Isolation of a GABA receptor from bovine brain using a benzodiazepine affinity column

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Benzodiazepine receptor solubilized from bovine cortical membranes was bound to a new benzodiazepine affinity column, the synthesis of which is described. Bio-specific elution with the benzodiazepine compound chlorazepate resulted in the elution of fractions highly enriched in specific binding for the GABA receptor agonist muscimol. Specific activity for [^{3}H]muscimol binding was > 1.3 nmol/mg protein. It is shown that [^{3}H]flunitrazepam binding activity can be recovered by removal of chlorazepate from the purified fraction. These results strongly support a model which suggests that the 2 binding sites reside on the same physical entity.

GABA receptor *Benzodiazepine receptor* *Isolation* *Affinity chromatography*

1. INTRODUCTION

A large number of observations on brain membrane-bound and soluble benzodiazepine (BZ) and Na^+-independent GABA receptors have led to the concept of a GABA–BZ ionophore complex. This complex is thought to mediate many of the sedative, anxiolytic and anticonvulsant actions of BZ, barbiturates and related compounds (review [1]). Evidence for an association of the two activities in detergent extracts of brain membranes has been obtained [2–5] but it remains to be established that the two activities are indeed located on the same physical entity.

We report here of the use of a BZ affinity column which purifies the high-affinity binding sites for both the specific GABA receptor agonist, muscimol, and the BZ ligand flunitrazepam.

2. MATERIALS AND METHODS

To synthesize the BZ affinity gel, 20 ml adipic dihydrazide agarose (Sigma) was reacted for 3 h at room temperature with 4.2 g iodoacetate in 40 ml total vol. at pH 5.0. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (1 g) was included to catalyze the reaction. The gel was washed and reacted overnight at room temperature with 144 mg of the BZ ligand Ro 7-1986/1 in 100 mM Na^+-carbonate (pH 9.0)/5% ethanol (v/v). The gel was finally treated with 0.2% mercaptoethanol to stop the reaction and washed exhaustively. The concentration of the bound ligand was estimated from a UV difference spectrum of the affinity gel versus a control gel (treated identically but with the BZ ligand omitted).

BZ receptor was solubilized from the bovine cortex membranes by a procedure similar to that in [3], but the deoxycholate was not exchanged for Triton X-100. Protease inhibitors [5] were included to protect the receptor and to maximize its recovery. The soluble BZ receptor was applied to the affinity column (2.6 × 4.6 cm) at 60 ml/h. The column was washed overnight with 500 ml 50 mM NaCl/5 mM HEPES (pH 7.5)/0.1 mM EDTA/0.1% (w/v) Triton X-100. It was finally washed with 50 ml of 20 mM Na^+-phosphate (pH 7.5)/0.1% Triton X-100. The receptor was eluted with 10 mM Na^+-phosphate (pH 7.5)/10 mM chlorazepate/0.1% Triton X-100.

[^{3}H]Flunitrazepam (72 Ci/mmol) and [^{3}H]mus-

Abbreviations: GABA, γ-aminobutyric acid; BZ, benzodiazepine; EDTA, ethylene-diamine-*N,N,N',N'*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; SDS–PAGE, sodium dodecylsulphate–polyacrylamide gel electrophoresis

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cimol (9.5 Ci/mmol) were from the Radiochemical Centre (Amersham). Both ligand binding activities were assayed by a polyethylene glycol precipitation/filtration method [6]. The receptor was diluted for assay into a medium containing 10 mM Na^+-phosphate (pH 7.5)/0.1 mM EDTA/0.1% Triton X-100.

SDS–PAGE was performed as in [7] with a 10% acrylamide gel. The purified protein was precipitated with 12% trichloroacetic acid prior to electrophoresis.

3. RESULTS AND DISCUSSION

Fig.1 shows the chemical structure of the BZ affinity column used in this work. The BZ ligand preserves a positive charge in the covalently bound form on the alkylated amino-group. The final concentration of immobilized BZ ligand on the gel was 0.3–0.5 μmol/ml gel (3 prep.). Application of an affinity column with a similar spacer arm, but with the BZ delorazepam, has been reported [8].

