BINDING OF [^{3}H]ETHYL-β-CARBOLINE-3-CARBOXYLATE TO BRAIN BENZODIAZEPINE RECEPTORS

Effect of drugs and anions

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1. Introduction

Ethyl-β-carboline-3-carboxylate (β-CCE) has been isolated from brain and urine extracts and found to potently inhibit the binding of [^{3}H]benzodiazepines to specific receptor sites in the brain [1]. Although β-CCE (or a closely related derivative) was initially postulated to be an endogenous ligand of the benzodiazepine receptor [1], subsequent studies strongly suggest [2] that this compound (as well as its methyl ester and 3-carboxylic acid derivatives) is formed artifactually during the extraction and isolation procedures.

Nonetheless, the extremely high affinity of β-CCE and related compounds in displacing [^{3}H]benzodiazepines from receptor sites in the central nervous system [3,4] coupled with recent reports that β-CCE and related β-carbolines are specific antagonists of many of the pharmacologic actions of benzodiazepines [5–9], suggests that these compounds may be valuable tools for studying the regulation of the benzodiazepine receptor.

It was suggested [3,10] that there may be significant differences in the regulation of the benzodiazepine receptor when it is occupied by an 'antagonist' such as β-CCE, rather than an 'agonist' (e.g., diazepam). In [3,10] binding of β-[^{3}H]carbolines was enhanced only slightly or not at all by γ-aminobutyric acid (GABA). In contrast, under similar experimental conditions, GABA and GABA-mimetic agents (e.g., muscimol) markedly enhanced the apparent affinity of [^{3}H]benzodiazepines for the benzodiazepine receptor [11]. These observations prompted us to examine the effect of other drugs and anions known to enhance the apparent affinity of [^{3}H]benzodiazepines for benzodiazepine receptors on the binding of β-[^{3}H]-CCE. We now report that in contrast to the changes in affinity of [^{3}H]benzodiazepines elicited by halide ions [12], barbiturates [13–15], and pyrazolopyridines [16,17], the apparent affinity of β-[^{3}H]CCE is unaffected by these agents. Furthermore, Scatchard analysis of β-[^{3}H]CCE binding to cerebral cortical and cerebellar membranes revealed a significantly greater number of binding sites than was observed with either [^{3}H]diazepam or [^{3}H]flunitrazepam, suggesting that at low concentrations benzodiazepines selectively label a subpopulation of the receptors labelled with β-[^{3}H]CCE. Alternatively, β-[^{3}H]CCE may bind to sites that are distinct from those labelled with [^{3}H]-benzodiazepines.

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2. Materials and methods

Adult, male Sprague-Dawley rats (Taconic Farms, Germantown NY) were killed by decapitation. Osmotically shocked membrane fragments were prepared from cerebral cortex (pooled parietal, temporal, occiptal and frontal cortices) or cerebellum by disruption with a Polytron (Brinkmann Instruments, setting 6, 15 s) in 100 vol. potassium phosphate buffer (50 mM, pH 7.4). The tissue was centrifuged at 20 000 × *g* for 20 min and reconstituted in an equal

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volume of buffer. The tissue was washed and centrifuged a total of 5 times, and reconstituted following the final centrifugation in 60 vol. homogenization buffer. Incubation conditions and methodology for the determination of [^{3}H]benzodiazepine or β-[^{3}H]-CCE binding were performed essentially as in [18] in a total volume of 1.5 ml. In experiments comparing either the kinetic properties of [^{3}H]benzodiazepines and β-CCE or the effects of drugs on the binding of these radioligands, aliquots of the same tissue sample were used.

Specific binding was defined as the difference between binding in the absence (total binding) and presence (non-specific binding) of a large molar excess of non-radioactive diazepam (3 μM) or methyl-β-carboline-3-carboxylate (β-CCM) (3 μM). No statistically significant differences in the non-specific binding of either radioligand were observed when either diazepam or β-CCM were used to determine the levels of non-specific binding (e.g., [^{3}H]diazepam + β-CCM = [^{3}H]-diazepam + diazepam). The ratio of specific to non-specific binding was, however, consistently lower when β-CCE was used as a ligand compared with either [^{3}H]diazepam or [^{3}H]flunitrazepam.

[^{3}H]Diazepam (spec. act. 87.6 Ci/mmol) and [^{3}H]-flunitrazepam (spec. act. 87.9 Ci/mmol) were purchased from New England Nuclear (Boston MA). β-[^{3}H]CCE (spec. act. 24 Ci/mmol) was obtained from Amersham (Arlington Heights IL). Diazepam was a gift of Hoffmann-LaRoche (Nutley NJ) and SQ 65, 396 was donated by Squibb Labs. (Princeton NJ). Methyl-β-carboline-3-carboxylate and (+)-dimethylbutylbarbituric acid [(+)-DMBB] were synthesized as in [9,14]. Sodium pentobarbital was purchased from Abbott Labs. (Chicago IL). All other materials were obtained from standard commercial sources.

