent with the functional data showing sensitivity of $GABA_B$ receptors to the allosteric action of CGP7930 in this area.

Very recently, Kerr et al. (2002) reported that in rat neocortical slices the arylalkylamines fendiline, prenylamine and F551 reversibly enhanced the hyperpolarizing effects of GABA and $GABA_B$ receptor agonists, thus behaving as a new class of positive allosteric modulators. Like CGP7930, these compounds increased both agonist potencies and maximal responses. Their effects on $GABA_B$ receptor coupled to cyclic AMP have not yet been investigated.

In conclusion, the present study shows that CGP7930 positively modulates brain $GABA_B$ receptors coupled to either stimulation or inhibition of cyclic AMP signalling, thus providing the first example of positive allosteric regulation of neurotransmitter receptor dually coupled to cyclic AMP by a synthetic molecule. Moreover, this study demonstrates the utility of the adenylyl cyclase assay system to investigate the allosteric modulation of $GABA_B$ receptors in the brain.

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References

Bowery, N.G., Bettler, B., Froestl, W., Gallagher, J.P., Marshall, F., Raiteri, M., Bonner, T.I., Enna, S.J., 2002. International Union of Pharmacology: XXXIII. Mammalian γ-aminobutyric $acid_B$ receptors: structure and function. Pharmacol. Rev. 54, 247–264.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Couve, A., Filippov, A.K., Connolly, C.N., Bettler, B., Brown, D.A., Moss, S.J., 1998. Intracellular retention of $GABA_B$ receptors. J. Biol. Chem. 273, 23367–26361.

Cunningham, M.D., Enna, S.J., 1996. Evidence for pharmacologically distinct $GABA_B$ receptors associated with cAMP production in rat brain. Brain Res. 720, 220–224.

Enna, S.J., Karbon, E.W., 1984. $GABA_B$ receptors and transmitter-stimulated cAMP accumulation in rat brain. Neuropharmacology 23, 821–822.

Fleming, W.W., Westfall, D.P., De La Lande, S., Jellet, L.B., 1972. Log-normal distribution of equieffective doses of norepinephrine and acetylcholine in several tissues. J. Pharmacol. Exp. Ther. 181, 339–345.

Galvez, T., Parmentier, M.-L., Joly, C., Malitschek, B., Kaupmann, K., Kuhn, R., Bittiger, H., Froestl, W., Bettler, B., Pin, J.-P., 1999. Mutagenesis and modeling of the $GABA_B$ receptor extracellular domain support a venus flytrap mechanism for ligand binding. J. Biol. Chem. 274, 13362–13369.

Galvez, T., Duthey, B., Kniazeff, J., Blahos, J., Rovelli, G., Bettler, B., Prezeau, L., Pin, J.-P., 2001. Allosteric interactions between GB1 and GB2 subunits are required for optimal $GABA_B$ receptor function. EMBO J. 20, 2152–2159.

Green, A., Walls, S., Wise, A., Green, R.H., Martin, A.K., Marshall, F.H., 2000. Characterization of [^{3}H]-CGP54626A binding to heterodimeric $GABA_B$ receptors stably expressed in mammalian cells. Br. J. Pharmacol. 131, 1766–1774.

Hall, D.A., 2000. Modeling the functional effects of allosteric modulators at pharmacological receptors: an extension of the two-state model of receptor activation. Mol. Pharmacol. 58, 1412–1423.

Hill, D.R., 1985. $GABA_B$ receptor modulation of adenylate cyclase activity in rat brain slices. Br. J. Pharmacol. 84, 249–257.

Hill, D.R., Dolphin, A.C., 1984. Modulation of adenylate cyclase activity by $GABA_B$ receptors. Neuropharmacology 23, 829–830.

Jones, K.A., Borowsky, B., Tamm, J.A., Craig, D.A., Durkin, M.M., Dai, M., Yao, W.-J., Johnson, M., Gunwaldsen, C., Huang, L.-Y., Tang, C., Shen, Q., Salon, J.A., Morse, K., Laz, T., Smith, K.E., Nagarathnam, D., Noble, S.A., Branchek, T.A., Gerard, C., 1998. $GABA_B$ receptors function as a heteromeric assembly of the subunits $GABA_B$R1 and $GABA_B$R2. Nature 396, 674–678.

