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ORIGINAL INVESTIGATION

Guy Griebel · Ghislaine Perrault · Valérie Letang
Patrick Granger · Patrick Avenet · Hans Schoemaker
David J. Sanger

New evidence that the pharmacological effects of benzodiazepine receptor ligands can be associated with activities at different BZ (ω) receptor subtypes

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Abstract *Rationale:* It has been suggested that different BZ (ω) receptor subtypes may mediate distinct behavioural effects of BZ receptor ligands. *Objective:* The present study examined this hypothesis further. *Methods:* The antagonism exerted by the selective BZ_1 (ω_1) receptor antagonist β-CCT on the pharmacological effects of the selective BZ_1 (ω_1) receptor agonist zolpidem and the non-selective BZ (ω) receptor agonist diazepam in behavioural, biochemical and electrophysiological experiments was assessed. *Results:* β-CCT which was devoid of activity per se, antagonized the effects of the non-selective BZ (ω) receptor full agonist diazepam and the selective BZ_1 (ω_1) receptor full agonist zolpidem against seizures produced by isoniazid, but β-CCT failed to affect their action on seizures produced by pentylenetetrazole (PTZ), suggesting that BZ_2 (ω_2) receptors may be primarily involved in the convulsant action of PTZ. In the light/dark test, β-CCT abolished the anxiolytic-like action of diazepam. In tests designed to investigate the central depressant activity of drugs, β-CCT antagonized the sedative effects of diazepam and zolpidem, but failed to modify clearly the myorelaxant effects of diazepam. These differences may be related to the selectivity of β-CCT for BZ_1 (ω_1) sites as indicated by the preferential displacement of [^{3}H]flumazenil in BZ_1 (ω_1)-enriched structures as compared to BZ_2 (ω_2)-enriched structures in the mouse. In in vitro experiments, β-CCT antagonized the potentiation of the GABA-induced Cl^- current produced by zolpidem in HEK cells expressing the $\alpha_1\beta_2\gamma_2$ receptor or in cerebellar Purkinje neurones, while it failed to modify the diazepam potentiation at either $\alpha_3\beta_2\gamma_2$ or $\alpha_5\beta_3\gamma_2$ receptor subtypes. *Conclusion:* These results are consistent with the hypothesis that BZ_1 (ω_1) receptors play an important role in the anxiolytic and sedative/hypnotic effects of BZ (ω) receptor ligands, whereas activity at BZ_2 (ω_2) sites might be associated primarily with muscle relaxation.

Key words Anxiety · Benzodiazepine · β-CCT · BZ (ω) receptor · Convulsions · Diazepam · In vivo binding · Mice · Myorelaxation · Sedation · Zolpidem

G. Griebel (✉) · G. Perrault · V. Letang · P. Granger · P. Avenet
H. Schoemaker · D.J. Sanger
CNS Research Department, Synthélabo Recherche,
31, avenue Paul Vaillant-Couturier, F-92220 Bagneux, France
e-mail: ggriebel@bagneux.synthelabo.fr
Fax: +33-1-45-36-20-70

Introduction

Benzodiazepines (BZs) produce their pharmacological effects by allosterically modulating the action of GABA via specific recognition sites on the $GABA_A$ receptor called BZ_1 and BZ_2 (Squires et al. 1979; Sieghart and Schuster 1984) or ω_1 and ω_2, respectively (Langer and Arbilla 1988). $GABA_A$ receptors have a pentameric structure formed by the assembly of subunits from at least five different subunit families (α_{1-6}, β_{1-3}, γ_{1-3}, ρ_{1-2} and δ_1). It is now widely acknowledged that $GABA_A$ receptors bearing the BZ_1 (ω_1) recognition site correspond to receptors containing the α_1 subunit, while those bearing the BZ_2 (ω_2) site correspond to an heterogeneous population of $GABA_A$ receptors containing α_2, α_3 or α_5 subunits (for reviews, see Luddens et al. 1995; Sieghart 1995).

The heterogeneity of BZ (ω) receptors has prompted speculation that a particular behavioural response might be associated with action at a defined receptor subtype. The functional role of BZ (ω) receptor subtypes was initially based on the distinct pharmacological profile of the selective BZ_1 (ω_1) receptor agonist CL218,872 suggesting that BZ_1 (ω_1) receptors may mediate anticonvulsant and anxiolytic effects of BZ (ω) receptor agonists, whereas activity at BZ_2 (ω_2) sites might be associated primarily with central depressant effects (Lippa et al. 1979; Squires et al. 1979). However, this hypothesis was questioned by findings with the selective BZ_1 (ω_1) receptor agonist zolpidem which was found to display sedative activity at doses lower than those producing anticonvulsant, ataxic or myorelaxant effects (Depoortere et al. 1986; Perrault et al. 1990). In addition, the idea that BZ_1 (ω_1) receptors may mediate the anxiolytic action of BZs has also been challenged by several

studies showing that zolpidem either produced weaker anxiolytic-like effects than BZs (Depoortere et al. 1986; Griebel et al. 1996a, 1996b) or was devoid of such activity (Sanger and Zivkovic 1988; Perrault et al. 1990; Sanger 1995; Griebel et al. 1996c), depending on the test used. It was further suggested that the preferential sedative properties of zolpidem may have prevented the behavioural expression of anti-anxiety activity. In addition, the pharmacological effects observed with the selective BZ_1 (ω_1) receptor antagonist and β-carboline derivative β-CCT (β-carboline-3-carboxylate *t*-butyl ester) support further the idea that BZ_1 (ω_1) receptors mediate a subset of behavioural effects of BZs. In rats, β-CCT antagonized the anticonvulsant and anxiolytic-like activity of diazepam, but unlike flumazenil did not block its myorelaxant effects, thereby suggesting that BZ_2 (ω_2) receptor might be responsible for the muscle relaxation produced by BZs (Shannon et al. 1984). Binding studies indicate that zolpidem and β-CCT exhibit high binding affinity for α_1-containing receptors, whereas they show moderate to no affinity for α_2, α_3 and α_5 containing receptors (Pritchett and Seeburg 1990; Faure-Halley et al. 1993; Cox et al. 1995). In electrophysiological experiments, zolpidem potentiated GABA-induced Cl^- currents with greater efficacy in recombinant receptors expressing $\alpha_1\beta_2\gamma_2$ subunits than in those containing $\alpha_3\beta_2\gamma_2$ subunits, and showed no effect in cells expressing $\alpha_5\beta_3\gamma_2$ subunits, thereby confirming its functional selectivity for BZ_1 (ω_1) receptors (Besnard et al. 1997).