3. Results

The binding of [^{3}H]diazepam, at 0.88 nM (~0.1 K_d-units) was significantly enhanced in the presence of 20–1000 mM NaCl. The EC_{50} (concentration required to elicit a half-maximum enhancement) for NaCl was estimated to be 30 mM. The enhancement of [^{3}H]-diazepam binding by NaCl was a result of an increase in the apparent affinity of the radioligand for receptor (unpublished observations and [12]) rather than a change in receptor number. In contrast, NaCl did not significantly enhance β-[^{3}H]CCE binding over 1–1000 mM (fig.1). The barbiturates pentobarbital and (+)-

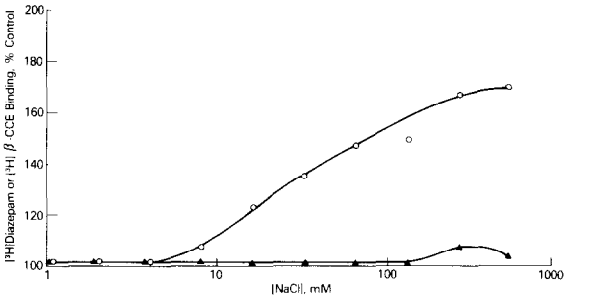

Fig.1. Effect of chloride on β-[^{3}H]CCE and [^{3}H]diazepam binding. The effect of NaCl on the binding of β-[^{3}H]CCE (▲) and [^{3}H]diazepam (○) was studied in cerebral cortical membranes, washed 5 times in 100 vol. potassium phosphate buffer (pH 7.4) as in section 2. Values represent the % increase in binding compared to chloride-free incubations and are taken from a representative experiment. The experiment was repeated 3 times with identical results. Experiments were done with β-[^{3}H]CCE and [^{3}H]diazepam at ~0.1 K_d units, 0.1 nM and 0.88 nM, respectively.

DMBB and the pyrazolopyridine SQ 65,396, significantly enhanced [^{3}H]diazepam binding in a NaCl-enriched buffer with EC_{50} values of 45,36 and 0.28 μM, respectively. In contrast, these drugs did not elicit a statistically significant increase in the binding of β-[^{3}H]CCE (fig.2). The effects of pentobarbital

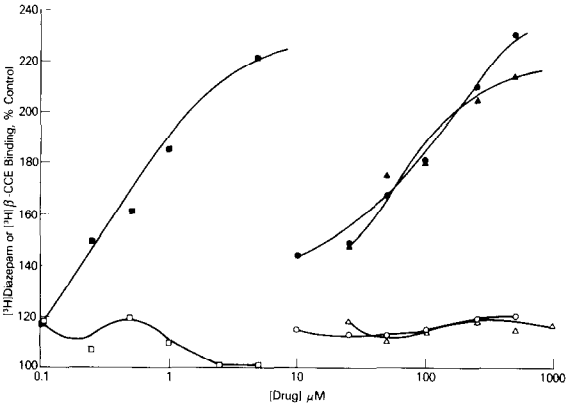

Fig.2. Effects of barbiturates and pyrazolopyridines on β-[^{3}H]CCE binding. In contrast to [^{3}H]diazepam binding (closed symbols), no statistically significant enhancements were observed for drugs on β-[^{3}H]CCE binding (open symbols): (□,■) SQ 65,396; (○,●) sodium pentobarbital; (△,▲) (+)-DMBB. Assay conditions were identical to those in fig.1, except for the addition of 150 mM NaCl to the incubations in order to optimize the effects of barbiturates and SQ 65,396. Values represent the % increase over 'no-drug' control incubations and are taken from a typical experiment, which was repeated at least 3 times with similar results.

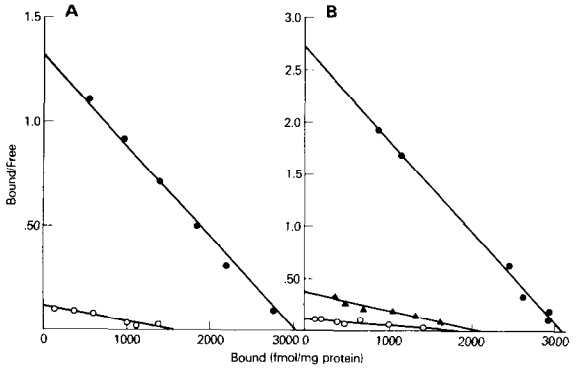

Fig.3. Scatchard analysis of β-[^{3}H]CCE and [^{3}H]diazepam binding. (A) Scatchard analysis of [^{3}H]diazepam (○) and β-[^{3}H]CCE (●) binding using cerebral cortical membranes washed 5 times. (B) Scatchard analysis comparing the binding of [^{3}H]diazepam (○) and [^{3}H]flunitrazepam (▲) to β-[^{3}H]CCE (●) in identically prepared cerebellar membranes. Assay conditions were as in section 2. Binding parameters are summarized in table 1. The binding capacity for β-[^{3}H]CCE was significantly greater than [^{3}H]diazepam or [^{3}H]flunitrazepam in all experiments (see table 1).

