PURIFICATION OF A γ-AMINOBUTYRIC ACID RECEPTOR-LIKE CONSTITUENT OF BOVINE BRAIN

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1. Introduction

Brain membranes have specific sites for GABA binding in the absence of sodium. These sites are of high and low affinities [1–6]. The binding material containing the high affinity sites has been solubilized with either lysolecithins or detergent and partially purified [7,8]. A proteolipid has also been partially purified from rat brain which showed a site of low affinity when tested in an organic medium [6]. Since the availability of the pure receptor could help to solve problems about the presence of several GABA binding sites and their relationship to the membrane binding sites for agonists [9], benzodiazepines [10], GABA-modulin [3], and any other ligand, we undertook the purification of the receptor.

We report the isolation of a hydrophobic peptide that is chromatographically and electrophoretically homogeneous and that binds GABA specifically with high and low affinities in organic or aqueous medium.

2. Materials and methods

2.1. *Purification of the receptor-like peptide*

Adult bovine brain was minced with 3 vol. cold acetone in a blender. The mixture was filtered and the cake extracted twice with acetone. The cake was then extracted 3 times with 2 vol. each of petroleum ether (b.p. 50–70°C) and filtered. The ether extracts were pooled and evaporated to dryness. The residue was suspended in 600 ml C/M (1:2, v/v), 60 ml of 1 M NaOH were added, incubated for 4 h at 45°C and neutralized with acetic acid. To partition [11], 600 ml C and 240 ml water were added, mixed and the upper phase discarded. The lower phase was washed twice with theoretical upper phase, and evaporated to dryness. The residue was suspended in 100 ml C/M/water (60:30:4.5, by vol.) and dialyzed in cellophane sacs against 10 × 3 vol. same mixture for 3 days. The outside fluids were evaporated to dryness, suspended in 100 ml C/M/water (60:30:4.5, by vol.) and passed through a DEAE–Sephadex column (2 × 40 cm, acetate form) equilibrated with the same solvent. The column was washed with 2 vol. solvent mixture and the non-retained material was evaporated to dryness. The residue was solubilized in 200 ml methanol at 55°C, cooled at 0°C and the cholesterol crystals were centrifuged off in the cold. This operation was repeated until no more cholesterol crystals were formed. The solution was evaporated to dryness, the residue dissolved in C/M/water (60:30:4.5, by vol.) and passed through a column of silica gel H (3 × 30 cm) equilibrated with this solvent mixture. The column was eluted with 5 vol. same solvent collected into fractions of 5 ml each. The fractions with binding activity were pooled and evaporated to dryness. The residue was solubilized in C/M/water (25:50:15, by vol.) and passed through a column of silica gel H (1.3 × 15 cm) equilibrated with the same solvent. Three volumes of this mixture were passed through the column and collected into fractions of 1 ml each. The fractions with binding activity were pooled, evaporated and subjected to TLC on washed silica gel H. The following developing mixtures were successively used: (A) C/M/15 N ammonia (60:30:4.5, by vol.); (B) C/M/glacial acetic acid (10:10:3, by vol.); (C) C/M/water (5:10:3, by vol.); and (D) C:M (2:1, v/v). A strip of each plate was cut out and stained with ninhydrin, or

Abbreviations: GABA, γ-aminobutyric acid; C, chloroform; M, methanol; TLC, thin-layer chromatography; IC_{50}, concentration causing 50% inhibition of specific [^{3}H]GABA binding

charred with sulphuric acid. The silica from the remainder plate fragment was scrapped off into fractions and eluted with C/M/water (60:30:4.5, by vol.). The eluates were evaporated to dryness and the material partitioned [11]. The lower phases were washed twice with theoretical upper phase.

