PUTATIVE BENZODIAZEPINE RECEPTOR: A PROTEIN SOLUBILISED FROM BRAIN

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Received 6 June 1979

1. Introduction

The benzodiazepine tranquillising drugs are believed to produce their pharmacological effects by affecting synaptic processes [1] but the exact site and mode of this action remain unknown. However, the binding of [^{3}H]diazepam to rat [2,3] and human [4] brain membranes has been demonstrated; its affinity and those calculated for other drugs in the series were shown to correspond to their clinical effectiveness [2,4]. An approach to identifying such a putative membrane receptor is to solubilise it in non-ionic detergent medium; this facilitates its biochemical analysis, as has proven fruitful with the peripheral nicotinic acetylcholine receptor [5]. We show here that the benzodiazepine-binding structure (provisionally referred to as the receptor) can be obtained in such a soluble condition with retention of its ligand binding activity and specificity. Evidence bearing upon its molecular constitution is also given.

2. Materials and methods

2.1. *Chemicals*

[*N-methyl*-^{3}H]Diazepam (47 Ci/mmol) and [*N-methyl*-^{3}H]flunitrazepam (31 Ci/mmol) were from the Radiochemical Centre, Amersham. Their unlabelled versions were gifts from Roche Products Ltd. Leupeptin and pepstatin were kindly provided by the USA-Japan Coop. Cancer Res. Prog. Triton X-100 was from Rohm and Haas, and all the other chemicals from Sigma.

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2.2. *Preparation of brain membranes and their solubilisation*

Whole brain cortex was removed from Sprague-Dawley rats (~250 g body wt) and homogenised in 10 vol. ice-cold sucrose (0.32 M) containing protease inhibitors [6], i.e., 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 200 μg/ml bacitracin, 1 mM benzamidine–HCl, 0.1 mM phenylmethane sulphonyl fluoride (PMSF) and 20 μg/ml soybean trypsin inhibitor. The homogenate was centrifuged at 2×10^4 *g* . min; the pellet (P_1) was discarded and the supernatant was recentrifuged at either 1.5×10^6 *g* . min (for P_2 fraction) or 6×10^6 *g* . min (for $P_2 + P_3$). The membranes were lysed by resuspension in 0.05 M Tris–HCl (pH 7.4) containing the inhibitors (as above). The receptor was either assayed in the membrane-bound state or solubilised by adding Triton X-100 to the final concentration noted in the text (using 8 ml medium/g original tissue wet wt), followed by brief homogenisation (Polytron; setting 4 (maximal setting is 10) for 20 s) and shaking for 1 h at 4°C. The resultant extract was spun at 6×10^6 *g* . min. The supernatant and pellets (the latter redispersed in 0.05 M Tris–HCl buffer, pH 7.4) were assayed for [^{3}H]ligand binding. In some cases the membranes were treated by a modification of the method reported [7] to remove an endogenous protein inhibitor: the lysed P_2 fraction was frozen and stored for 1 day at –20°C, thawed, extracted in 0.01% Triton X-100 at 4°C for 10 min and centrifuged at 1.5×10^6 *g* . min, before extraction as above. Protein contents were determined [5] on all samples (after complete solubilisation in 0.75 M NaOH) by the Folin-Lowry method.

2.3. *Benzodiazepine binding assays*

The binding assay on membranes (lysed P_2, or

$P_2 + P_3$, fraction) followed fairly closely that in [2], using an initial incubation at 22°C (15 min) and a final one at 4°C (30 min), but without dilution of the suspension thereafter before applying it to a glass fibre filter and then washing with 2 × 5 ml ice-cold 0.05 M Tris–HCl buffer (pH 7.4). For assays on the solubilised fraction, the samples were diluted with extraction medium so as to contain < 1 mg protein/ml and [^{3}H]ligand was added (to the concentration stated), with incubation for 15 min at room temperature and then on ice for 30 min. Samples (100 μl) were then applied to Whatman DE81 filters which were held in a PVC washing assembly (joined to a vacuum manifold) designed to give free access of wash liquid up to the edge of the filter disc. After 4 min for equilibration suction was applied and then 2 washes, each with 10 ml ice-cold 0.05 M Tris–HCl buffer (pH 7.4) containing 0.2% Triton X-100, were given rapidly. The filters were dried and the radioactivity counted by liquid scintillation at 30–35% efficiency, using a toluene-based mixture [5] containing 10% Soluene (Packard). The specific binding was the total binding minus the non-specific binding; the latter was measured with the unlabelled ligand (3 μM for diazepam and 20 μM for flunitrazepam) present in the incubation medium. All samples were incubated in triplicate.

