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# Does ethyl-β-carboline-3-carboxylate interact with mouse brain benzodiazepine receptors in vivo?

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β-Carboline-3-carboxylic acid ethyl ester (β-CEE) has been recently isolated from human urine and brain extracts from different animal species (Braestrup et al 1980). On account of its selective affinity for benzodiazepine (BDZ) binding sites, in the nanomolar range, β-CEE has been related to an endogenous ligand for BDZ receptors in the brain (Braestrup et al 1980).

Experimental data in animals indicate that β-CEE has pharmacological activity opposite to that of diazepam (Jones & Oakley 1981), it produces a dose-dependent increase of the 50% protective dose (PD50) of diazepam against leptazol (pentetrazol)-induced convulsions and it lowers the seizure threshold in mice (Tenen & Hirsch 1980). Recently, Hirsch & Lydigsen (1981) reported that β-CEE displaces [$^3$H]flunitrazepam from mouse brain benzodiazepine receptors in vivo. In the present study we investigated whether β-CEE displaces [$^3$H]diazepam bound to mouse brain, when given at doses required to antagonize the antileptazol effect of diazepam.

Treatment schedules were as reported by Tenen & Hirsch (1980) and the presence of convulsions was evaluated in a separate group of mice. Female CD mice (Charles River, Italy), 25–30 g, were pre-treated with either 0·9% NaCl (saline) or β-CEE, 10 mg kg$^{-1}$ intravenously, and 5 min later intraperitoneally with vehicle or diazepam at two different doses. Twenty minutes later, the animals were injected intravenously with 35 mg kg$^{-1}$ of leptazol or 25 μCi [$^3$H]diazepam (S.A. 87.6 Ci mmol$^{-1}$, New England Nuclear). In vivo [$^3$H]diazepam binding was assayed according to Williamson et al (1978). Mice were injected in the lateral tail vein with 25 μCi of [$^3$H]diazepam in 0·2 ml of saline, and decapitated 1 min after injection. Brain and cerebellum were immediately removed, hemisected and homogenized in 50 volumes of ice-cold Tris HCl buffer (50 nM, pH 7·4) using an Ultra-Turrax TP18-10 (20 s, full speed). One half of the tissue was homogenized in Tris-HCl buffer containing 3 μM diazepam, and incubated at 0 °C for at least 30 min, to determine non-specific binding. 0·5 ml aliquots of the tissue homogenate were filtered through Whatman GF/B filters, washed twice on the filters with 5 ml of ice-cold Tris-HCl buffer and counted in 10 ml Dioxane scintillator (Supelchem).

Percentage of specific binding is defined as the amount of radioactivity specifically retained on the filter divided by the total amount of radioactivity present in the tissue aliquot (homogenate) × 100. In these conditions, β-CEE did not significantly affect brain diazepam levels (Table 1), indicating that its antagonism of diazepam's effect cannot be explained on pharmacokinetic grounds.

As shown in Table 2, diazepam produced dose-dependent occupancy of BDZ binding sites in mouse brain.

* Correspondence.

Table 1. Brain levels (μg $g^{-1}$) of diazepam and desmethyldiazepam 20 min after i.p. injection of diazepam.

| Pretreatment (mg $kg^{-1}$ i.v.) | Treatment (mg $kg^{-1}$ i.p.) | Diazepam | Desmethyl-diazepam |
|---|---|---|---|
| Saline | Diazepam (0·32) | 17·13 s.d. 7·4 | 55·14 s.d. 11·94 |
| β-CEE (10) | Diazepam (0·32) | 10·80 s.d. 2·39 | 40·78 s.d. 6·64 |
| Saline | Diazepam (3·8) | 43·42 s.d. 15·77 | 223 s.d. 31 |
| β-CEE (10) | Diazepam (3·8) | 27·61 s.d. 4·9 | 210·27 s.d. 38·4 |

Data are means with s.d. of 4 animals per group. The differences were not significant by Student's *t*-test. Diazepam and desmethyl-diazepam were determined by g.l.c. as described by Caccia et al (1980).
β-CEE was injected in 10 ml $kg^{-1}$ of saline plus few drops of Tween 80.