The aim of the present study was to investigate further the functional role of BZ (ω) receptor subtypes by studying the antagonism exerted by the selective BZ_1 (ω_1) receptor antagonist β-CCT on the pharmacological effects of zolpidem and the non-selective BZ (ω) receptor agonist diazepam in behavioural, biochemical and electrophysiological experiments. Behavioural procedures were designed to assess the anticonvulsant, anxiolytic-like, sedative and myorelaxant effects of BZ (ω) receptor agonists. In vivo binding studies of displacement of [^{3}H]flumazenil in different brain areas in the mouse and electrophysiological experiments using cell lines expressing different α subunits, were performed to confirm the regional and functional selectivity of β-CCT for $GABA_A$ receptors containing the α_1 receptor subtype.

Material and methods

Animals

Male CD1 (isoniazid-induced convulsions, actimeter and loaded grid test), OF1 (pentylenetetrazole-induced convulsions and in vivo binding) and BALB/c (light/dark test) mice weighing 18–25 g were supplied by Charles River (Saint-Aubin-les-Elbeuf, France) and Iffa Credo (L'Arbresle, France). CD1 and OF1 mice were housed in groups of 20, and BALB/c mice were housed in groups of six. In the electrophysiological experiments, cerebellar slices were obtained from Sprague-Dawley rats (5–9 days old, Charles River). All animals were maintained under standard laboratory conditions (21–22°C, relative humidity 40–55%) with free access to food and water. They were kept on a 12:12-h light-dark cycle with light onset at 6 a.m.

Drugs

The compounds used were diazepam, flunitrazepam, zolpidem, β-CCT, flumazenil (synthesized by the chemistry department, Synthélabo Recherche), isoniazid, pentylenetetrazole and GABA (Sigma Chemicals, St Louis, Mo., USA). In in vivo experiments, the drugs were prepared as solutions or suspensions in physiological saline containing Tween 80 (0.1%), while in in vitro studies they were diluted in DMSO which had a final concentration of 0.08%. All doses are expressed as the bases. In in vivo experiments, the drugs were administered intraperitoneally (IP) in a constant volume of 20 ml/kg 30 min before experiments were carried out. [^{3}H]-Flumazenil was purchased from New England Nuclear (Boston, Mass., USA).

Isoniazid-induced convulsions

Isoniazid (800 mg/kg, SC) was administered simultaneously with the test drugs. The anticonvulsant effect was assessed by measuring the latency to the appearance of the first convulsion. Isoniazid inhibits glutamic acid decarboxylase, the enzyme that catalyzes the synthesis of GABA from glutamic acid. The maximal delay in onset of isoniazid-induced seizures produced by a test compound may therefore be taken as an index of increased GABAergic function and has been proposed as an in vivo measure of the intrinsic activity of BZ (ω) receptor ligands at $GABA_A$ receptors (Mao et al. 1975). Data were analyzed using a two-way (dose×pre-treatment) ANOVA followed by Dunnett's *t*-test. ED_{50} values were calculated by linear regression.

Pentylenetetrazole-induced convulsions

At 30 min after injection of the test drugs or the vehicle, mice were given a SC injection of 125 mg/kg pentylenetetrazole (Swinyard et al. 1989). The occurrence of tonic extension of the hindlimbs was noted during the 30-min period which followed. This behaviour was chosen because it was the final response displayed before death occurred.ED_{50} values were calculated by the probit method of Litchfield and Wilcoxon (1949).

Light/dark test

This well-validated mouse model of anxiety is based on that described by Misslin et al. (1989). The apparatus consisted of two polyvinylchloride boxes (20×20×14 cm) covered with Plexiglas. One of these boxes was darkened. A neon tube fixed on the ceiling provided the room illumination so that the light intensity in the centre of the illuminated box was 150 lux. An opaque plastic tunnel (5×7×10 cm) separated the dark box from the illuminated one. At the beginning of the experiment, a mouse was placed in the illuminated box, facing the tunnel. Recording started when the animal entered the tunnel for the first time. The apparatus was equipped with infrared beams and sensors capable of recording the following three parameters during a 4-min period: (a) time spent by the mouse in the lit box; (b) attempts at entry into the lit box followed by avoidance responses. This includes stretched attend posture (the mouse stretches forward and retracts to original position) (Rodgers 1997); (c) total number of tunnel crossings. Data were analyzed using the non-parametric Kruskal-Wallis test.

Actimeter

Sedative activity was assessed in square, clear Plexiglas boxes (22×27×10 cm) equipped with infrared beams and sensors. They were placed in sound attenuated cupboards. Horizontal locomotor activity was quantified as total number of beams crossed during a 10-min period. Data were analyzed using two-way (dose×pre-treatment) ANOVA followed by Dunnett's *t*-test. ED_{50} values were calculated by linear regression.

Loaded grid test

This test was used to examine the myorelaxant properties of the drugs (Fleury 1957). During the pre-test, mice were held by their tails and allowed to grip with their front paws a metal grid to which increasing weights were attached. Animals which continued to grip 30 g for 10 s were selected for the experiment. Several hours later, each mouse was given a second pre-test and was then injected with the test drugs or vehicle. Thirty minutes later, each animal was re-tested and the maximum weight tolerated was recorded. Data were analyzed using two-way (dose×pre-treatment) ANOVA followed by Dunnett's *t*-test. ED_{50} values were calculated by linear regression.