2.2. *Binding determinations*

2.2.1. In organic medium

Material from ~100 mg fresh tissue was dissolved in 0.6 ml C/M (2:1, v/v/). [^{3}H]GABA (New England Nuclear, 43 Ci/mmol) was added in 5 μl water when the lowest concentrations of ligand (5–10 nM) were assayed. For higher concentrations of the ligand, 10 nM [^{3}H]GABA plus different amounts of non-radioactive GABA to reach final concentrations between 20 nM and 50 mM of the total ligand, were added. After incubation at 22°C for 20 min, 1 ml water was added and the mixture vigorously shaken and centrifuged. The upper phases were discarded, the lower phases were washed 3 times with 1 ml theoretical upper phase, evaporated to dryness in a vial and their radioactivities measured.

2.2.2. In aqueous medium

This method was used with the homogeneous receptor. Preparations in intermediate stages of purification contained too much lipid to allow the use of this method. The peptide (60 μg) was suspended by sonication (5 s) in 0.6 ml/1.6 mM Tris–HCl buffer (pH 7.4). Radioactive and non-radioactive GABA were added as indicated above. The mixture was incubated at 22°C for 20 min and 1 ml C/M (2:1, v/v) added. The lower phase was washed 3 times with 1 ml theoretical upper phase, evaporated to dryness and its radioactivity was measured. For procedures in organic and aqueous medium, controls for zero incubation time and incubations in the presence of 50 mM of non-radioactive GABA were run to discount the non-displaced [^{3}H]GABA binding. Determinations were done in triplicate. The average deviations were <10%. This partition method [11] was tested for the separation of free and bound ligands. Results showed that <0.5% of the original ligand remained free in the lower phase which in every case was discounted with the appropiate control. It was also tested for the dissociation of the bound-receptor ligand; after 3 washings practically no more [^{3}H]GABA passed to the upper phase.

2.3. *Electrophoresis*

This was performed on 0.1% sodium dodecyl sulphate–10% polyacrylamide according to [12]. Fixing, staining with Coomassie blue and destaining were carried out according to [13].

Table 1
Purification of the GABA receptor-like constituent of bovine brain

Preparation	pmol GABA bound/ g fresh brain[b]	pmol GABA bound/ mg protein[c]
Total brain[a]	8.55	0.071
Ether extract	8.55	2.13
Unsaponifiable	8.45	7.04
Dialyzable	8.04	44.46
Not retained by DEAE–Sephadex	7.93	88.11
Methanol soluble	7.81	97.60
Eluted from silica colums	4.47	112.00

[a] The values corresponding to total brain were calculated from the values of the ether extract because this solvent extracted quantitatively the binding activity

[b] The binding activity lost at each step of purification was found in the corresponding discarded fraction

[c] The protein was determined according to [14] except for the material at the material at the final step in which the protein was determined by the biuret method [15]

The incubation conditions for [^{3}H]GABA binding were as indicated in section 2.2, organic medium. Values shown were obtained in the presence of 20 nM [^{3}H]GABA

3. Results and discussion

The results of the different steps of purification (table 1) show that the peptide has solubility properties of a proteolipid but at variance with most proteolipids so far described [16], the receptor is dialyzable and is not denaturated by being subjected to mild saponification or evaporation to dryness. The final recovery of binding capacity was 40–50% of that in the ether extract. The binding activity lost at each step of purification was found in the corresponding discarded fractions; the purification data did not suggest in any step the presence of activators or inhibitors in the ether starting preparation. Judging from the fold-purification this peptide constitutes <0.1% of the brain proteins. At several steps the material was partitioned [11] and washed to eliminate hydrophilic substances. The strongly hydrophobic property of the peptide made possible the use of the partition to eliminate free [^{3}H]GABA without loss of binding material. In addition the receptor–GABA complex formed with high affinity was not readily dissociable in the chloroformic phase and the radioactivity remained practically constant after 3 washings. This property of the complex was also observed when higher GABA concentrations were used. This allowed the measurement of low affinity, very dissociable, sites. The amount of GABA bound to the peptide/g total brain (8.55 pmol) was of the same order but somewhat less than the 20 pmol/g total brain reported with disrupted and washed membranes [17].

