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A Three-State Model of the Benzodiazepine Receptor Explains the Interactions Between the Benzodiazepine Antagonist Ro 15-1788, Benzodiazepine Tranquilizers, β-Carbolines, and Phenobarbitone

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Summary. The potent benzodiazepine receptor ligands β-carboline-3-carboxylic acid ethyl ester (β-CCE) and the corresponding methylester (β-CCM) administered i.v. depressed segmental dorsal root potentials in spinal cats, reversed the prolongation of dorsal root potentials by phenobarbitone, and abolished the depression of a motor performance task induced by phenobarbitone in mice; β-CCE enhanced the low-frequency facilitation of pyramidal population spikes in the hippocampus of anaesthetized rats. These effects of β-carbolines reflect a depression of GABAergic synaptic transmission and, thus, are diametrically opposed to the enhancing action of benzodiazepine tranquilizers. The specific benzodiazepine antagonist, Ro 15-1788, while not affecting dorsal root potentials, hippocampal population spikes or phenobarbitone-induced motor performance depression, abolished the effects of β-CCE on the three parameters and similar effects of β-CCM on the spinal cord and motor performance.

A three-state model of the benzodiazepine receptor is proposed in which benzodiazepine tranquilizers act as agonists enhancing the function of the benzodiazepine receptor as a coupling unit between GABA receptor and chloride channel, β-carbolines act as "inverse agonists" reducing this coupling function, and Ro 15-1788 represents a competitive antagonist blocking both the enhancing effect of agonists and the depressant effect of "inverse agonists" on GABAergic synaptic transmission.

Key words: Benzodiazepine antagonists – Ro 15-1788 – β-Carboline-3-Carboxylates – Phenobarbitone – GABAergic transmission

Introduction

Several structurally differing ligands with high affinity for benzodiazepine receptors have been found to act as benzodiazepine antagonists. The imidazobenzodiazepine Ro 15-1788 prevents or blocks selectively centrally mediated actions of benzodiazepine tranquilizers and lacks intrinsic effects in most neuropharmacological procedures (Hunkeler et al. 1981; Möhler et al. 1981; Polc et al. 1981a; Bonetti et al. 1982). Similar effects were described for phenylpyrazoloquinolinones (Bernard et al. 1981). In contrast, ethyl- and methyl-β-carboline-3-carboxylate (β-CCE and β-CCM, Braestrup et al. 1980) antagonize the effects of benzodiazepine tranquilizers at dose levels which produce behavioural and electrophysiological effects that are opposite to those of benzodiazepines (Oakley and Jones 1980; Tenen and Hirsch 1980; Cepeda et al. 1981; Cowen et al. 1981; Jones and Oakley 1981; O'Brien et al. 1981; Polc et al. 1981b; Braestrup et al. 1982).

Nutt et al. (1982) recently showed that Ro 15-1788 blocks not only the effect of diazepam on pentetrazole seizure thresholds in rats and on GABA-induced depolarization in isolated cervical sympathetic ganglia, but also the opposite effect of β-CCE on these two parameters. They suggested that these three drugs interacted with the benzodiazepine receptor to stabilize one of two possible functional (active and inactive) states. The aim of the present investigation was to study the interaction between Ro 15-1788 and β-carbolines a) on two central GABAergic synapses previously shown to be affected by benzodiazepines and b) in a test situation in which the GABA receptor function is altered by phenobarbitone, thought to affect the chloride ionophore portion of the GABA receptor-effector complex (Olsen 1981). A three-state model of the benzodiazepine receptor is proposed to explain the findings.

Materials and Methods

Cat Spinal Cord. The general set-up of the cat spinal cord preparation was described elsewhere (Polc et al. 1981a). A left dorsal rootlet L_7 was mounted on a bipolar Ag-AgCl electrode for DC recording of dorsal root potentials. The central end of the severed ipsilateral dorsal root S_1 was placed on a bipolar electrode for supramaximal stimulation (5–10 mA, single 0.05 ms pulses at 0.5 Hz). After amplification, eight consecutive dorsal root potentials were averaged, displayed on an oscilloscope and recorded photographically.