In vivo binding

Experiments on in vivo binding of [^{3}H]-flumazenil to the mouse brain were performed as described by Goeders and Kuhar (1985) and Benavides et al. (1988). [^{3}H]-Flumazenil (specific activity, 92 Ci/mmol) was prepared in physiological saline and injected at a dose of 2.5 µCi/mouse via the tail vein in a constant volume of 0.2 ml. Animals were killed by decapitation 5 min later and their brains were rapidly removed and dissected. For the determination of non-specific binding, mice were pretreated with flunitrazepam (5 mg/kg, IP) 30 min prior to the radioligand injection. Cerebral cortex, cerebellum, striatum, spinal cord and hippocampus were dissected out on ice, weighed and solubilized in 0.5 ml Soluene (Packard, Downers Grove, Ill., USA) at 50°C before the addition of 10 ml scintillation cocktail.

Electrophysiological experiments

In order to compare the functional properties of β-CCT at BZ (ω) receptors, we performed whole-cell patch-clamp experiments with HEK293 cells stably expressing recombinant $\alpha_1\beta_2\gamma_2$, $\alpha_3\beta_2\gamma_2$ or $\alpha_5\beta_3\gamma_2$ $GABA_A$ receptors and with cerebellar Purkinje neurons known to express the α_1, β_2, and γ_2 receptor subunits. It is now well established that diazepam potently but non-selectively potentiates the Cl^- current at $\alpha_1\beta_2\gamma_2$, $\alpha_3\beta_2\gamma_2$ and $\alpha_5\beta_3\gamma_2$ $GABA_A$ receptors, whereas zolpidem potently acts only at α_1-containing receptors (Besnard et al. 1997). We therefore evaluated the efficacy of β-CCT to antagonize the potentiation induced by zolpidem at $\alpha_1\beta_2\gamma_2$ receptors and that by diazepam at α_3 and α_5 receptors.

Cell preparations

Cell lines expressing rat $\alpha_1\beta_2\gamma_2$, $\alpha_5\beta_3\gamma_2$ or $\alpha_3\beta_2\gamma_2$ $GABA_A$ receptor combinations were maintained in appropriate culture conditions (Besnard et al. 1997). Cells were scraped from the culture flask and transferred to a plastic-bottomed patch-clamp recording chamber in which they settled. The chambers were placed on the stage of an inverted microscope (Olympus IMT2) equipped with Hoffman optics (Modulation Contrast, New York, USA) and the cells viewed at a total magnification of ×400. A polyethylene tube (opening 500 µm) connected to a solution distributor was moved to within 3 mm of the cell under investigation and allowed fast superfusion of solutions (3–5 ml/min).

To record from Purkinje neurons in cerebellar slices, rats were anaesthetized with halothane (4%, 2 min), decapitated and the cerebellum was dissected out and removed from the meninges under a dissecting microscope in oxygenated aCSF (95% O_2/5% CO_2). The cerebellum was then embedded in agar 2% at a temperature just below 37°C. The agar piece containing the cerebellum was glued on the Perspex stage of a vibroslicer (Campden 752, Campden Instruments Ltd, Loughborough, UK) and slices of 180–200 µm were prepared. Slices were allowed to recover for 1 h in an oxygenated aCSF at room temperature and then transferred into a glass-bottomed recording chamber where they were maintained by a grid of nylon threads glued to a U-shaped platinum frame. Cells were viewed using an upright microscope (Nikon, Optiphot 2) equipped with Normaski optics and an infrared videocamera (Hamamatsu C2400, Hamamatsu Photonics K.K., Sunayama-Cho, Japan). Purkinje cells were identified by their large size and their localization in the cerebellar cortex between the molecular and the granular cell layers. Patch clamping was performed 20–40 µm within the slice. Slices were permanently superfused with Hepes buffered solution (H-aCSF) using a multiway perfusion system (ML/M SPS 8, List-Medical, Darmstadt, Germany) which also allowed drug application.

Patch-clamp

The whole-cell configuration of the patch-clamp technique was used. Pipettes were pulled from thick-walled borosilicate glass capillaries (Phymep, Paris, France) on a two-stage puller and had a resistance of 5–10 MΩ when filled with the pipette solution. Pipettes were brought into contact with the cells with a 3D piezoelectric micromanipulator (Burleigh PCS1000, Optilas, Evry, France). Whole-cell currents were recorded with an Axopatch 1D (Axon Instruments) connected to a 386 DX personal computer driven by pClamp software (Axon Instruments). For the measurement of GABA-induced currents, cells were clamped at –20 mV and the current recorded on videotape through a PCM (Vetter, Rebersburg, USA) for off-line analysis or directly digitized at 25 Hz by using the Axotape software. Cl^- currents were elicited by concentrations of GABA giving 5–10% of the maximal response, i.e. 0.3 µM for $\alpha_1\beta_2\gamma_2$ transfected cells and 1 µM for Purkinje neurons, $\alpha_3\beta_2\gamma_2$ and $\alpha_5\beta_3\gamma_2$ transfected cells. For each cell, the potentiations were first measured by comparing the Cl^- current obtained in response to GABA alone with that obtained in the presence of zolpidem or diazepam and then antagonized by co-application of β-CCT.

Data analysis and statistics

The positive modulatory effects of zolpidem and diazepam were evaluated by their absolute potentiation factors P_Z and P_D and calculated as follow: $P_Z=I_{GZ}/I_G$ and $P_D=I_{GD}/I_G$, where I_G is the current induced by GABA alone and I_{GZ}, I_{GD} the currents induced by GABA in presence of zolpidem and diazepam, respectively. The percentage inhibition by β-CCT of the potentiation was determined by the following equation:

$$Inh(\%) = 100 * \left[1 - \frac{(P_{GMB} - 1)}{(P_{GM} - 1)}\right]$$

where P_{GM} and P_{GMB} are the absolute potentiation factors of the modulator in the absence or in presence of β-CCT, respectively.

