Volume 219, number 2, 301–305 FEB 04929 July 1987

Benzodiazepine agonists protect a histidine residue from modification by diethyl pyrocarbonate whereas propyl β-carboline does not

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Received 8 May 1987

The pH sensitivity of benzodiazepine binding suggests that a histidine residue may be present in, or close to the benzodiazepine binding site. This was confirmed by the selective modification of histidine residues using diethyl pyrocarbonate which was found to block both benzodiazepine and β-carboline binding. In order to assess whether this histidine residue is located in or adjacent to the benzodiazepine and β-carboline binding sites, experiments were performed using either benzodiazepine or β-carboline to protect against diethyl pyrocarbonate treatment. It was found that benzodiazepine agonists, but not propyl β-carboline protect the benzodiazepine binding sites from diethyl pyrocarbonate modification.

Benzodiazepine; β-Carboline; Binding site; pH sensitivity; Diethyl pyrocarbonate; Histidine residue

1. INTRODUCTION

Benzodiazepines and β-carbolines can modulate the GABAergic transmission by binding to the Bdz receptor located on the multisubunit Bdz-GABA A receptor-chloride channel complex. Amongst Bdz and β-carbolines three classes of ligands have been characterised: (i) agonists, enhancing the GABAergic transmission; (ii) inverse agonists diminishing GABAergic transmission; and (iii) antagonists, blocking the effects of both agonists and inverse agonists by competitive inhibition [1]. It is established that DEP blocks the Bdz [2–4] and β-carboline binding to the Bdz-GABA A receptor-chloride channel complex without acting on the other binding sites [5]. Flurazepam, a Bdz agonist, protects the Bdz and β-carboline binding from DEP treatment [5], indicating that this reagent acts on an amino acid residue either in the Bdz binding site or its surroundings. This action of DEP has been attributed to modification of either a histidine or a tyrosine residue [5,6].

We present here further evidence that a histidine residue is located near or at the Bdz binding site as shown by the pH dependency of Bdz antagonist Ro 15-1788 binding. Furthermore, the antagonist B-CCP does not protect Ro 15-1788 binding from DEP modification, whereas the Bdz agonist Flu does.

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Abbreviations: DEP, diethyl pyrocarbonate; Bdz, benzodiazepine; B-CCP, propyl β-carboline-3-carboxylate; B-CCM, methyl β-carboline-3-carboxylate; Flu, flunitrazepam; GABA, gamma-amino butyric acid

2. MATERIALS AND METHODS

[*N-methyl*-^{3}H]Ro 15-1788 (87 Ci/mmol) and [*ethyl*-2-^{3}H]ethyl-β-carboline-3-carboxylate (50 Ci/mmol) were from New England Nuclear. [*N-methyl*-^{3}H]Flunitrazepam (65 Ci/mmol) was from CEA, Saclay, France. Clonazepam, Flu and

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

Ro 15-1788 were kindly provided by Dr Willy Haefely, Hoffmann-La Roche, Basel. B-CCM and B-CCP were gifts from Dr Robert H. Dodd, ICSN CNRS Gif-sur-Yvette. Absolute DEP from Fluka was stored at 4°C and diluted 34-fold in ethanol just before use. All buffers, incubations and rinsing were at 4°C unless otherwise stated. The Tris-phosphate buffer was prepared by adjusting the pH of a 50 mM Tris base solution with H_3PO_4.

Membrane preparation and [^{3}H]ligand binding were performed as described [7]. Briefly male Wistar rats (200–250 g) were decapitated and the cerebral cortex dissected, homogenised (Polytron + Potter Elvehjem) in 50 mM Tris-HCl, pH 7.4 (8.33 ml/g fresh tissue), and centrifuged for 3 min at 400 × g. The supernatant was centrifuged for 20 min at 20000 × g and the pellet was resuspended in Tris-HCl (6 ml/g original tissue) and recentrifuged for 20 min at 20000 × g. The resulting pellet was again suspended, under the same conditions, frozen and stored at −30°C. Protein was estimated by the method of Lowry et al. [8]. For binding experiments, membranes were diluted (75–100 μg protein/ml) in the indicated buffer at the required pH (the pH of the incubation medium remained unchanged after adding membranes), and incubated for 1 h with ligands in a total volume of 1 ml. Non-specific binding was determined at each pH in the presence of 1 μM clonazepam. Incubations were stopped by adding 3 ml of ice cold incubation buffer to each tube and filtering under vacuum through Whatman GF/B glass fiber filters. The tube was rinsed once and the filter 3 times with 3 ml ice cold buffer. Bound radioactivity retained on the filters was counted in 10 ml of Aquasol scintillation solution with an LKB Wallace 1215 Rackbeta 2 counter.