Drug effects were evaluated in the following way. The area of dorsal root potentials was measured planimetrically during a stable control period of 20 min and taken as 100%. The values measured 5–10 min after drug injection were expressed as per cent deviation from predrug values. The paired *t*-test was used for statistical analysis. Ro 15-1788 (aqueous microsuspension), β-CCE and β-CCM (dissolved in 0.1 ml/kg glycerolformal) were injected into the right femoral vein. Glycerolformal had no effect on spinal cord activities.

Rat Hippocampus. Male albino SPF Fü rats, weighing 300 g, were anaesthetized with urethane-chloralose (1.2 g/kg – 65 mg/kg i.p). A femoral vein and a femoral artery were cannulated for drug injections and for monitoring of the arterial blood pressure. Body temperature was kept at 37° C

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by means of a thermostatically controlled heating pad. Bipolar stainless steel stimulating electrodes (100 kΩ) were stereotaxically placed into the right fimbria hippocampi. Tungsten microelectrodes (5 MΩ) were used for recording population spikes from the pyramidal cell layer of the CA 1 region in the ipsilateral dorsal hippocampus. The pyramidal cell layer was identified by the depth from cortical surface (2.0 – 2.5 mm), the firing pattern of single units and the responses evoked by fimbrial stimulation. Population spikes evoked by single supramaximal stimuli (1 – 2 mA, 0.1 ms pulses) were amplified (band pass 10 Hz – 3 kHz) and recorded on magnetic tape. Amplitudes of population spikes were measured after analog-digital conversion (40.96 ms sweep, 10 μs resolution) using a DATALAB DL 4000. Facilitation of population spikes was induced by volleys of low frequency (2 Hz) stimulation of 6 s duration. The amount of low-frequency facilitation prior to drug administration was determined and taken as 100%. Postdrug low-frequency facilitation was then calculated for each animal. Drug effects were evaluated 5 min after injection. The paired *t*-test was used for statistical analysis. Ro 15-1788 was available as an aqueous microsuspension and β-CCE was dissolved in glycerolformal. The injection volume was 0.3 ml/kg.

Mouse Horizontal Wire Test. Albino SPF Fü mice of either sex, weighing 18 – 20 g, were lifted by the tail and allowed to grasp a horizontally strung wire with the forepaws and then released (Courvoisier 1956; Bonetti et al. 1982). The number of animals out of a total of 10 per treatment group was determined which did not grasp the wire with the forepaws or did not actively grasp the wire with at least one hindpaw within 3 s. In control animals administered acacia this number was consistently found to be zero; an impairment of grasping in 5 or more out of 10 mice is statistically significant (Chi-Square distribution). Phenobarbitone, L-cycloserine, meprobamate, β-CCE, β-CCM and Ro 15-1788 were given as suspensions in acacia by the routes indicated below.

Drugs Used. The following drugs were synthesized in our research department: Ro 15-1788, β-CCE, β-CCM, and L-cycloserine by Drs. Field, Hunkeler and Kyburz. Sodium phenobarbitone was purchased from Merck, Darmstadt, FRG, and meprobamate from Siegfried AG, Zofingen, Switzerland.

Results

Cat Spinal Cord

The interaction between Ro 15-1788 and β-CCE on segmental dorsal root potentials, which are at least partly mediated by axoaxonal GABAergic synapses on the primary afferent terminals in the spinal cord (Nicoll and Alger 1979), was studied in 5 cats. Five minutes after injection of 0.3 mg/kg β-CCE i.v. the area of dorsal root potentials was significantly reduced to 75.8% ± 5.3 S.E.M. of controls ($P < 0.05$). Similar effects were obtained in two cats with 0.1 mg/kg β-CCM i.v. In two further cats 1 and 3 mg/kg β-CCE i.v. depressed dorsal root potentials to about 60%; the higher dose induced spontaneous dorsal root potentials as seen with high doses of picrotoxin and bicuculline (Besson et al. 1971; McMillan and Mokha 1982) suggesting the induction of spinal seizures. Ro 15-1788 (0.3 mg/kg) administered i.v. 5 min after β-CCE