β-CEE, at a dose of 10 mg $kg^{-1}$, which reportedly raises the PD 50 of diazepam of leptazol-induced convulsions from 0·32 to 3·8 mg $kg^{-1}$ (Hirsch & Lydigsen 1981), did not significantly occupy mouse brain BDZ receptors labelled with tracer amounts of [$^3$H]diazepam; and consequently, does not seem to affect diazepam occupancy of BDZ receptors when either 0·32 or 3·8 mg $kg^{-1}$ of diazepam were given to mice. Similar results were obtained in cerebellum (data not reported).

When given at higher dose (32 mg $kg^{-1}$ i.v. 25 min before [$^3$H]diazepam, β-CEE displaces [$^3$H]diazepam binding from mouse brain and cerebellum (40 and 60%, respectively), confirming the data reported by Hirsch & Lydigsen (1981). The greater displacing effect found by these authors may be explained by their different experimental conditions, principally the fact that they gave β-CEE 10 min before [$^3$H]flunitrazepam. On account of the rapid metabolism of β-CEE, 10 min after i.v. injection is the time at which its pharmacological effect is maximum (Jones & Oakley 1981). However, the aim of our experiments was to find the possible sites of antagonism by β-CEE of diazepam's antileptazol effect. Therefore we gave β-CEE 5 min before diazepam, time at which it effectively antagonizes the activity of 0.32 mg $kg^{-1}$ of diazepam, as shown in Table 2.

The present data clearly show that in similar pharmacological conditions (protection against leptazol convulsions), obtained with either saline + 0·32 mg $kg^{-1}$ diazepam or β-CEE + 3·8 mg $kg^{-1}$ diazepam, a different proportion (39 and 58% respectively) of BDZ binding sites was occupied. However, different pharmacological conditions (protection or no protection against leptazol-induced convulsions), obtained with either saline + 0·32 mg $kg^{-1}$ diazepam or the same dose of diazepam plus 10 mg $kg^{-1}$ β-CEE, resulted in the same fraction (39%) of BDZ receptors being occupied by diazepam. This suggests that no simple correlation can be established between the pharmacological activity of benzodiazepines and their occupancy of BDZ binding sites labelled in vivo with tracer amounts of [$^3$H]diazepam. It is therefore tempting to speculate that the BDZ binding sites responsible for their anticonvulsant action, at which presumably β-CEE selectively interacts, represent only a small fraction of total BDZ receptors in the brain, or preferentially involve a particular class of BDZ receptors that are not revealed by the present approach.

Table 2. Effect of pretreatment with either saline or β-CEE on in vivo diazepam occupancy of mouse brain receptors.

| Pretreatment (mg $kg^{-1}$, i.v.) | Treatment (mg $kg^{-1}$, i.v.) | Leptazol-induced convulsions (no. of mice) | Percentage [$^3$H]diazepam bound | Percentage receptor occupancy |
|---|---|---|---|---|
| Saline | Vehicle | 5/6 | 26·2 s.d. 5·4 (10) | — |
| β-CEE (10) | Vehicle | 6/6 | 22·9 s.d. 5·9[a] (9) | — |
| Saline | Diazepam (0·32) | 5/17* | 16·1 s.d. 4·8[b] (7) | 39 |
| β-CEE (10) | Diazepam (0·32) | 16/18** | 15·9 s.d. 1·5[b,c] (6) | 39 |
| Saline | Diazepam (3·8) | 0/6 | 12·4 s.d. 3·0[b,d] (6) | 53 |
| β-CEE (10) | Diazepam (3·8) | 4/12* | 10·9 s.d. 2·5[b,c] (9) | 58 |

Data are mean values with s.d.; number of mice is given in parenthesis.
* Different from controls $P = 0·05$ } Fisher's exact test.
** Different from saline $P = 0·001$ }
(a) no. significantly different from controls
(b) $P < 0·01$ different from controls } Mann Whitney Rank Sum Test.
(c) no. significantly different from saline
(d) $P < 0·05$ different from diazepam (0·32)

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