INVOLVEMENT OF TYROSYL RESIDUES IN THE BINDING OF BENZODIAZEPINES TO THEIR BRAIN RECEPTORS

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1. Introduction

Chemical modification of functional groups in receptors for neurotransmitters and neuromodulators can shed light on the fine structure of the receptor binding site(s). No information is yet available on the identity of amino acid residues in the binding site(s) of brain benzodiazepine receptors. Evidence was provided in [1] for the involvement of a tyrosyl residue in the binding of indoles and benzodiazepines to human serum albumin. It is thus pertinent to investigate whether tyrosine also plays a role in the binding of benzodiazepines to their brain receptors. Here, we report that the chemical modification of both membrane-bound and solubilized brain benzodiazepine receptors by tetranitromethane and by *N*-acetylimidazole results in loss of the ability of the receptors to bind [^{3}H]flunitrazepam ([^{3}H]FNZ). Our results strongly suggest that tyrosine is involved in the binding of benzodiazepines to their receptors in the brain.

2. Experimental

2.1. *Chemicals*

[^{3}H]Flunitrazepam ([^{3}H]FNZ, 84.8 Ci/mmol) was obtained from New England Nuclear (Boston MA). Sodium dexoxycholate (DOC) and tetranitromethane (TNM) were obtained from Fluka (Buchs). Diazepam was donated by Assia (Jerusalem) nitrazepam was donated by Ikapharm (Kfar Saba) and RO-5-4864 was the gift of Dr H. Möhler (F. Hoffmann-La Roche, Basel). γ-Aminobutyric acid (GABA), *N*-ethylmaleimide (NEM) and *p*-chloromercuriphenylsulfonic acid (PCMPS) were purchased from Sigma (St Louis MO). Imidazole was from British Drug Houses (Poole) and acetic anhydride was from Frutarom (Haifa). *N*-Acetylimidazole was synthesized by reacting imidazole with acetic anhydride and evaporating the excess of acetic anhydride under vacuum. Sephadex G-75 was obtained from Pharmacia (Uppsala). All other chemicals were of analytical grade.

2.2. *Methods*

Crude washed membranes were prepared from calf cerebral cortex as in [2]. The membranes were suspended in 50 mM Tris–HCl (pH 8.0) or in 50 mM Tris–HCl (pH 6.5). Benzodiazepine receptors were solubilized by 0.5% DOC in 25 mM Tris–HCl (pH 8.0) as in [3]. [^{3}H]FNZ binding (final conc. 5 nM) was assayed at 0°C using the filtration procedure for particulate receptors and the combined ammonium sulfate precipitation and filtration procedure for soluble receptors [3].

Nitration with TNM at pH 8 (unless otherwise indicated) was according to [4]. Aliquots of TNM in ethanol were added to the receptor preparations to yield a final TNM concentration of 2.5–20 mM, while keeping ethanol at <5%. The appropriate amount of ethanol without TNM was added to control aliquots. Incubation with gentle mixing was carried out at constant pH at room temperature. Nitration of particulate receptor preparations was followed by direct dilution (20-fold) of the treated aliquots into the assay mixture at 0°C. Nitration of soluble receptors was terminated by separating the reagent from the receptor molecules on a Sephadex G-75 column pre-equilibrated with 0.25% DOC, 25 mM Tris–HCl (pH 7.2). The columns were prepared in 3 ml plastic syringes and the receptor was eluted by centrifugation at 1000 rev./min for 3 min at 4°C. The same gel filtration procedure was also employed to separate the solubilized receptor from various ligands used in protection experiments, as described below.

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Acetylation with *N*-acetylimidazole was done as in [5]. Membranes were prepared in 50 mM sodium borate buffer (pH 7.5) and incubated with 10 mM *N*-acetylimidazole at room temperature, with gentle mixing. The reaction was followed by 20-fold dilution of aliquots into the assay mixture at 0°C. Deacetylation was performed by incubating the acetylated membranes with 80 mM hydroxylamine at 30°C for various times up to 2 h.

Alkylation with NEM (5 mM) or PCMPS (1 mM) was carried out for 30 min at room temperature in the presence of 60 mM NaCl, 25 mM Tris–HCl (pH 7.2).