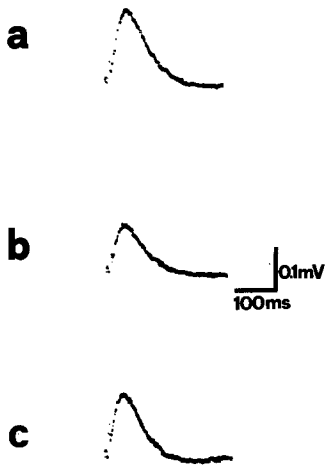

Fig. 1 a–c. Depression of segmental dorsal root potentials by β-CCE and its reversal by Ro 15-1788 in a spinal cat. Shown are averaged responses in a predrug control period (**a**), responses obtained 5 min after 0.3 mg/kg β-CCE i.v. (**b**), and responses recorded 5 min after 0.3 mg/kg Ro 15-1788 i.v. injected immediately after **b** (**c**)

(0.3 mg/kg i.v.), virtually abolished the β-CCE effect by increasing the area of dorsal root potentials to 93.6% ± 6.1 S.E.M. A typical example of the antagonism of the two drugs is shown in Fig. 1. In three additional experiments, 10 mg/kg phenobarbitone i.v. prolonged dorsal root potentials and this effect was consistently blocked by 0.3 mg/kg β-CCE or β-CCM i.v. Ro 15-1788 (0.3 mg/kg i.v.) reversed this effect of β-carbolines (data not shown).

Rat Hippocampus

Fimbria stimulation at 2 Hz and supramaximal intensity for evoking a population spike from CA 1 pyramidal cells consistently led to the appearance of a second population spike after 4 to 6 stimuli; in some experiments even a third population spike appeared after 5 s. This low-frequency facilitation was suggested to be due to a progressive reduction of GABAergic recurrent inhibition of pyramidal cells in the hippocampus (Ben-Ari et al. 1979). As shown for the last response in a volley in a typical experiment (Fig. 2), 1 mg/kg β-CCE i.v. clearly enhanced low-frequency facilitation of the second spike; in addition a third population spike appeared. The amplitude of the first population spike was only marginally enhanced. Total low-frequency facilitation of the second spike, i. e. the sum of the amplitudes of all 12 responses in a volley, was enhanced in all 5 animals studied (to 214% ± 46 of controls; 2 $P < 0.01$). Ro 15-1788 (1 mg/kg i.v.) abolished the effect of β-CCE (Fig. 2) by reducing the total low-frequency facilitation of the second population spike to control values (91% ± 15.5). A third population spike was no longer visible. Ro 15-1788, given alone at this dose, had no effect on population spikes in 3 rats (data not shown).

Mouse Horizontal Wire Test

As illustrated in Fig. 3, the impairment of performance induced by 100 mg/kg phenobarbitone i.p. was abolished by 100 mg/kg β-CCE i.v. and β-CCM in the subconvulsive dose of 4 mg/kg i.v. Ro 15-1788 fully reversed the effect of β-CCE and β-CCM when given 5 min afterwards (5 and 3 mg/kg p.o., respectively). The same effect was obtained with 0.3 mg/kg