Modification by DEP and protection experiments were performed essentially as in [5]. Membranes were diluted 10-fold in 20 mM sodium phosphate, pH 6, buffer containing 0.2 M NaCl, centrifuged 10 min at 29000 × g, and the pellet was resuspended in the same buffer at 1–2 mg protein/ml. For protection experiments, ligand at a final concentration of 1 μM was preincubated with the membranes for 5 min at 20°C before adding DEP. DEP was added at a final concentration of 1 mM, and after a 15 min incubation at 20°C, the reaction was stopped by a 16-fold dilution in ice cold 50 mM Tris-HCl, pH 7.5, followed by a 10 min centrifugation at 29000 × g and the pellet was resuspended in 35 ml of Tris-HCl buffer. This was repeated 6 times to remove remaining DEP and protective ligands. The final resuspension was made in 18 ml Tris-HCl, and the treated membranes were tested for [^{3}H]Ro 15-1788 binding as described. Control membranes were treated in an identical manner but without the addition of DEP.

3. RESULTS

3.1. *pH studies*

The pH sensitivity of [^{3}H]Ro 15-1788 binding at a final concentration of 0.8 nM is shown in fig.1. Maximum binding occurred at pH 7.5, with a 57% decrease at pH 5.5 and a 55% decrease at pH 10.5. Since Ro 15-1788 has no pK_a in this pH range (Fleury, B., personal communication, and [9]), changes in its binding can be attributed to modification of its binding sites.

The reversibility of this pH effect on Bdz binding was tested by diluting the membranes 10-fold in Tris-phosphate buffer at pH 5.5, 7.5 and 10.5. At different incubation times aliquots were taken, centrifuged for 10 min at 29000 × g and the pellet resuspended in Tris-phosphate buffer, pH 7.5 (75–100 μg protein/ml), was tested for [^{3}H]Flu

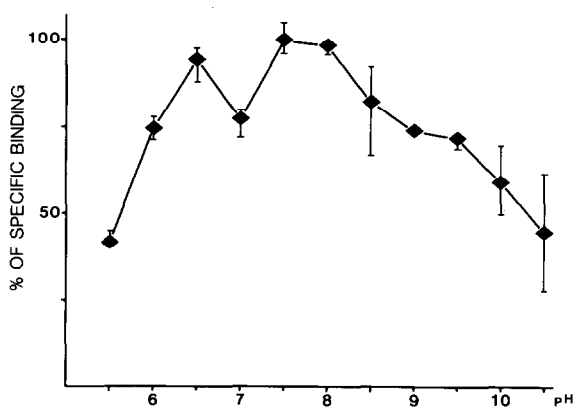

Fig.1. Specific binding of 0.8 nM of [^{3}H]Ro 15-1788 in 50 mM Tris-phosphate buffer at pH from 5.5 to 10.5 expressed in % of the specific binding at pH 7.5. The results are the means of two experiments. Non-specific binding was determined in the presence of 1 μM clonazepam and was below 3% of the total binding. Assays were carried out in triplicate. Buffer of the same pH was used to incubate and to rinse the filters.

and [^{3}H]B-CCE binding as described. No decrease in binding with time was observed, indicating that no denaturation of Bdz and β-carboline binding sites occurred at either pH 5.5 or pH 10.5. The pH sensitivity does not therefore result from a denaturation of the Bdz receptor macromolecule.

Increasing the ionic strength of binding buffer, by adding 0.45 M NaCl, which is known to diminish electrostatic interactions between surface residues of proteins [10], did not change the pH dependency of the binding between pH 7.5 and 5.5 (not shown).