3. Results and discussion

Reaction of both particulate and solubilized calf cortex preparations with TNM resulted in a gradual loss of [^{3}H]FNZ-binding capacity (fig.1,2). Very little [^{3}H]FNZ binding was detected after 1 h incubation with ≥5 mM TNM, at pH 8.0. Binding of [^{3}H]FNZ to control aliquots treated with ethanol alone was not significantly altered even after 3 h (fig.1). Binding capacity of solubilized receptors was less stable under these experimental conditions (fig.2A, see also [3]); the effect of TNM in these preparations was even more pronounced and 2.5 mM TNM caused a 50% reduction in binding relative to control within 20 min (fig.2B).

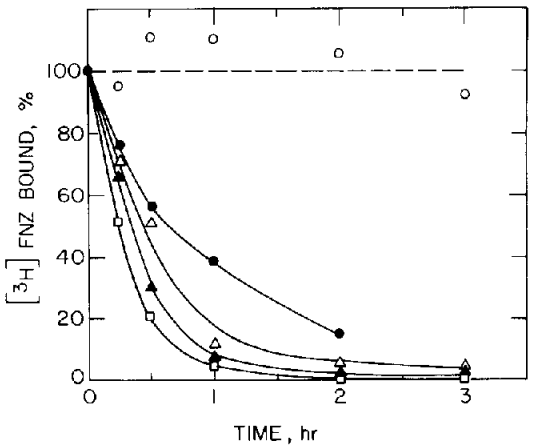

Fig.1. Effect of incubation with TNM on the subsequently measured [^{3}H]FNZ-binding capacity of a particulate benzodizepine receptor preparation. The incubation with TNM was carried out a room temperature at pH 8 for the indicated times in the absence of TNM (○——○), or in the presence of 2.5 mM TNM (●——●), 5 mM TNM (△——△), 10 mM TNM (▲——▲) or 20 mM TNM (□——□).

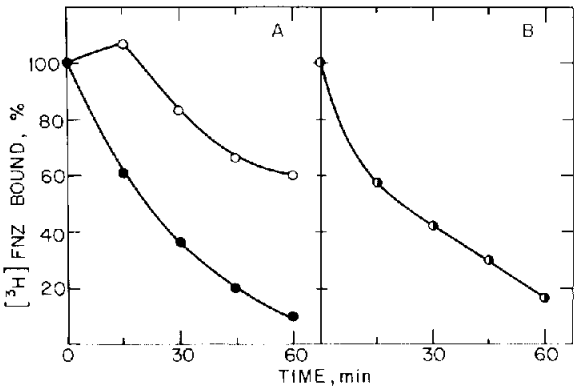

Fig.2. Effect of incubation with TNM on the subsequently measured [^{3}H]FNZ-binding capacity of DOC-solubilized benzodiazepine receptor preparation. The incubation with TNM was carried out at room temperature at pH 8.0 for the indicated times: (A) (○——○), no TNM present; (●——●), incubation in the presence of 2.5 nM TNM; (B) Binding capacity relative to control, calculated from (A).

We have tested the ability of benzodiazepines and of GABA, which has been reported to interact with the benzodiazepine receptor [3,6], to protect against the TNM effect (table 1). Incubation with diazepam protected against the loss of binding activity induced by TNM, whereas the pharmacologically inactive ben-

Table 1
Effect of various ligands on the modification of benzodiazepine receptors by TNM

Ligand	Residual activity (%)	
	Exp. I	Exp. II
	48	34
5×10^{-6} M Diazepam	69	80
10^{-5} M GABA	58	55
5×10^{-6} M Diazepam + 10^{-5} M GABA	111	106
5×10^{-6} M Nitrazepam + 10^{-5} M GABA	–	118
10^{-4} M RO-5-4864	46	35

Aliquots of a DOC-solubilized benzodiazepine receptor preparation were incubated with the indicated ligands for 60 min at 0°C. TNM was than added to final conc. 2.5 mM (exp. I) or 5.0 mM (exp. II) and incubation was further carried on for 30 min at room temperature. Aliquots of the receptor were separated from the ligands and from TNM as in section 2.2. Values are presented as percent of [^{3}H]FNZ-binding activity, assayed subsequently, relative to control without TNM

zodiazepine, RO-5-4864, was not effective. GABA also provided some protection against nitration; combinations of GABA and a pharmacologically active benzodiazepine (diazepam or nitrazepam) were most effective (table1).