Results

Behavioural experiments

Isoniazid-induced convulsions

Table 1 shows that β-CCT alone did not significantly increase the latencies to isoniazid-induced convulsions up to 60 mg/kg. Both diazepam [$F(11,107)=78.6$, $P<0.001$] and zolpidem [$F(11,108)=71.9$, $P<0.001$] showed anticonvulsant activity, producing dose-related antagonism of clonic convulsions induced by injection of isoniazid (Fig. 1 and Table 2). Post-hoc analysis revealed that the latencies to convulsions were significantly increased by both drugs from 1 mg/kg. In the presence of β-CCT (30 mg/kg), the dose-response curves for both zolpidem and diazepam were shifted to the right, indicating the blockade of their anticonvulsant activity.

Pentylenetetrazole-induced convulsions

β-CCT (3–60 mg/kg) alone did not block the convulsant effects of 125 mg/kg pentylenetetrazole (Table 1). Diazepam and zolpidem produced a dose-related increase in the number of mice protected with ED_{50} values of 0.7 and 11 mg/kg, respectively (Fig. 2 and Table 2). β-CCT (30 mg/kg) did not modify the anticonvulsant effects of diazepam and zolpidem, while in the presence of 5 mg/kg flumazenil the ED_{50} values for diazepam and zolpidem were increased approximately 3-fold and 4-fold.

Light/dark test

β-CCT (3–60 mg/kg) alone had no significant effect on the behaviour of mice in the light/dark test (Table 1). Diazepam displayed clear anxiolytic-like effects, increasing the time spent by mice in the bright area [K=47.37, P<0.001] and the total number of tunnel crossings [K=43.99, P<0.001], while decreasing the number of aborted attempts at entry into the lit box [K=45.12, P<0.001] (Fig. 3). All anxiolytic-like effects of diazepam were fully antagonized by 30 mg/kg β-CCT.

Table 1 Effects of β-CCT alone in the behavioural tests used in this study. Data represent mean±SEM. n=10–15

Test	Parameter	β-CCT (mg/kg, IP-30')					
		0	3	10	30	60	Statistics
Isoniazid-induced convulsions	Latency to convulsions (min)	18.4±0.8	19.6±1.1	21.4±1.1	21.7±1.0	23.0±1.5	F=2.3, P=0.06
Pentylenetetrazole-induced convulsions	Percent inhibition	0	0	0	0	0	ED_{50}>60
Light/dark	Time in lit box (s)	4.8±3.8	7.4±7.2	1.6±1.6	4.6±4.6	29.3±18.8	K=5.5, P=0.23
	Attempts	20.1±1.8	19.0±3.8	15.5±2.5	16.7±3.0	16.1±2.7	K=4.9, P=0.3
	Tunnel crossings	3.0±1.4	2.8±1.6	1.2±0.2	2.0±1.0	4.2±1.7	K=2.1, P=0.71
Actimeter	Beams crossed	307±22	375±39	337±25	375±39	370±43	F=1.6, P=0.21
Loaded grid	Weight carried (g)	30±1.1	29±1.2	27±1.1	28±1.1	28.5±1.3	F=0.9, P=0.46

Table 2 ED_{50} values for anticonvulsant and central depressant effects, and minimal effective dose (*MED*) for anxiolytic-like activity calculated from dose-response functions. *NT* indicates not tested, *ND* not determined (the dose-response curve did not fit to the regression analysis within 95% confidence limits)

	ED_{50} (mg/kg, IP)				MED (mg/kg, IP)
	Anticonvulsant activity		Central depressant effects		Anxiolytic-like effects
	Pentylenetetrazole-induced convulsions	Isoniazid-induced convulsions	Actimeter	Loaded grid	Light/dark test
β-CCT	>60	>60	>60	>60	>60
Diazepam	0.7±0.4	2.4±2.0	8.4±2.1	1.1±0.6	2.5
Zolpidem	11.6±8.2	1.8±0.6	2.5±0.6	NT	NT
Diazepam+β-CCT (30 mg/kg)	0.6±0.4	10±ND	13.6±6.3	2.2±0.5	>5
Zolpidem+β-CCT (30 mg/kg)	17.0±12.0	10±ND	>10	NT	NT
Diazepam+flumazenil (5 mg/kg)	2.0±1.1	NT	NT	7.4±1.1	NT
Zolpidem+flumazenil (5 mg/kg)	43.6±104.9	NT	NT	NT	NT

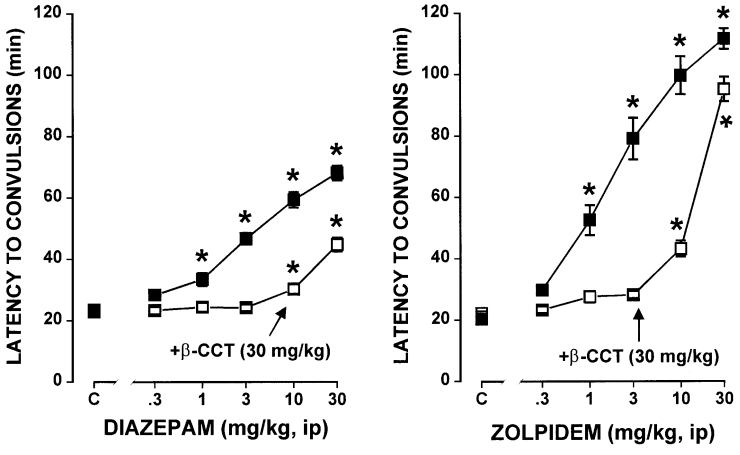

Fig. 1 The anticonvulsant effects of diazepam or zolpidem alone (*filled squares*) or in combination with β-CCT (*open squares*) against clonic seizures produced in mice by 800 mg/kg, SC of isoniazid. Data represent mean±SEM. *P<0.05 (Dunnett's t-test). n=10