Binding constants of [^{3}H]Ro 15-1788 at pH 7.5 and 5.5 in Tris-phosphate buffer were investigated by saturation experiments (fig.2). A 46% decrease in B_{max} (number of sites) was seen between pH 7.5 and pH 5.5, which is in good agreement with the 57% decrease found for ([^{3}H]Ro 15-1788) = 0.8 nM binding (fig.1). No effect on K_d (affinity) was observed.

3.2. *Modification experiments*

Incubating membranes at pH 6 with 1 mM DEP for 15 min at 20°C was found to block 67% of [^{3}H]Ro 15-1788 binding sites without changing the K_d (figs 3 and 4).

Data pertaining to protection by Flu are shown in fig.3. No decrease in B_{max} was observed when 1 μM flunitrazepam was included in the incubation, confirming the protective potency of Bdz agonists. A control consisting of membranes incubated with Flu alone showed that the ligand was

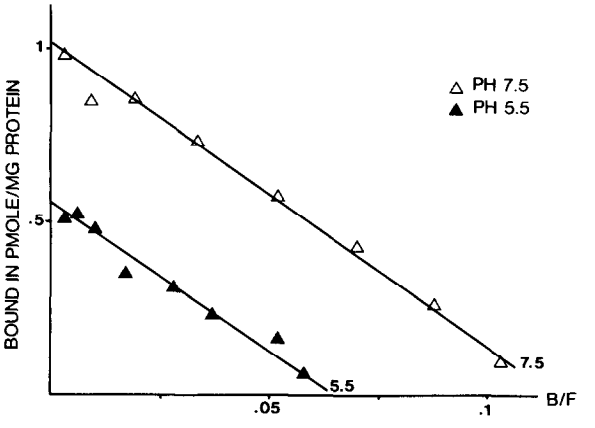

Fig.2. Eadie-Hofstee analysis of [^{3}H]Ro 15-1788 binding at pH 7.5 (△) and at pH 5.5 (▲) in Tris-phosphate buffer. Each point was made in duplicate. Buffer of the same pH was used to incubate and to rinse the filters.

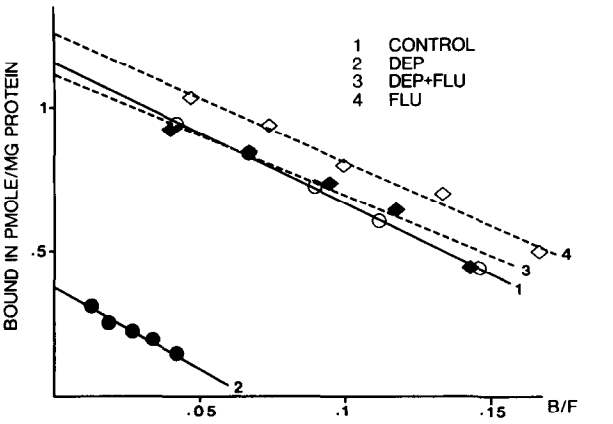

Fig.3. Eadie-Hofstee analysis of [^{3}H]Ro 15-1788 binding to: (○) control membranes; (●) DEP-treated membranes; (♦) DEP + Flu-treated membranes; (◇) membranes incubated with Flu. For protection 1 μM Flu was incubated 5 min before adding DEP. Modification by 1 mM DEP was performed 15 min at 20°C. [^{3}H]Ro 15-1788 binding was tested after modification. This experiment was repeated four times with similar results.