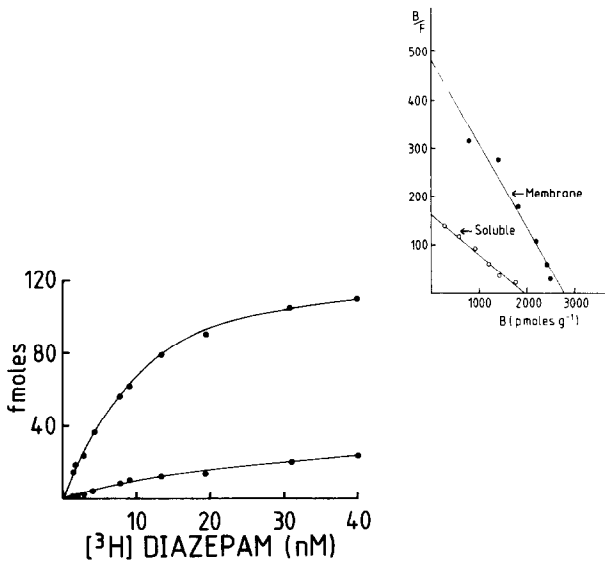

Fig.1. Binding of [^{3}H]diazepam to cortex membrane fraction ($P_2 + P_3$) solubilised in 0.7% Triton X-100. Each point is the mean of triplicates, expressed as fmol bound/100 μl extract. The lower curve shows the control points for non-specific binding (i.e., in the presence of 3 μM unlabelled diazepam); the upper curve represents the specific binding obtained after correction for that control. Insert: Scatchard plot for the specific binding of [^{3}H]flunitrazepam to cortex membrane fraction (P_2), intact (•), or after solubilisation in 0.7% Triton X-100 (○).

3. Results

3.1. *Binding sites in brain membranes*

Specific binding of [^{3}H]diazepam to rat brain membranes was observed and the findings [2,3] were confirmed: at pH 7.4 (4°C) the sites in the crude synaptosomal (lysed P_2) membrane fraction (or that plus the microsomal fraction P_3) exhibited a dissociation constant (K_d) of 3.4 (± 0.6) nM, with the drug, for a single, saturable class of sites as seen in the Scatchard plot (using [^{3}H]diazepam at ⩽ 100 nM). Studies with [^{3}H]flunitrazepam [8,9] also gave evidence for a single, specific, high affinity (K_d 6 nM) binding site (fig.1, insert). The values found from Scatchard plots (table 1, lines 1,2) agree fairly well with the results (K_d 2.5–5.3 nM; 0.81–1.45 pmol/mg protein) of similar measurements made with diazepam on rat or human membranes [3,4,10]. However, others [8,9] have observed a lower specific activity (0.1–0.5 pmol/mg protein) but higher affinity ($K_d \sim$ 1 nM) for flunitrazepam in rat brain membranes. The binding of flunitrazepam and diazepam found here were mutually exclusive, again indicating a specific site.

3.2. *Solubilisation*

Homogenisation and shaking of the membranes in the detergent Triton X-100 was able to extract actively-binding material into solution: the extent of this increased up to ~0.7% (v/v), and was then constant up to 2% detergent (table 2, column 2). Extension of the period of extraction to 3 h or to 15 h did not increase the yield. It was necessary to add inhibitors of proteases, using a set of these recently found effective in preventing proteolysis of cholinesterases in Triton X-100 extractions of muscle [6]; when these were omitted the extracted receptor measured was much less, and when partly omitted a partial decrease occurred (table 2, column 3).