The homogeneity and the peptide nature of the receptor isolated by the method outlined were indicated by the followings results: The material run on TLC with 4 different solvent mixtures showed a single band when charred with sulphuric acid (fig.1) or stained with ninhydrin (not shown). These bands coincided between themselves and with that having binding activity (fig.1). After gel electrophoresis and staining with Coomassie blue, the peptide also showed a single band with of M_r ~11 000 (fig.2). Treatment with protease (Sigma Chemicals, type VI) caused a decrease of 90% of the specific GABA binding to the highest affinity site of the receptor peptide (table 2).

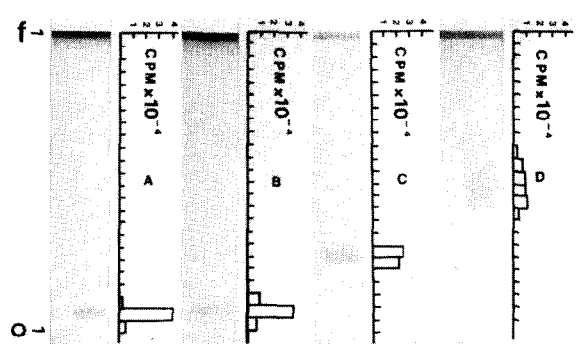

Fig.1. Thin-layer chromatography of the peptide receptor (60 μg). Developing solvent mixtures, A–D, as indicated in section 2.1. The spots were exposed by charring with 50% sulphuric acid at 140°C. Specific [^{3}H]GABA binding of the peptide in cpm × 10^{-4}.

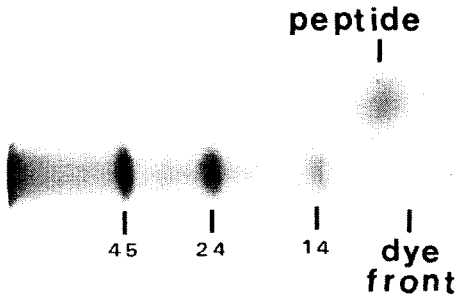

Fig.2. SDS–polyacrylamide gel electrophoresis of the peptide receptor. Bottom: protein markers, ovoalbumin, 45 000 M_r; trypsinogen, 24 000 M_r, and lysozyme, 14 000 M_r. Top: peptide receptor (120 μg).

The curve of the GABA binding to the peptide showed 4 dissociation constants. This was so whether the binding was carried out in organic or aqueous medium (fig.3). The K_d and B_m values were calculated from Scatchard analysis of each independent satura-

Table 2
Effect of the protease on the specific [^{3}H]GABA binding to the highest affinity site of the receptor peptide

Additions[a]	Specific binding[b]
Peptide (50 μg)	2761 cpm
Peptide (50 μg) + protease (50 μg)	128 cpm
Peptide (50 μg) + protease (50 μg), zero time	2710 cpm
Protease (50 μg)	0

[a] The indicated additions were incubated in 0.2 ml 0.05 M Tris–acetate buffer (pH 7.1) for 10 h at 37°C. The digestion was stopped by adding 20 vol. C/M (2:1, v/v) and partitioned. The lower phase was separated and evaporated to dryness

[b] The residue obtained from the lower phase was tested for binding activity in organic medium (see section 2.2) with 15 nM [^{3}H]GABA