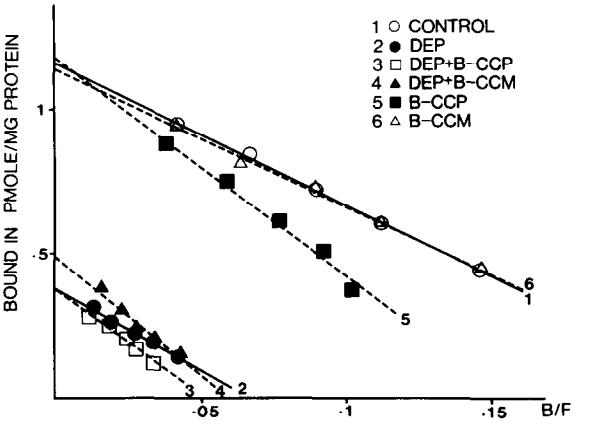

Fig.4. Eadie-Hofstee analysis of [^{3}H]Ro 15-1788 binding to: (○) control membranes; (●) DEP-treated membranes; (□) DEP + B-CCP-treated membranes; (▲) DEP + B-CCM-treated membranes; (■) membranes incubated with B-CCP; (△) membranes incubated with B-CCM. (For clarity: line 1 is solid and line 6 broken.) For protection experiments 1 μM B-CCM or 1 μM B-CCP were preincubated 5 min at 20°C before DEP addition. This experiment was repeated three times with similar results.

completely removed by the washing procedures.

Data pertaining to protection by β-carbolines are shown in fig.4. Under the same conditions B-CCP (1 μM) did not protect the Bdz binding site against DEP modification a 67% decrease in B_{max} being observed in either its presence or its absence. In addition 1 μM B-CCM gave only a very slight protection (58% decrease in B_{max} as opposed to a 67% decrease for DEP or DEP + B-CCP). The slight increase in K_d observed when β-carbolines were present was probably due to the incomplete removal of the ligands as a similar increase was found with control membranes incubated with B-CCP alone.

Thin layer chromatography and spectrophotometry were used to verify that DEP did not react with B-CCP, B-CCM or Flu.

4. DISCUSSION

In this study we were specifically interested in investigating modifications of the Bdz binding site and it was therefore considered important to use an antagonist, [^{3}H]Ro 15-1788, for binding, instead of an agonist. Agonists binding is far more susceptible than antagonists binding to allosterical modulations [1] and is therefore more sensitive to modifications of the Bdz receptor macromolecule occurring away from the binding site.

The pH sensitivity of [^{3}H]Ro 15-1788 binding was found to be similar to that previously reported for the agonist [^{3}H]diazepam [11]. Since we have shown that the pH effect is reversible and is not therefore caused by receptor denaturation, we have concluded that this pH sensitivity is the result of changes in the Bdz binding site probably caused by the protonation or deprotonation of amino acid residues at or near the site. The decrease in binding observed between pH 7.5 and 10.5 could be due to numerous amino acid residues since many of them have pK_a values in this range. Histidine, however, is the only amino acid which has a side chain pK_a, in solution, of between pH 7.5 and 5.5. In addition the absence of shift in acidic pH sensitivity with increased ionic strength, indicates that the pK_a of the amino acid residue, on which protonation occurs, is not very different from its value in solution. The most likely explanation for the decrease in binding observed over this pH range therefore is that it is caused by the protonation of a histidyl residue.

The protonation of this putative histidine residue results in a decrease of B_{max} (fig.2), indicating that the residue is near to or in the Bdz binding site, as the modification of a residue further away from the site would be expected to disturb it less and to result in a K_d rather than a B_{max} modification.

The results obtained with DEP also support the presence of a histidine residue in the Bdz binding site or near to it. DEP, under the conditions used, is a highly selective reagent for histidine residues [12] and this coupled with the observed pH effects, makes its action through a tyrosine residue very unlikely. DEP treatment of the Bdz receptor complex has been shown previously to modify the binding of the agonist [^{3}H]diazepam. The fact that we have obtained similar results using the antagonist [^{3}H]Ro 15-1788, strongly indicates that the reagent modifies the Bdz binding site itself and is not acting at a site which could allosterically affect agonists binding. The absence of protection by β-carbolines from DEP treatment implies that the histidine residue, although important for β-carboline binding [5], is not directly part of the β-carboline binding site.

The protection afforded by Bdz agonists Flu and flurazepam can be interpreted in at least two ways. The histidine residue modified by DEP may be part of the Bdz binding site and thus would be protected sterically by Bdz binding. This residue may therefore be a part of the Bdz binding site which is distinct from the β-carboline binding site. Alternatively, the histidine residue may be in a region of the Bdz receptor macromolecule which allosterically interacts with the Bdz binding site. In contrast to the binding of antagonists (B-CCP) or partial inverse agonists (B-CCM), agonist binding might stabilise the receptor in a conformation in which DEP is no longer able to react with the histidine residue.

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