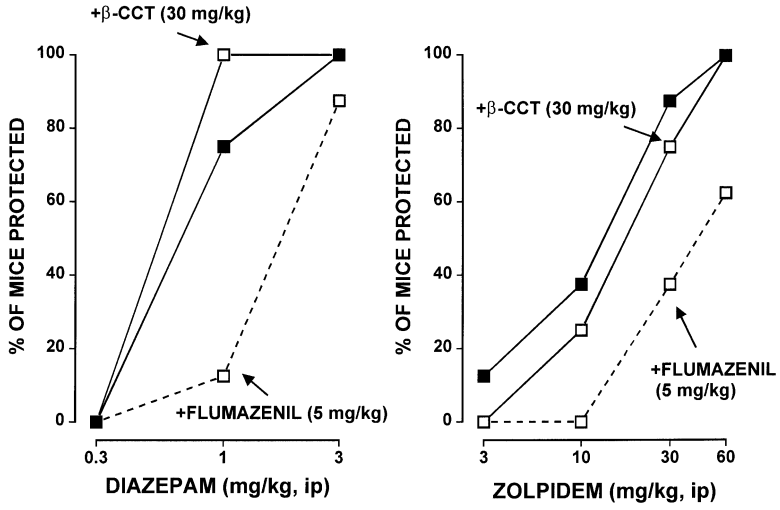

Fig. 2 The anticonvulsant effects of diazepam and zolpidem alone (*filled squares*) or in combination with β-CCT or flumazenil (*open squares*) against tonic seizures produced in mice by pentylenetetrazole (125 mg/kg, SC). Results are shown as the percentage inhibition of the convulsions (i.e. number of mice not convulsing/number of animal tested×100). *n*=10

The interaction between zolpidem and β-CCT was not performed as zolpidem was found inactive in this test in a previous study (Griebel et al. 1996b).

Actimeter

β-CCT (3–60 mg/kg) alone did not significantly modify spontaneous locomotor activity (Table 1), whereas both diazepam [$F(9,70)=11.71$, $P<0.001$] and zolpidem [$F(9,75)=3.38$, $P<0.01$] dose-dependently reduced the number of beams crossed with ED_{50} values of 8.4 and 2.5 mg/kg, respectively. Figure 4 shows that the dose-effect curves of diazepam and zolpidem were shifted significantly to the right by 30 mg/kg β-CCT (ED_{50}=13.6 and >10 mg/kg, respectively). β-CCT fully reversed the decrease in locomotor activity produced by zolpidem. In contrast, the β-carboline failed to block the hypolocomotor effects of diazepam at the highest dose (30 mg/kg), indicating weaker antagonism than that observed with zolpidem.

Loaded grid test

β-CCT (3–60 mg/kg) alone was without effect in this test (Table 1). Figure 5 shows that diazepam [$F(9,90)=149.3$, $P<0.001$] produced a dose-related reduction in the maximum tolerated weight, an effect which was antagonized by 5 mg/kg flumazenil. β-CCT (30 mg/kg) produced some degree of antagonism, reversing weakly, but significantly, the effects of 3 mg/kg only of diazepam [$F(9,90)=102.9$, $P<0.001$].

In vivo [^{3}H]flumazenil binding in the mouse brain

Figure 6 shows that at the dose of 30 mg/kg β-CCT decreased the amount of radioactivity measured in all five structures. Specific binding was inhibited with different efficacies, depending on the structure. Thus, the greatest

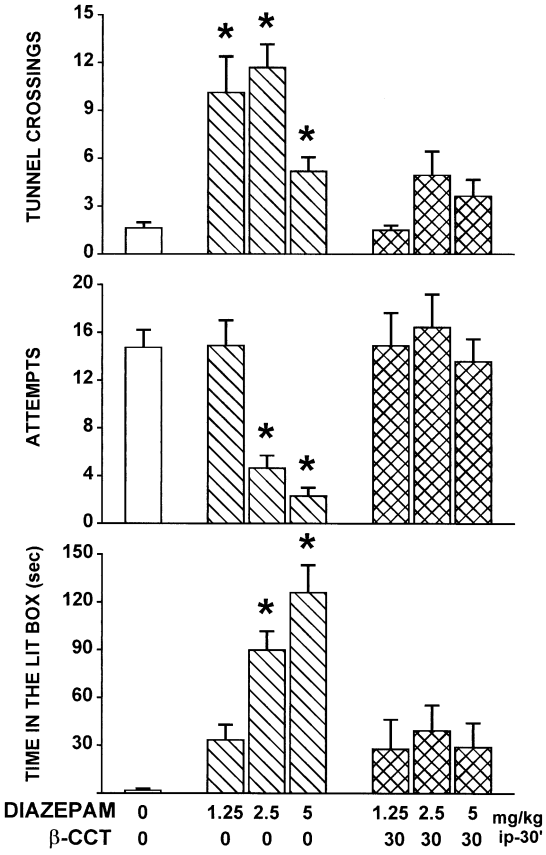

Fig. 3 Effects of diazepam alone or in combination with β-CCT in the light/dark anxiety model in mice. Data represent mean±SEM. *$P<0.05$ (Kruskal-Wallis test). *n*=15

inhibitions of in vivo [^{3}H]flumazenil binding (96 and 90%) were observed in the cerebellum and the cerebral cortex, respectively. In the striatum, hippocampus and spinal cord, the specific binding was decreased by 80, 61 and 60%, respectively.

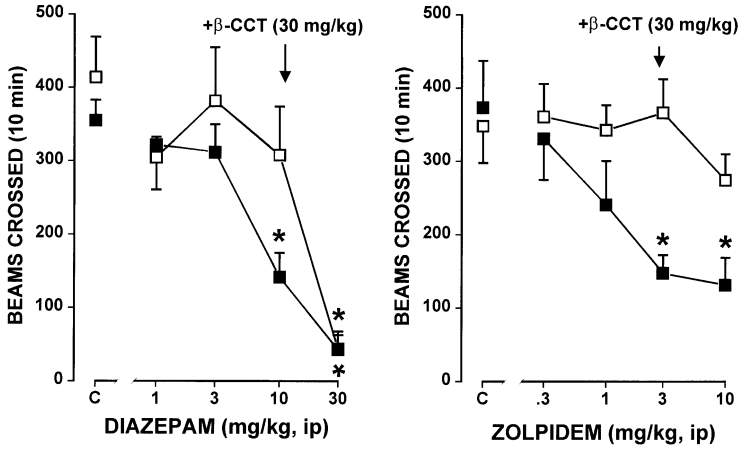

Fig. 4 Effects of diazepam or zolpidem alone (*filled squares*) or in combination with β-CCT (*open squares*) on spontaneous activity of mice in the actimeter test. Data represent mean±SEM. **P*<0.05 (Dunnett's *t*-test). *n*=10