When the deoxycholate-solubilized receptor from bovine brain cortex was applied to the affinity column, >87% of the specific [^{3}H]flunitrazepam binding sites were retained by the column; <5% of the bound BZ binding sites were lost during the subsequent extensive washing of the column. Bio-specific elution was achieved by the water-soluble BZ ligand, chlorazepate. The resulting fractions could not be assayed directly for [^{3}H]-flunitrazepam binding activity due to the presence of a high concentration of chlorazepate. However, these fractions were found to be highly enriched in specific [^{3}H]muscimol binding sites. The yield of [^{3}H]muscimol binding activity was 14–20% of the applied soluble extract. This corresponded to 600 pmol [^{3}H]muscimol binding sites (measured at saturation) in 30 ml total vol. The protein determination was difficult due to both the low concentration of protein and the presence of chlorazepate which interfered with the protein assay [9]. [^{3}H]-Muscimol binding was saturable (fig.2A). Non-specific binding was very low (<3% of the total

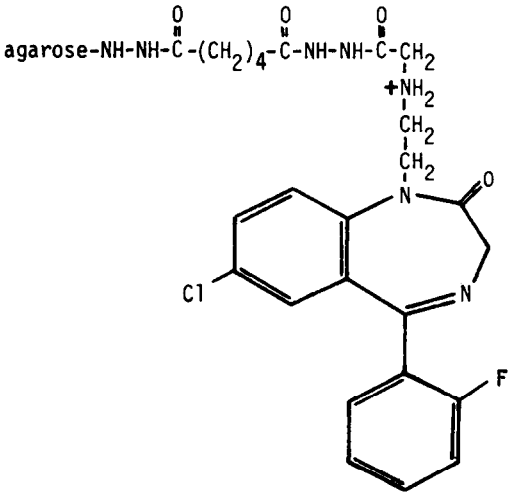

Fig.1. Structure of the immobilized benzodiazepine affinity gel used.

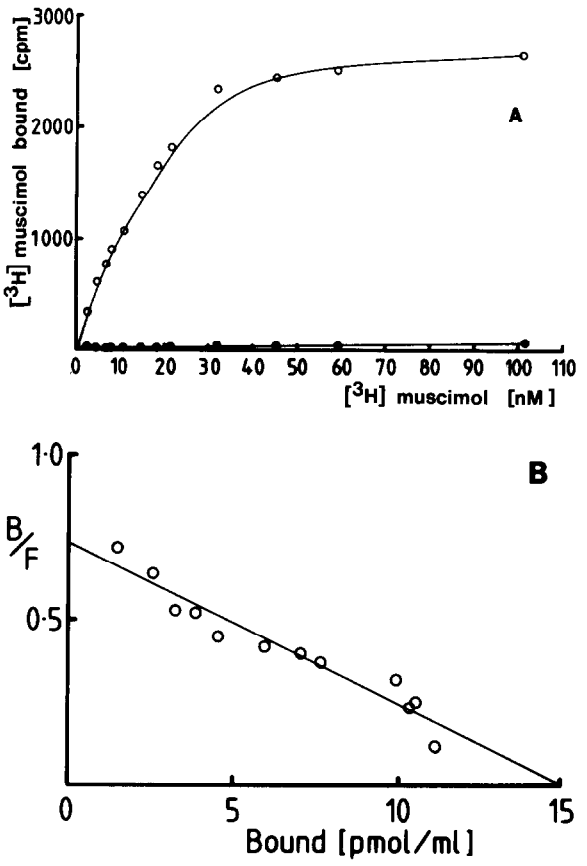

Fig.2. (A) Binding of [^{3}H]muscimol to the purified fraction. The receptor was incubated with [^{3}H]muscimol for 30 min at 0°C. Non-specific binding was measured in the presence of a 1000-fold excess of unlabelled muscimol: (○) specific binding; (•) non-specific binding. (B) Scatchard plot of the [^{3}H]muscimol binding to the purified component.

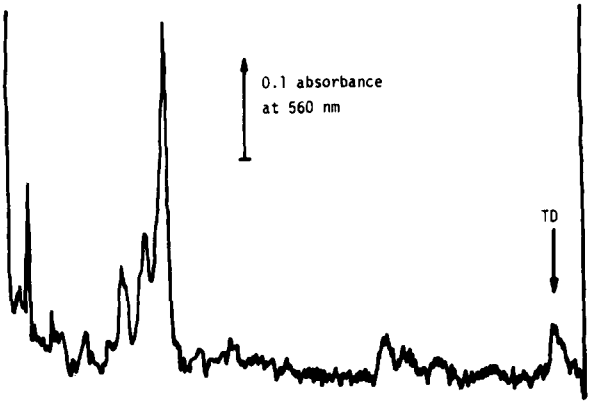

Fig.3. Scan of a SDS–polyacrylamide gel (10%) of the purified complex recovered from the DEAE–cellulose column stained with Coomassie blue; TD, position of the tracking dye.

binding at 100 nM). A Scatchard plot of the saturation curve (fig.2B) showed the presence of a single class of [^{3}H]muscimol binding sites. The K_d-values found in 3 different preparations were from 16–25 nM.