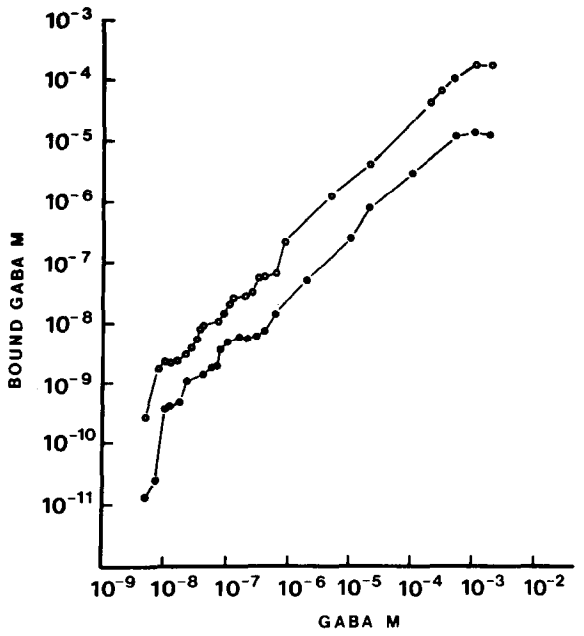

Fig.3. Saturation curves of the specific binding of [^{3}H]GABA to homogeneous peptide (60 μg). The binding activity was measured as in section 2.2. Organic medium (○——○); aqueous medium (●——●). The experiments in both media were repeated twice with practically identical results.

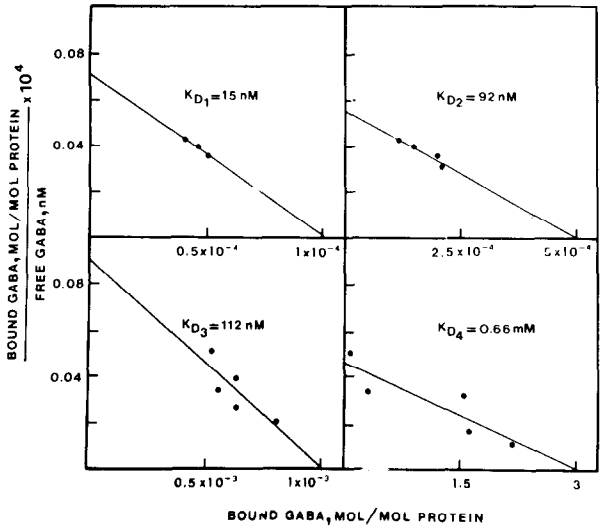

Fig.4. Scatchard plots of the four sites of specific [^{3}H]GABA binding to homogeneous peptide, in aqueous medium. The data shown are those in fig.3. The regression line at each independent site drawn through the experimental points was computer-fitted. The correlation coefficients were, for site K_{d_1} 0.97; site K_{d_2} 0.94; site K_{d_3} 0.81; and site K_{d_4} 0.90.

tion curve using the points nearest to the maximal binding (fig.4). The values for K_d were 15 nM, 90 nM, 112 nM and 0.66 mM. Those for B_m were 1×10^{-4}, 5×10^{-4}, 1×10^{-3} and 3 mol GABA/mol peptide. The amounts of GABA bound to each site were widely different, the 3 sites of high affinity amounting to ~0.1% of the total binding activity of the peptide. A great difference of binding capacity between high and low affinity sites were also found in membranes [5].

Further evidence on the possible identity between this peptide and the receptor were the characteristics of displacement of [^{3}H]GABA from the high affinity site by muscimol (IC_{50} 3 nM), GABA (IC_{50} 99 nM), and imidazoleacetate (IC_{50} 790 nM). The IC_{50} values found with the purified peptide are in the range of values reported by other authors with membranes or less purified materials. For (+) bicuculline we found an IC_{50} value of >500 μM which supports the conclusion of those authors who indicate that binding sites for this antagonist and GABA might differ (see [9]). In addition, the specific [^{3}H]GABA binding to the other 3 sites, measured at [^{3}H]GABA concentrations of 6×10^{-8}, 4×10^{-7} and 4×10^{-4} M, were inhibited by 80–86% when muscimol was added at the same concentrations of GABA indicated for each site (not shown).

Perhaps the most interesting finding so far obtained with the purified peptide is that it showed 4 binding sites whose K_d are similar to those described in membranes which are: 32 nM [1], 16 and 130 nM [2], 20 and 110 nM [3], 21 nM [4] and 30 μM [6], There is fair coincidence between the values of these K_d and those found for the peptide (see above). Whether the different binding sites are expressions of different molecules that purify in approximately the same proportions as they are in the membranes or of differences in conformation or aggregation of a single primary structure, the receptor appears to maintain in solution the same properties it has in membranes.

Acknowledgements

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