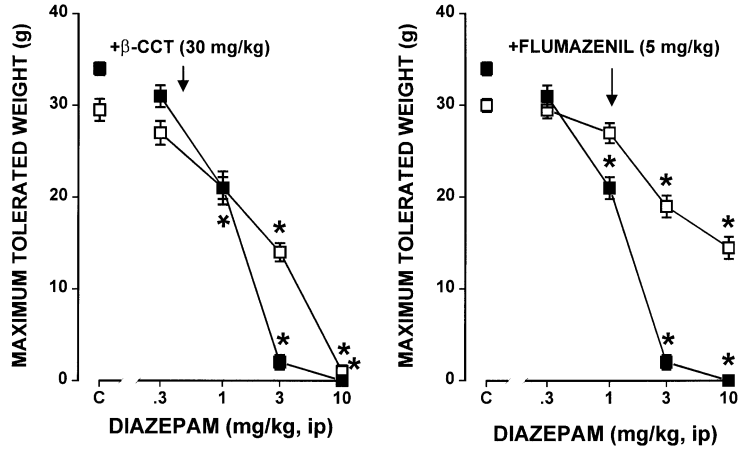

Fig. 5 Effects of diazepam alone (*filled squares*) or in combination with β-CCT (*open squares*) on muscle strength in the loaded grid test in mice. Data represent mean±SEM. **P*<0.05 (Dunnett's *t*-test). *n*=10

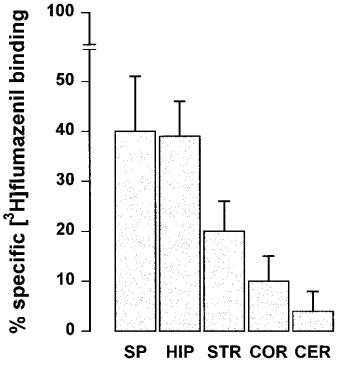

Fig. 6 Inhibition of the in vivo [^{3}H]flumazenil binding by 30 mg/kg of β-CCT to central BZ binding sites in the mouse spinal cord (*SP*), hippocampus (*HIP*), striatum (*STR*), cerebral cortex (*COR*) and cerebellum (*CER*). The drug was administered IP 30 min before death. Each bar represents the mean±SEM of data obtained from five animals

Electrophysiological studies

In HEK cells expressing the $\alpha_1\beta_2\gamma_2$ receptor combination, 1 μM of zolpidem potentiated the GABA-induced Cl^- current by a factor of 3.82±0.31 (*n*=7). As shown in Fig. 7, the co-application of β-CCT at concentrations of 0.01, 0.1 and 1 μM led to an antagonism of this potentiation by 25.5±3.6, 71.1±4.9 and 91.6±2.4%, respectively. In stably transfected cell lines expressing $\alpha_5\beta_3\gamma_2$ or $\alpha_3\beta_2\gamma_2$ receptor combinations, 1 μM diazepam potentiated the current responses GABA by a factor of 1.92±0.17 (*n*=3) and 4.22±0.25 (*n*=4), respectively. Co-application of β-CCT 0.01 μM did not antagonize the diazepam potentiation at either $\alpha_5\beta_3\gamma_2$ or $\alpha_3\beta_2\gamma_2$ receptor subtypes. The antagonist properties of β-CCT began to be clearly measurable at the concentration of 0.1 μM, reducing by 24±2.1 and 28±5.7% the diazepam potentiation at the α_3- and α_5-containing recombinant receptors, respectively. Even at 1 μM (the highest concentration tested), β-CCT was unable to antagonize more than 53.25±3.3 and 41.3±2.8% of the positive modulatory effect of diazepam at $\alpha_5\beta_3\gamma_2$ or $\alpha_3\beta_2\gamma_2$ receptor subtypes.

In Purkinje cells recorded from cerebellar slices, 1 μM zolpidem potentiated the Cl^- current induced by 1 μM GABA by a factor of 3.5±0.20 (*n*=26). Co-application of 0.01, 0.1 and 1 μM β-CCT led to an inhibition of, respectively, 32±6 (*n*=3), 70±8 (*n*=5) and 73±2% (*n*=6) of the positive modulatory effect of zolpidem. As shown in Fig. 7, β-CCT antagonized the potentiation of zolpidem with the same efficacy and potency in Purkinje cells as in stably transfected $\alpha_1\beta_2\gamma_2$ recombinant receptors.

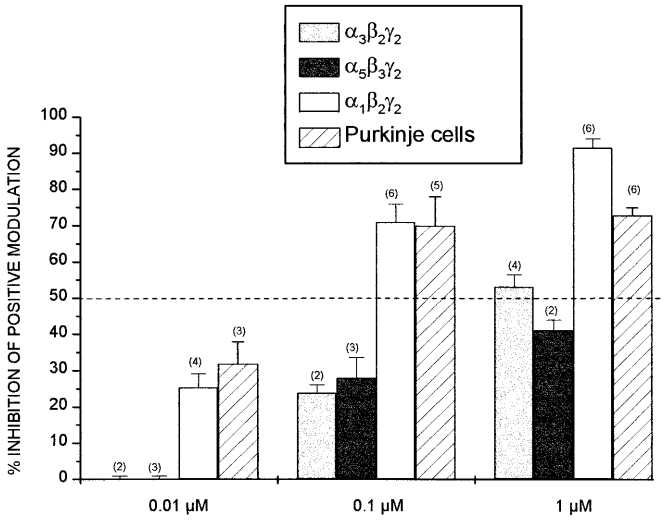

Fig. 7 The potentiation by zolpidem or diazepam of the GABA-induced Cl^- currents were antagonized by β-CCT in a receptor subtype dependent manner. For each cell, the potentiations were first measured by comparing the Cl^- current obtained in response to GABA alone with that obtained in the presence of zolpidem ($\alpha_1\beta_2\gamma_2$ and Purkinje cells) or diazepam ($\alpha_5\beta_3\gamma_2$and $\alpha_3\beta_2\gamma_2$) and then antagonized by co-application of β-CCT. Values are expressed as mean±SEM The number of cells is indicated in *brackets*

Discussion

The results of the present experiments showed that the preferential BZ_1 (ω_1) receptor antagonist β-CCT antagonized some but not all behavioural effects of the non-selective BZ (ω) receptor full agonist diazepam and the selective BZ_1 (ω_1) receptor full agonist zolpidem. These findings corroborate further the idea that the different BZ (ω) receptor subtypes may subserve different neural functions and that the behavioural effects of BZs may be mediated by different subtypes (Shannon et al. 1984; Zivkovic et al. 1992; Sanger et al. 1994).