The assay used relies upon the retention of the

Table 1
Binding of [^{3}H]flunitrazepam by rat cortex membranes (P_2) and by their 0.7% Triton X-100 extracts

Receptor state	K_d (nM)[a]	pmol/mg protein	
		At 20 nM ligand[b]	At satn.[a]
Membrane bound:			
unwashed	6 ± 0.1	2.1	2.7 ± 0.07
washed[c]	6 ± 0.2	2.2	2.6 ± 0.14
Solubilised	12 ± 0.4[d]	1.1	1.8[d] ± 0.06

[a] The mean ± SEM of values derived from the slopes and the intercepts of Scatchard plots, as illustrated in fig.1, from 4 separate experiments
[b] Values are the means from 2 independent experiments, each using pooled tissues from 3 rats
[c] In these experiments the membranes were (after centrifugation but prior to subsequent extraction) initially frozen, stored for 1 day at −20°C, thawed and washed with 0.01% Triton X-100 at 4°C for 10 min. Values not significantly different from these were obtained when fresh (not freeze-thawed) membranes were extracted similarly in 0.01% Triton X-100
[d] The membranes used for extraction were frozen, thawed and washed as noted in [c]. However, identical values were obtained in a parallel experiment using membranes which were not pretreated in this manner

Table 2
Extract of benzodiazepine binding sites from the membranes into aqueous detergent solutions

Triton X-100[a] (%)	Relative binding (soluble)[b]	
	Protected	Unprotected
0.1	0.02	0
0.3	0.36	–
0.5	0.83	0.32
0.7	1.00	0.59
1.5	0.97	0.35
2.0	0.93	0.33

[a] The membrane fraction was extracted for 1 h at 4°C with Triton X-100 present at the final concentration shown
[b] The relative binding of [^{3}H]benzodiazepine (under standard conditions) in the solubilised fraction, taking the value found in the extraction in 0.7% Triton X-100 as the reference

Assays were with 24 nM [^{3}H]flunitrazepam of [^{3}H]diazepam; the measured radioactivity corresponding to the relative value 1.0 was ~1000 cpm (specific binding)/assay. The mean for two independent extractions (each using 3–5 rats) is given. For the experiments of the second column, the full set of protease inhibitors listed under section 2 was present throughout. In the experiments of the last column, much less protection was given (leupeptin, bacitracin, PMSF and benzamidine were omitted), resulting in decreased yields of active binding sites. Separate experiments using membranes showed that none of the protease inhibitors used reduced the binding of [^{3}H]flunitrazepam in the standard assay conditions

solubilised acidic membrane proteins, but not free benzodiazepines, on a DEAE-cellulose disc, as applied [5] to the muscle acetylcholine receptor. Saturable binding of [^{3}H]diazepam and [^{3}H]flunitrazepam to the solubilised receptor was observed. The non-specific background (taken as the binding in the presence of a large excess of the unlabelled ligand) was a conveniently small fraction of the total; values found with $\leqslant$ 40 nM [^{3}H]diazepam are shown in fig.1, and for [^{3}H]flunitrazepam, the non-specific background was always still lower.

Scatchard plots (as in fig.1, insert) again showed a single class of benzodiazepine binding sites in the soluble extract; the affinity for either ligand was reduced in the soluble form to 12 nM. The total recovery of the membrane binding sites in the solublised form was 60–70% after a single detergent treatment, when calculated for the total number of sites at saturation with ligand in each case. Excess diazepam or flunitrazepam inhibited the binding of the other ligand (table 3).

Pre-treatment of the membranes by freezing, thawing and washing in 0.01% Triton X-100, was recently described as removing an endogenous protein inhibitor of [^{3}H]diazepam binding to brain membranes [7]. This was also applied here (table 1): the very dilute detergent wash step removed no receptor and did not increase the detectable number of

receptor sites in the membranes. The specific activity, with or without this pre-treatment, was reduced in the solubilisation to ~66% of the washed membrane value (table 1).

3.3. *Molecular properties of the solubilised component*

The specific binding activity was completely stable for ⩾ 1 day at 4°C. On brief exposure to heat it was abolished, and the same was true after a short treatment with either of two proteases (table 3). The action of trypsin here could be completely blocked by trypsin inhibitors; as expected the action of pronase was not affected. Incubation with phospholipase A_2 had no effect (table 3). Hence, the soluble binding activity appears to depend upon the integrity of a protein.