SDS–PAGE was performed and the gels stained by the Coomassie blue or a silver [10] method. By comparison with protein standards, it was confirmed in both staining methods that the protein content of the [^{3}H]muscimol binding fractions was very low ($< 15\ \mu g/ml$). The specific binding activity can therefore be estimated to be > 1300 pmol/mg protein. Both staining methods showed a single major band in the region of M_r 53 000. A scan of a typical preparation of the purified receptor is shown in fig. 3.

Thus we have isolated saturable [^{3}H]muscimol binding activity with a BZ affinity column by means of bio-specific elution with a BZ ligand. This observation provides strong evidence for a tight association of GABA and BZ binding sites that can be preserved during isolation. That such an association in one protein or molecular complex is characteristic of a native structure from the original membrane is indicated by the finding that the 2 binding sites possess an identical target size in the brain membranes [5,11].

There have been two reports on the use of BZ affinity columns for the purification of the BZ receptor [2,8]. However, in [2], bio-specific elution and high specific activity, as reported here, have not been obtained; in the other case [8], co-purification of a GABA binding site was not shown.

The ratio of the 2 classes of binding site was examined after the removal of chlorazepate. This was approached by binding the receptor to either a hydroxyapatite or a DEAE-cellulose column, followed by washing of the column and elution of the protein with 0.8 M Na^+-phosphate and 0.6 M KCl, respectively. With both methods specific flunitrazepam binding is recovered in the purified material. Specific [^{3}H]muscimol and [^{3}H]flunitrazepam binding were measured close to saturation; i.e., at 30 nM and 25 nM, respectively. The ratio of [^{3}H]muscimol binding to that of [^{3}H]flunitrazepam was 3.2 for the material resulting from the hydroxyapatite column and (5 expt.) for that recovered from the ion-exchange column 2.9–3.5. However, these results should be viewed with caution at this preliminary stage: this is due to possible residual contamination by chlorazepate, to possible losses of the specifically bound ligand (especially [^{3}H]muscimol) during the filter washing in the assay, and to the possibility of differential inactivation of the 2 binding sites during the procedures involved. For these reasons, the exact ratio of the 2 binding sites on the purified complex remains to be established.

Recent experiments have shown that chlorazepate can indeed be removed completely from the present preparation. Results on the subunit and oligomeric structure and the pharmacological properties of this purified BZ–GABA receptor complex will be presented in full elsewhere.

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REFERENCES

[1] Olsen, R.W. (1981) J. Neurochem. 37, 1–13.
[2] Gavish, M. and Snyder, S.H. (1981) Proc. Natl. Acad. Sci. USA 78, 1939–1942.
[3] Stephenson, F.A., Watkins, A.E. and Olsen, R.W. (1982) Eur. J. Biochem. 123, 291–298.

[4] Chang, L.-R. and Barnard, E.A. (1982) J. Neurochem. in press.
[5] Lang, B., Barnard, E.A., Chang, L.-R. and Dolly, J.O. (1979) FEBS Lett. 104, 149–153.
[6] Gavish, M., Chang, R.S.L. and Snyder, S.H. (1979) Life Sci. 25, 783–790.
[7] Douglas, M. and Butow, R.A. (1976) Proc. Natl. Acad. Sci. USA 73, 1083–1086.
[8] Martini, C., Lucacchini, A., Ronca, G., Hrelia, S. and Rossi, C.A. (1982) J. Neurochem. 38, 15–19.
[9] Bensadoun, A. and Weinstein, D. (1976) Anal. Biochem. 70, 241–250.
[10] Morrisey, J.H. (1981) Anal. Biochem. 117, 307–310.
[11] Chang, L.-R., Barnard, E.A., Lo, M.M.S. and Dolly, J.O. (1981) FEBS Lett. 126, 309–312.