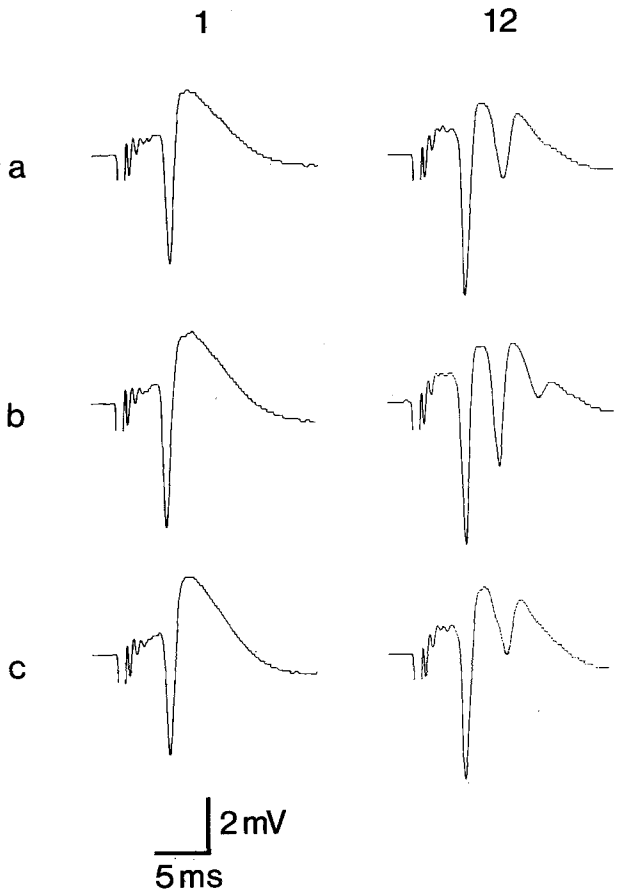

Fig. 2 a–c. Increase by β-CCE of the second CA 1 hippocampal population spike and its reversal by Ro 15-1788 in an anaesthetized rat. Depicted are tracings of single digitized responses to the first (1) and the twelfth (12) fimbrial stimulus in a 2 Hz train prior to (**a**), 5 min after 1 mg/kg β-CCE i.v. (**b**), and 5 min after 1 mg/kg Ro 15-1788 i.v., injected immediately after a second injection of β-CCE (1 mg/kg) 1 h after **b** (**c**)

Ro 15-1788 i.v. (data not shown). Ro 15-1788 had no effect on performance of undrugged and phenobarbitone-treated animals. The impairment of performance induced by the GABA-transaminase inhibitor L-cycloserine (100 mg/kg i.p.), but not that induced by meprobamate (250 mg/kg i.p.), was abolished by 4 mg/kg β-CCM i.v. (results not shown).

Discussion

The present results show that β-CCE and β-CCM depress GABAergic transmission in the cat spinal cord as measured by dorsal root potentials and, thus, produce an effect which is opposite to that seen with benzodiazepine tranquilizers (see Haefely et al. 1981a). β-CCE enhanced the low-frequency facilitation of CA 1 pyramidal cell activity in the rat hippocampus; the compound has previously been shown to antagonize the inhibitory effect of iontophoretically applied GABA on this facilitation (Polc et al. 1981b). In contrast to β-CCE, benzodiazepine tranquilizers were found to depress pyramidal cell activity and potentiate the inhibitory action of GABA (see Haefely et al. 1981a; Polc et al. 1981b; Ahlquist et al. 1982).

At first sight, these effects of β-CCE (and β-CCM) might be explained by assuming either that the β-carbolines inhibit the effect of an endogenous benzodiazepine-like ligand present at the benzodiazepine receptor or that β-carbolines produce excitatory effects that are unrelated to the benzodiazepine receptor, but perhaps connected to the carboline structure. In fact, β-CCE, which binds to central benzodiazepine binding sites with high affinity, reduces various effects of benzodiazepines (Tenen and Hirsch 1980; Cowen et al. 1981; Nutt et al. 1982), thus acting as a benzodiazepine antagonist, but also has proconvulsive properties (Oakley and Jones 1980; Cepeda et al. 1981; Nutt et al. 1982). It is, however, unlikely that β-CCE produces its effects on the spinal cord and hippocampus by inhibiting the effect of an