When administered alone up to 60 mg/kg, β-CCT behaved as a neutral ligand as it was devoid of activity in all behavioural procedures used in this study. The drug was without anticonvulsant or proconvulsant actions against pentylenetetrazole- or isoniazid-induced seizures. Similarly, in the light/dark test, β-CCT failed to modify any behavioural measure indicating that it exhibited neither anxiolytic- nor anxiogenic-like effects. Finally, in the tests for motor activity (actimeter and loaded grid), the compound did not impair spontaneous locomotion or muscle strength.

In the pentylenetetrazole- and isoniazid-induced seizure tests, β-CCT dose-dependently antagonized the effects of diazepam and zolpidem on the latency to clonic seizures produced by isoniazid, but it failed to affect the action of these compounds on the tonic seizures produced by pentylenetetrazole. The anticonvulsant effects of diazepam and zolpidem against pentylenetetrazole were reversed by the non-selective BZ (ω) receptor antagonist flumazenil. Based on the selectivity of β-CCT for the BZ_1 (ω_1) receptor subtype, these findings suggest that distinct BZ (ω) receptor subtypes may be involved in the convulsant effects of pentylenetetrazole and isoniazid. This idea is strengthened by the observation that diazepam antagonized pentylenetetrazole-induced seizures at lower doses than those protecting against isoniazid, whereas zolpidem blocked isoniazid-induced convulsions at much lower doses than those inhibiting the convulsant effects of pentylenetetrazole.

The lack of interaction between β-CCT and diazepam in the pentylenetetrazole convulsion model contrasts with the results obtained by Shannon et al. (1984) who showed that β-CCT (10 and 30 mg/kg, IP) blocked the anticonvulsant effects of diazepam (1 mg/kg, IP) in the pentylenetetrazole model. However, the use of a lower dose of the convulsant agent (80 mg/kg versus 125 mg/kg in this study) which produces clonic but not always tonic convulsions and/or a different administration route (IP versus SC in this study) may at least in part account for this discrepancy. In contrast, the present findings with β-CCT in the convulsion models fit well with previous studies showing that the selective BZ_1 (ω_1) agonist zolpidem is about 7 times less potent against pentylenetetrazole convulsions than against isoniazid (Depoortere et al. 1986; Zivkovic et al. 1988; Perrault et al. 1990). Conversely, non-selective BZ (ω) receptor agonists such as triazolam, flunitrazepam, brotizolam, zopiclone or midazolam were 4–24 times more potent in antagonizing pentylenetetrazole-induced, than isoniazid-induced convulsions. These findings led to the suggestion that BZ_1 (ω_1)-selective ligands are more efficacious than non-selective BZ (ω) ligands in inducing effects which involve GABAergic mechanisms. Indeed, convulsions induced by isoniazid, which is an inhibitor of glutamic acid decarboxylase, the enzyme involved in the GABA synthesis, have been proposed to be an index of intrinsic activity at $GABA_A$ receptors (Mao et al. 1975). Similar differences between selective and non-selective BZ (ω) receptor ligands have been observed by Sanger (1995), who showed that several non-selective BZ (ω) full (e.g. chlordiazepoxide, clorazepate, alprazolam) and partial (e.g. bretazenil, Ro 19-8022) agonists antagonized the pentylenetetrazole discriminative stimulus, while the BZ_1 (ω_1) selective ligands abecarnil, zaleplon, zolpidem, CL 218,872 and alpidem produced only partial antagonism. Together, these findings suggest that pentylenetetrazole-induced convulsions may be associated with an action at BZ_2 (ω_2) receptor subtypes.

In the light/dark anxiety model, β-CCT completely abolished the anxiolytic-like action of diazepam over the entire dose-range (1.25–5 mg/kg). The β-carboline reversed the effects of diazepam on both the spatio-temporal (i.e. the time spent by mice in the lit box and the number of tunnel crossings) and the more ethologically derived (i.e. attempts at entry into the lit box followed by withdrawal responses) measures of anxiety. This result confirms previous findings with β-CCT showing that the drug (10 mg/kg, IP) blocked the rate-increasing effects of diazepam on punished behaviour in rats (Shannon et al. 1984). Although these data are entirely consistent with the hypothesis that BZ_1 (ω_1) receptors play a crucial role in anxiety, they need

to be confirmed in other tests of anxiety. This hypothesis which was initially proposed by Lippa and colleagues (1979) on the basis of the finding that the BZ_1 (ω_1) receptor partial agonist CL 218,872 exerted anxiolytic-like effects in the punished drinking test in rats, has been challenged by a number of other results showing that BZ_1 (ω_1)-selective compounds have weaker anxiolytic-like effects than non-selective BZ (ω) agonists (Depoortere et al. 1986; Stephens et al. 1993; Jones et al. 1994; Griebel et al. 1996b) or are even devoid of such activity (Sanger and Zivkovic 1988; Perrault et al. 1990; Sanger 1995; Griebel et al. 1996c) in a variety of anxiety models. However, these latter studies used mainly BZ_1 (ω_1) receptor ligands with high intrinsic activity (e.g. zolpidem, zaleplon or abecarnil) as was demonstrated by in vitro and in vivo data. For example, zolpidem and zaleplon displayed a very large increase in the latency to clonic seizures produced by isoniazid (Perrault et al. 1990; Sanger et al. 1996). Furthermore, abecarnil enhanced the function of GABA-coupled chloride channel with a potency and efficacy even greater than the full agonist diazepam as revealed by the action on [^{35}S]TBPS binding in the rat and the mouse (Serra et al. 1993). As a result, these drugs have a marked propensity to decrease spontaneous locomotor activity. Taken together, these results led to the suggestion that the anxiolytic-like effects of the BZ_1 (ω_1) receptor ligands may have been partially or completely masked by behavioural sedation, depending on the anxiety test employed.