(500 μM) were also examined on the binding of both β-[^{3}H]CCE (1 nM) and [^{3}H]flunitrazepam (1 nM) at 37°C in sodium phosphate buffer containing 150 mM NaCl. Under these conditions, the binding of β-[^{3}H]-CCE was not enhanced while a significant increase in [^{3}H]flunitrazepam binding (73%) was observed. The K_d of β-[^{3}H]CCE, like that of benzodiazepines [19] was increased ~7-fold at 37°C compared to incubations done at 0–4°C (unpublished).

Scatchard analyses of the binding of β-[^{3}H]CCE, [^{3}H]diazepam, and [^{3}H]flunitrazepam to cerebral cortical and cerebellar membranes (fig.3, table 1) demonstrated that the maximum number of binding sites (B_{max}) observed with β-[^{3}H]CCE is significantly greater than the number of sites observed with either [^{3}H]diazepam or [^{3}H]flunitrazepam. The number of β-[^{3}H]CCE binding sites was almost 80% greater in the cerebellar membranes and 60% greater in cerebral cortical membranes (table 1) than were the number of [^{3}H]diazepam binding sites.

4. Discussion

The benzodiazepine receptor has been proposed to be a component of a larger receptor complex consisting of recognition sites for benzodiazepines and GABA, which are functionally (if not physically) coupled to a Cl^- channel [20–22].

The observation that GABA does not enhance (or enhances very weakly) β-[^{3}H]carboline binding [3,10] suggests that the binding of a compound which is a functional benzodiazepine antagonist [5–9] differs substantially from the binding of a benzodiazepine to its recognition site. Since it is hypothesized that pyrazolopyridines, barbiturates, and Cl^- increase the affinity of the benzodiazepine receptor at a distinct locus from GABA [13,23,24], we have examined the effects of agents which increase the apparent affinity of benzodiazepines at a chloride ionophore site (the 'barbiturate receptor', cf. [15]) on β-[^{3}H]CCE binding. Our results clearly demonstrate that, in contrast to the binding of [^{3}H]benzodiazepines, neither anesthetic nor convulsant barbiturates, pyrazolopyridines, nor anions alter the apparent affinity of the β-carbolines.

Perhaps the most interesting observation here is the very significant differences in the number or sites 'labelled' by β-[^{3}H]CCE compared with either [^{3}H]-diazepam or [^{3}H]flunitrazepam. In [3] no significant difference in receptor number was observed using

Table 1
Binding of β-[^{3}H]CCE, [^{3}H]diazepam and [^{3}H]flunitrazepam in cerebral cortex and cerebellum
(values represent the $\bar{x}$ ± SEM of 3–4 expt)

	Cerebral cortex			Cerebellum		
	B_{max}[d]	K_d[e]	r	B_{max}	K_d	r
β-[^{3}H]CCE	2505 ± 222	1.05 ± 0.04	0.997 ± 0.003	3178 ± 96	1.09 ± 0.3	0.983 ± 0.01
[^{3}H]Diazepam	1396 ± 145[a]	8.6 ± 0.56	0.998 ± 0.002	1939 ± 175[b]	11.2 ± 0.9	0.992 ± 0.002
[^{3}H]Flunitrazepam				2075 ± 371[c]	3.7 ± 0.6	0.994 ± 0.002

[a] $P < 0.02$; [b] $P < 0.002$; [c] $P < 0.05$ compared to B_{max} of β-[^{3}H]CCE binding (by Student's t-test); [d] fmol/mg protein; [e] nM

[^{3}H]propyl-β-carboline-3-carboxylate and [^{3}H]flunitrazepam as ligands. Furthermore, it was concluded in [3] that the binding of these two ligands was mutually exclusive. Differences in tissue preparation, buffers or radioligand could account for this discrepancy. However, the marked differences in B_{max} observed between β-[^{3}H]CCE and [^{3}H]benzodiazepines (80% in cerebral cortex; 60% in cerebellum) in our study suggest that at low concentrations, the benzodiazepines may bind to a subpopulation of sites which are fully occupied by β-[^{3}H]CCE. Despite the high affinity of β-CCE for benzodiazepine receptors (and vice versa), it could also be hypothesized that β-[^{3}H]CCE binds to a distinct class of sites which benzodiazepines occupy only at very high concentrations. This hypothesis is supported by the observation that the non-specific binding of β-[^{3}H]CCE is very similar using either 3 μM diazepam or β-CCM. These sites may represent a distinct 'antagonist' site with a pharmacological profile similar to that of the 'agonist' site. In this regard, high affinity anti-estrogen binding sites that are distinct from the estrogen receptor itself have been reported [25].

The regulation of benzodiazepine receptors differs from other drug and neurotransmitter receptors in that rapid changes in receptor number can occur in vivo and in vitro after drug treatment or physiological manipulation (cf. [26]). It is possible that the observed differences in site number between [^{3}H]benzodiazepines and β-[^{3}H]CCE could be related to an 'inducible' population of cryptic binding sites that are labelled by β-[^{3}H]CCE. Subsequent investigations will undoubtedly clarify the nature of the apparent differences in regulation and site number observed between benzodiazepines and β-CCE.

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