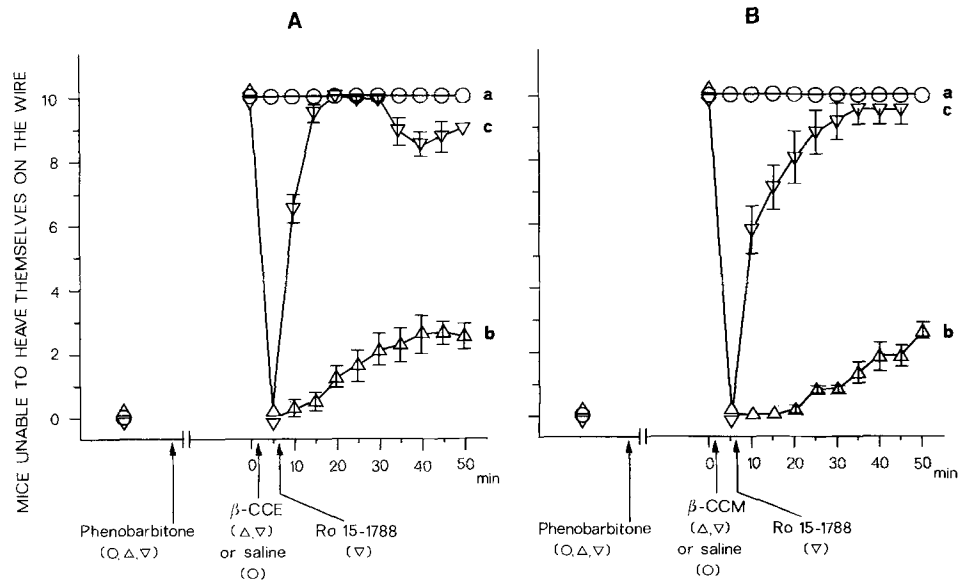

Fig. 3. Ro 15-1788 antagonizes the reversal induced by β-CCE (**A**) and β-CCM (**B**) of the depressant effect of phenobarbitone on performance of mice in the horizontal wire test. Phenobarbitone (100 mg/kg i.p.) was injected to 6 groups of 10 mice and depressed performance in all animals (*a*). In the 6 groups labelled *b*, β-CCE (100 mg/kg) or β-CCM (4 mg/kg) was injected i.v. as soon as the full phenobarbitone effect was reached. In the 6 groups labelled *c*, the injection of either β-carboline was followed 5 min later by Ro 15-1788 (5 mg/kg p.o. in A and 3 mg/kg p.o. in B). The symbols are the means ± S.E.M.

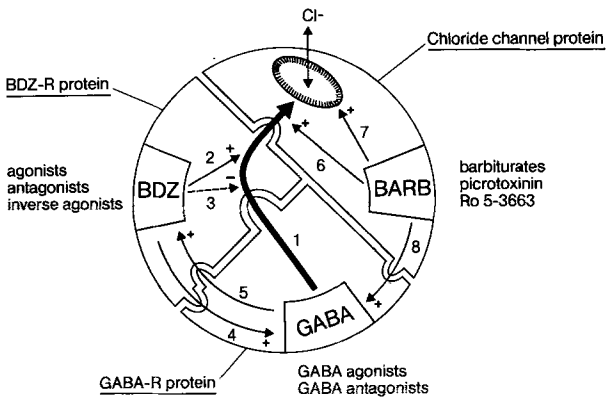

Fig. 4. Proposed model of the subsynaptic GABA receptor (GABA-R)-benzodiazepine receptor (BDZ-R)-chloride channel complex. The three proteins carry a binding domain for benzodiazepines, GABA, and barbiturates (BRB), respectively. Activation of the GABA receptor results in the opening of the chloride channel; in this process the benzodiazepine receptor is proposed to be involved as a coupling unit (1). Benzodiazepine agonists enhance (2), inverse agonists reduce (3) the coupling function. The benzodiazepine receptor also functions as a modulator of the affinity state of the GABA receptor; benzodiazepine agonists increase the affinity of the GABA receptor (4), while GABA agonists increase the binding of benzodiazepine agonists (5). Barbiturates enhance the coupling process (1) close to or at the chloride channel (6) and, in high concentrations, directly open the channel (7); they also seem to enhance the affinity of the GABA receptor (8)

endogenous benzodiazepine-like ligand, since the specific benzodiazepine receptor antagonist Ro 15-1788 had no intrinsic activity in the spinal cord (Polc et al. 1981a) and in the hippocampus, yet abolished the effects of β-CCE in the three experimental conditions investigated here. This strongly indicates that the effects of β-CCE found in this study are mediated by benzodiazepine receptors.