Sucrose density centrifugation of the active extract was performed to estimate the molecular size of the receptor. Lysed P_2 membranes from fresh rat brain were extracted and immediately applied to the gradient which contained 0.5% Triton X-100. Assays of all of the fractions revealed a single peak of activity centered at $s_{20,w}$ = 12.8 S (fig.2); a slight shoulder at 15 S was also observed.

Table 3
Effects of various different treatments upon the binding of [^{3}H]benzodiazepines to detergent-solubilised receptor

Treatment	Conc.	Binding[a] (% control)
None (control)	–	100%
Flunitrazepam[b]	3 × 10^{-6} M	1% ± 1%
Diazepam[b]	3 × 10^{-6} M	4% ± 3%
Trypsin (1 h, 37°C)	0.03%	5%
Inactivated trypsin[c]	0.03%	<1%
Treated pronase[c]	0.03%	<1%
Phospholipase A[d]	109 U/ml	95%
65°C, 5 min	–	<1%
90°C, 1 min	–	<1%

[a] The treatments were applied in 0.5% Triton X-100/0.05 M Tris–HCl (pH 7.4), prior to the standard assay (using 24 nM [^{3}H]flunitrazepam). For the treatments at 37°C, the control was also incubated at 37°C, for 1 h prior to assay. In the control 4300 cpm represented the 100% values

[b] Present during the assay; when the flunitrazepam was present 5 nM [^{3}H]diazepam was used for the assay of tests and controls

[c] The protease (used again here at 37°C, 1 h) had been, to inactivate trypsin, pretreated (37°C, 30 min) with a 30-fold excess of soybean trypsin inhibitor, or with 10^{-4} M DFP (for trypsin, second value) as a further control

[d] Phospholipase A_2 from bee venom plus 20 mM $CaCl_2$ was present for 1 h at 37°C prior to the binding measurement; the activity of this same sample of enzyme was determined using egg lecithin as substrate

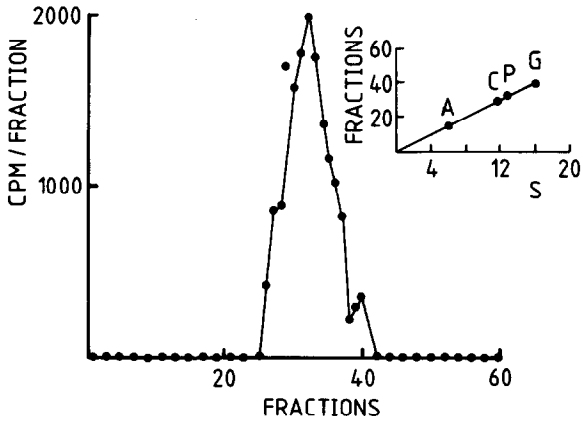

Fig.2. Density gradient sedimentation of the solubilised benzodiazepine-binding component. A 5–20% sucrose gradient was run as described [6] but with the sucrose made up in 0.5% Triton X-100/0.05 M Tris–HCl (pH 7.4). Fractions of 230 μl were collected, and 200 μl from each was taken for [^{3}H]flunitrazepam binding assay. Standards run in parallel were horse liver alcohol dehydrogenase (A), beef liver catalase (C) and *Escherichia coli* β-galactosidase (G); their $s_{20,w}$ values are plotted (in S) in the calibration plot (insert) used to determine the $s_{20,w}$ value at the receptor peak maximum (P).

4. Discussion

The solubilisation of the membrane-bound benzodiazepine binding component in a stable and active state opens up the way to its purification. Affinity chromatographic methods for this should be feasible. The possibility of the blockade by endogenous ligands of some of the binding sites in the membrane [7] was avoided by the pre-treatment with very dilute detergent, but the amounts of such inhibitors initially present probably are insufficient to interfere with benzodiazepine binding in the soluble state. The extent of receptor solubilisation is high; ligand affinities of the receptor in the presence of the solubilising detergent are about one-half of that in the membrane.

The evidence presented here indicates that a pro-

tein with $s_{20,w}$ = 12.8 S (when in detergent solution) is the benzodiazepine receptor.

Acknowledgements

This work was supported by a Programme Grant from the Medical Research Council (MRC). B.L. holds an MRC research studentship and L.R.C. a Taiwan National Science Council studentship. We thank Dr C. Braestrup (Roskilde, Denmark) for helpful initial discussion.

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