Karbon, E.W., Enna, S.J., 1985. Characterization of the relationship between γ-aminobutyric acid B agonists and transmitter-coupled cyclic nucleotide-generating systems in rat brain. Mol. Pharmacol. 27, 53–59.

Kaupmann, K., Malitschek, B., Schuler, V., Heid, J., Froestl, W., Beck, P., Mosbacher, J., Bischoff, S., Kulik, A., Shigemoto, R., Karschin, A., Bettler, B., 1998. $GABA_B$-receptor subtypes assemble into functional heteromeric complexes. Nature 396, 683–687.

Kerr, D.I.B., Ong, J., Puspawati, N.M., Prager, R.H., 2002. Arylalkylamines are a novel class of positive allosteric modulators at $GABA_B$ receptors in rat neocortex. Eur. J. Pharmacol. 451, 69–77.

Knight, A.R., Bowery, N.G., 1996. The pharmacology of adenylyl cyclase modulation by $GABA_B$ receptors in rat brain slices. Neuropharmacology 35, 703–712.

Kuner, R., Kohr, G., Grunewald, S., Eisenhardt, G., Bach, A., Kornau, H.-C., 1999. Role of heteromer formation in $GABA_B$ receptor function. Science 283, 74–77.

Margeta-Mitrovic, M., Jan, Y.N., Jan, L.Y., 2000. A trafficking checkpoint controls $GABA_B$ receptor heterodimerization. Neuron 27, 97–106.

Margeta-Mitrovic, M., Jan, Y.N., Jan, L.Y., 2001. Function of GB1 and GB2 subunits in G protein coupling of $GABA_B$ receptors. Proc. Natl. Acad. Sci. U. S. A. 98, 14649–14654.

Ng, G.Y.K., Clark, J., Coulombe, N., Ethier, N., Hebert, T.E.H., Sullivan, R., Kargman, S., Chateauneuf, A., Tsukamoto, N., McDonald, T., Whiting, P., Mezey, E., Johnson, M.P., Liu, Q., Kolakowski Jr., L.F., Evans, J.F., Bonner, T.I., O'Neill, G.P., 1999. Identification of a $GABA_B$ receptor subunit, gb2, required for functional $GABA_B$ receptor activity. J. Biol. Chem. 274, 7607–7610.

Olianas, M.C., Onali, P., 1999. $GABA_B$ receptor-mediated stimulation of adenylyl cyclase activity in membranes of rat olfactory bulb. Br. J. Pharmacol. 126, 657–664.

Onali, P., Olianas, M.C., 2001. βγ-mediated enhancement of corticotropin-releasing hormone-stimulated adenylyl cyclase activity by activation of γ-aminobutyric $acid_B$ receptors in membranes of rat frontal cortex. Biochem. Pharmacol. 62, 183–190.

Salomon, Y., Londos, D., Rodbell, M., 1974. A highly sensitive adenylate cyclase assay. Anal. Biochem. 58, 541–548.

Towbin, H., Staehelin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354.

Urwyler, S., Mosbacher, J., Lingenhoehl, K., Heid, J., Hofstetter, K., Froestl, W., Bettler, B., Kaupmann, K., 2001. 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. Mol. Pharmacol. 60, 963–971.

Watling, K.J., Bristow, D.R., 1986. $GABA_B$ receptor-mediated enhancement of vasoactive intestinal peptide-stimulated cyclic AMP production in slices of rat cerebral cortex. J. Neurochem. 46, 1756–1762.

White, J.H., Wise, A., Main, M.J., Green, A., Fraser, N.J., Disney, G.H., Barnes, A.A., Emson, P., Foord, S.M., Marshall, F.H., 1998. Heterodimerization is required for the formation of a functional $GABA_B$ receptor. Nature 396, 679–682.

Wojcik, W.J., Neff, N.H., 1984. γ-Aminobutyric acid B receptors are negatively coupled to adenylyl cyclase in brain, and in the cerebellum these receptors may be associated with granule cells. Mol. Pharmacol. 25, 24–28.