β-CCE and β-CCM reduced the effect of phenobarbitone and L-cycloserine, but not that of meprobamate, in the horizontal wire test in mice and the effect of phenobarbitone on the cat spinal cord. That this effect of β-carbolines was not due to an unspecific excitation is indicated by the fact that Ro 15-1788 blocked the barbiturate antagonistic action of the β-carbolines. Ro 15-1788 is inactive against the pharmacological effects of barbiturates (Polc et al. 1981a; Bonetti et al. 1982) and L-cycloserine. Therefore, the effects of β-carbolines on phenobarbitone-induced depression of motor performance in mice and prolongation of dorsal root potentials in the cat spinal cord must be mediated via benzodiazepine receptors.

The following model of the GABA receptor-benzodiazepine receptor-chloride channel complex is proposed to explain the present and earlier findings (Fig. 4). Stimulation of the GABA receptor induces a conformational change which triggers the opening of chloride channels; the benzodiazepine receptor is suggested to operate as a coupling unit between GABA receptor and chloride channel. It seems very probable that there is no need for the benzodiazepine binding domain of the coupling unit to be activated by an endogenous or exogenous benzodiazepine-like ligand in order to permit a normal coupling function, since Ro 15-1788 does not affect GABAergic synaptic transmission; accordingly, binding of Ro 15-1788 to the benzodiazepine receptor does not alter the function of the coupling process. Benzodiazepine tranquilizers (agonists) enhance the coupling function of the benzodiazepine receptor by inducing a change in its conformation or charge distribution; this benzodiazepine-induced state is, in addition, well known to increase the affinity of the GABA receptor for GABA (Costa and Guidotti 1979; Skerritt et al. 1982). Electrophysiologically, the benzodiazepine-induced increase of GABA affinity and/or enhancement of coupling function is reflected by an increased frequency of single chloride channel opening events in response to a given amount of GABA without changes in the mean channel open time, single channel conductivity or ion selectivity of the channel (Choi et al. 1981; Study and Barker 1981). β-Carbolines do not only block the access of benzodiazepine agonists to the binding site as one would expect if they were pure competitive antagonists. Rather, they also induce a change in the benzodiazepine receptor function which is, however, diametrically opposed to the change induced by benzodiazepine agonists and results in a depressed coupling function of the benzodiazepine receptor. Molecules acting in a similar way as β-carbolines might, therefore, be called "inverse agonists". Ro 15-1788 acts like a pure competitive antagonist in the traditional view, occupying the benzodiazepine binding domain without inducing a functionally relevant change of the benzodiazepine receptor, but blocking the access of both agonists and inverse agonists. Barbiturates at low and medium doses also enhance the GABA receptor function (Nicoll 1978; Macdonald and Barker 1979); in contrast to benzodiazepines they are believed to act rather on the chloride channel protein (Olsen 1981; Supavilai et al. 1982), and electrophysiologically their action is reflected by an increase of the mean open time of single chloride channels activated by the stimulation of the GABA receptor (Study and Barker 1980). L-Cycloserine markedly increases the level of GABA in the central nervous system and enhances synaptic events evoked by stimulation of GABAergic neurones (Haefely et al. 1979). Ro 15-1788 does not alter the effect of phenobarbitone or L-cycloserine because it fails to change the coupling and/or modulatory function of the benzodiazepine receptor. β-Carbolines decrease the effect of phenobarbitone and L-cycloserine because they reduce the coupling function of the benzodiazepine receptor, which should diminish the frequency of chloride channel opening events. This effect of β-carbolines is suppressed by Ro 15-1788. The effect of meprobamate, which does not act via the GABAergic system (Haefely et al. 1981b), was unaffected by β-CCM.

Based on the present evidence, ligands for the benzodiazepine receptor may belong to either of the following four categories: a) full agonists (as for instance benzodiazepine tranquilizers), b) pure antagonists (for all practical purposes: Ro 15-1788), c) antagonists with partial agonistic activity (such as phenylpyrazoloquinolinones), and d) antagonists with partial inverse agonistic activity (β-carbolines). It appears that in vitro binding studies are also able to detect these transitional properties of ligands by the so-called GABA shift: binding of pure and partial agonists to the specific benzodiazepine binding sites is increased to a differing degree by GABA, that of pure antagonists is unaffected, and that of inverse agonists is actually decreased by GABA (Möhler and Richards 1981; Braestrup et al. 1982; Gee and Yamamura 1982).

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