The analysis of the effects of β-CCT on the action of diazepam and zolpidem in the actimeter revealed that the β-carboline antagonized the decrease in spontaneous locomotor activity produced by 10 mg/kg of diazepam and by zolpidem from 3 mg/kg. These effects are usually considered to reflect the sedative/hypnotic activity of BZ (ω) receptor agonists. In contrast, unlike flumazenil, β-CCT failed to modify clearly the myorelaxant effects of diazepam in the loaded grid test. It is unclear why β-CCT failed to modify the myorelaxant effects of diazepam at 1 and 10 mg/kg, while blocking them at the intermediate dose of 3 mg/kg. It is possible that at doses higher than 30 mg/kg, β-CCT may have produced a clearer antagonism of these effects, thereby confirming its only moderate selectivity for BZ_1 (ω_1) receptors. It is interesting to note that a previous study using the inverted screen test for ataxia has also found little interaction between β-CCT (30 mg/kg), and diazepam (0.5–10 mg/kg) as the β-carboline did not significantly affect the diazepam-induced increase in the number of mice which fell from a wire mesh screen after its inversion (Shannon et al. 1984). Together, these findings are compatible with the idea that the sedative/hypnotic effects of BZ (ω) receptor agonists are produced by activity at BZ_1 (ω_1) sites, whereas the myorelaxant and ataxic effects presumably involve BZ_2 (ω_2) receptors (for review, see Sanger et al. 1994). This hypothesis is based mainly on the observation that the selective BZ_1 (ω_1) receptor full agonist zolpidem impairs performance in the loaded grid and rotarod tests at doses 17–18 times higher than those reducing exploratory activity, while non-selective BZ (ω) receptor full agonists reduce locomotor activity at doses similar to or higher than those producing a deficit in rotarod performance and in grip strength (Depoortere et al. 1986; Zivkovic et al. 1988; Perrault et al. 1990). Moreover, the present results are in line with previous data showing that abecarnil did not influence spinal reflexes and, in genetically spastic rats, did not affect muscle tone (Turski and Stephens 1993).

The present findings of a complete failure of β-CCT to block the hypolocomotor effects of diazepam at 30 mg/kg, while it blocked clearly these effects at 10 mg/kg appear somewhat unexpected. However, it is likely that in addition to sedation, muscle relaxation may have played a particularly important role in the deficits of performance observed in the actimeter at such a high dose of diazepam.

The in vivo interaction of β-CCT with mouse brain BZ binding sites, as labelled by IV injection of [^{3}H]flumazenil, showed that the drug prevented binding in several brain regions including the hippocampus, the striatum, the cerebral cortex and the cerebellum. However, β-CCT interacts preferentially with BZ_1 (ω_1)-enriched structures (i.e. cerebellum) than with BZ_2 (ω_2)-enriched structures (i.e. spinal cord, hippocampus, striatum). A similar profile was obtained in a previous study with the selective BZ_1 (ω_1) receptor agonist zolpidem (Benavides et al. 1988). In line with these data, the electrophysiological experiments showed that 0.01 μM β-CCT antagonized the potentiation of the GABA-induced Cl^- current produced by zolpidem in the HEK cells expressing the $\alpha_1\beta_2\gamma_2$ receptor or in cerebellar Purkinje neurons known to express this receptor subtype, while the same dose of β-CCT failed to modify the diazepam potentiation at neither $\alpha_3\beta_2\gamma_2$ nor $\alpha_5\beta_3\gamma_2$ receptor subtypes. Although at higher concentrations of β-CCT (i.e. 0.1 μM and 1 μM) there was some evidence of blockade of the effects of diazepam at these two receptors, it is important to note that even at 1 μM, this antagonism barely reached 50% of the positive modulatory effects of diazepam. Based on the findings that $GABA_A$ receptor containing α_1 subunits display BZ_1 (ω_1) pharmacology, whereas α_3- and α_5-containing $GABA_A$ receptors exhibit BZ_2 (ω_2) pharmacology (Pritchett and Seeburg 1990; Besnard et al. 1997), these results are in agreement with in vitro binding results showing that β-CCT has higher affinity for recombinant $GABA_A$ receptors containing α_1 subunits than for those containing α_3 or α_5 subunits (Cox et al. 1995).

In summary, the results of this study confirmed the selectivity of the β-carboline β-CCT for the BZ_1 (ω_1) receptor subtype. The drug antagonized the anticonvulsant effects of diazepam and zolpidem in the isoniazid test, but failed to block the effects of the former in the pentylenetetrazole test. In the light/dark test, β-CCT antagonized the anxiolytic-like effects of diazepam. Finally, the β-carboline reversed the sedative effects of diazepam and zolpidem, but did not modify the myorelaxant effects of diazepam. These findings thus provide additional evidence for the functional significance of BZ (ω) receptor subtypes. They are consistent with the hypothesis that BZ_1 (ω_1) receptors containing the α_1 subunit play an important role in the anxiolytic and sedative/hypnotic ef-

fects of BZ (ω) receptor ligands, whereas activity at BZ_2 (ω_2) sites on which β-CCT and zolpidem have weaker affinity, might be associated primarily with muscle relaxation. Further work with compounds that are selective for other subtypes than those containing the α_1 subunit or with genetically modified animals are necessary to evaluate the actual contribution (if any) of BZ_2 (ω_2) receptors in the anxiolytic-like effects of BZ (ω) receptor ligands.

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