TNM may also modify cysteine or, though less likely, tryptophan residues [7]. Nitration of tyrosine is expected to proceed much slower at pH 6.5 than at pH 8.0, whereas nitration of cysteine is efficient at the lower pH [4,7]. Tryptophan is not expected to react below pH 7 [7]. We have subjected an aliquot of a particulate receptor preparation to treatment with 5 mM TNM at pH 6.5, and found that loss of [^{3}H]-FNZ-binding activity, although significant, was much slower than at pH 8.0. Thus, 90% of the [^{3}H]FNZ-binding capacity was lost after 1 h at pH 8.0, while the corresponding value at pH 6.5 was 50%. This result argues against the involvement of cysteine or tryptophan in the reaction. In addition, we have incubated aliquots of a particulate receptor preparation for 1.5 h at room temperature in the presence of 1 mM PCMPS, 5 mM NEM or 5 mM TNM. [^{3}H]FNZ-binding, relative to control, was found to be 112%, 116% and 10%, respectively. The latter results render the possibility that TNM modifies a cysteine residue very unlikely.

To further support the hypothesis that a modified tyrosine is responsible for the inactivation of [^{3}H]-FNZ binding sites, we tested the effect of *N*-acetylimidazole, which acetylates phenolic groups [5], on the benzodiazepine receptor. Incubation with *N*-acetylimidazole led to a gradual loss of [^{3}H]FNZ-binding capacity, which was partially reversed by deacetylation with hydroxylamine (fig.3).

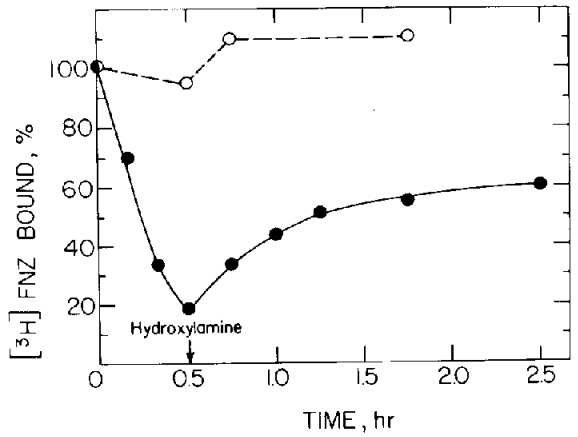

Fig.3. Effect of incubation with *N*-acetylimidazole on the subsequently measured [^{3}H]FNZ-binding capacity of a particulate benzodiazepine receptor preparation. Incubation with *N*-acetylimidazole was carried out at room temperature for 30 min with 10 mM reagent (•——•) or without it (○ —○). After 30 min (arrow) hydroxylamine (final conc. 80 mM) was added to both preparations.

Taken together, our results suggest that a tyrosyl residue (or residues) is located at, or in proximity to, the ligand binding site(s) of the brain receptors for benzodiazepines. It is plausible to assume that the modified tyrosine is exposed to the outer surface of the receptor since *N*-acetylimidazole was reported not to react with buried tyrosine residues [5]. In [1], a tyrosine residue was reported to be involved in the binding of benzodiazepines and indoles, e.g., L-tryptophan, to human serum albumin, and evidence was also that in the latter macromolecule the nitrated amino acid is not tryptophan. The similarity between the binding sites of serum albumin and brain benzodiazepine receptors is limited, since the affinity of benzodiazepines to brain receptors is several orders of magnitude higher than their affinity for serum albumin, and the binding of [^{3}H]FNZ to calf brain receptor is not displaced even by 10^{-2} M L-tryptophan.

The protection against the inactivation of solubilized receptors by TNM, provided by GABA and a combination of GABA and a benzodiazepine, also corroborates previous reports that the DOC-solubilized benzodiazepine receptor bears a GABA recognition site [3,8]. However, the relationship between the benzodiazepine and the GABA sites (e.g., do they reside in close proximity or on separate polypeptides) as well as direct evidence for the identity and the location of the tyrosyl residue(s) involved in ligand binding to the receptor, must await the purification of the receptor molecule.

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