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Two forms of the $GABA_A$ receptor distinguished by anion-exchange chromatography

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The $GABA_A$ receptor complex was solubilized from rat brain membranes in Triton X-100, enriched by 1012-S affinity chromatography, and subjected to DEAE anion-exchange chromatography. Two forms were distinguished by their differential elution during this HPLC with a KCl gradient. They displayed similar [^{3}H]muscimol- and [^{3}H]flunitrazepam-binding characteristics, as well as [^{3}H]flunitrazepam-binding inhibition by CL 218872. Rechromatography of these distinct ionic forms indicated that they were not in dynamic equilibrium during chromatography. Resolution of these two pharmacologically similar populations of $GABA_A$ receptor by anion-exchange HPLC suggests that they differ in charge densities, a condition which may reflect differing glycosylation or phosporylation states of the complex.

Aminobutyric acid receptor, γ-; Benzodiazepine; Receptor heterogeneity; Anion-exchange HPLC; CL 218872

1. INTRODUCTION

γ-Aminobutyric acid (GABA) is recognized as a major inhibitory neurotransmitter in the central nervous system [1]. GABA may affect neuronal membrane potentials by acting directly upon the $GABA_A$ receptor and its associated Cl^- channel complex. The gating of the Cl^- channel in the $GABA_A$ receptor is also influenced by benzodiazepine and barbiturate binding to specific receptor sites on the complex [2,3]. Additional GABA receptors not linked to benzodiazepine-binding sites are also prevalent in the nervous system, some of which constitute a separate class known as $GABA_B$ receptors [4]. Considerable progress has been made in the characterization of $GABA_A$ receptors purified from different species and regions of the nervous system (reviews [5,6]).

Consistent with data from other neurotransmitter receptor systems, there appears to be heterogeneity among the $GABA_A$ receptors. Early evidence for $GABA_A$ receptor heterogeneity was obtained in pharmacological studies of the associated benzodiazepine-binding site. Thus, the triazolopyridazine, CL 218872, and β-carbolines have relatively high affinity for the type I benzodiazepine-binding site of $GABA_A$ receptors, while these ligands interact less effectively with this site on the type II receptor [7,8]. The two subclasses of benzodiazepine-binding sites which are coupled to $GABA_A$ receptors have differential ontogenies and distributions in the adult nervous system [8–11] as well as differential sensitivity to thermal inactivation in the presence of certain ions or GABA [12]. The type I receptor is less easily extracted from membranes with detergent and is reported to have characteristics which correlate with a postsynaptic element, while type II may be more concentrated on presynaptic endings [13,14].

Partially homologous cDNA molecules corresponding to the 50 kDa α- and 55 kDa β-subunits of the $GABA_A$ receptor have been isolated from bovine brain [15]. Photolabeling studies using [^{3}H]flunitrazepam have suggested that a 59 kDa subunit polypeptide may be associated with the CL 218872-insensitive, type II receptor in some tissues [16]. We have identified a similar α-like $GABA_A$

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subunit in type II-enriched receptor preparations purified from neonatal cortex and spinal cord [17]. Recently, Levitan et al. [18] have reported three distinct α-like subunit cDNAs in bovine brain which code for subunits they have designated $\alpha 1$, $\alpha 2$ and $\alpha 3$. The distribution of transcripts for the three α-subunits displays regional differences in the nervous system [18].

Beyond subunit composition, receptor heterogeneity may be derived from the degree and quality of glycosylation [19–21] and/or phosphorylation [22]. Receptor subtypes which differ in these respects may be distinguishable by the density of ionized groups within the receptor complex. There have been no reports of non-denatured $GABA_A$ receptor subtypes which vary in terms of their ionic constitution, although [^{3}H]flunitrazepam-photolabeled polypeptides are reported to migrate to three different positions during isoelectric focusing of receptor solubilized in urea [23].

Here, we have examined $GABA_A$ receptor, solubilized and partially purified from rat brain membranes, for evidence of ionic heterogeneity as inferred from mobility during DEAE anion-exchange HPLC.

2. EXPERIMENTAL

2.1. *Receptor preparation*

For each experiment, 70 freshly dissected rat brains were homogenized in a Waring blender in 1 l ice-cold H_2O containing 0.32 M sucrose, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM Na_2EDTA, 0.5 mM dithiothreitol and 40 mg/l of ovoinhibitor brought to pH 7.4 with Tris. The tissue was further homogenized using a glass Potter-Elvehjem homogenizer and sedimented at 1000 × g for 25 min. The supernatant fluid was sedimented at 25000 × g for 25 min and the pellets, designated P1, resuspended in 500 ml of the homogenizing buffer without sucrose and phenylmethylsulfonyl fluoride. The suspension was sedimented at 8000 × g for 20 min and the supernatant with soft upper pellets collected and sedimented at 48000 × g for 20 min. The pellets were resuspended in the same medium using a Polytron, resedimented at 48000 × g, and resuspended in 20 mM Tris-citrate (pH 7.4) with 1 mM Na_2EDTA and 0.5 mM dithiothreitol. The membranes were sedimented again at 48000 × g. The pellets, designated P2, were resuspended with 450 ml solubilization buffer containing 20 mM Tris-citrate (pH 7.4), 1% Triton X-100 (membrane biochemistry grade, Boehringer Mannheim), 1 mM Na_2EDTA, 0.5 mM dithiothreitol and 100 mg/l bacitracin. The mixture was stirred for 30 min on ice and sedimented at 48000 × g for 3 h to remove detergent-insoluble membrane components.

2.2. *1012-S affinity chromatography*

The affinity column of 1012-S-acetamide adipic hydrazide bound to Sepharose 4B was synthesized following the procedure of Taguchi and Kuriyama [24] using 1012-S graciously provided by Dr Kentaro Hirai (Shionogi Research Laboratories, Osaka). A 25 ml bed volume gel was prepared from 7.5 g CNBr-activated Sepharose 4B and 40 mg 1012-S. A similar column was also prepared substituting 60 mg glutamate for 1012-S.

The columns were equilibrated at 4°C with buffer consisting of 50 mM Tris-citrate (pH 7.2) with 0.1 mM Na_2EDTA and 0.1% Triton X-100. The 400-ml samples of solubilized receptor were passed in series over the glutamate column followed by the 1012-S column at a rate of 12 ml/h. More than 90% of the benzodiazepine-binding sites passed through the glutamate column. The high affinity of 1012-S for the benzodiazepine-binding site precluded assay of this site with radioligand following specific elution with 1012-S. To permit assay of the benzodiazepine-binding site through subsequent anion-exchange chromatography, $GABA_A$ receptor was eluted from the 1012-S affinity column with 140 ml of 1 M NaSCN in equilibration buffer, rather than with 1012-S. These affinity column eluates were dialyzed vs three changes, 4 l/change, of 20 mM Tris-citrate (pH 7.2), containing 0.04 Na_2EDTA and 0.04% Triton X-100 (dialysis buffer) to reduce the NaSCN concentration.

2.3. *Anion-exchange HPLC*

Following dialysis, affinity-purified receptor was concentrated from 100 to 30 ml using a Novacell/Filtron membrane of 100 kDa cutoff. This concentrated sample, or in some cases 10 ml solubilized membrane protein, was applied in 2-ml aliquots at a rate of 1 ml/min to a Mono-Q DEAE ion-exchange column (Pharmacia, 0.5 × 25 cm) using a Gilson gradient HPLC system. The column was washed with 20 ml of 50 mM Tris-citrate (pH 7.2) with 10% sucrose and 0.1 mM Na_2EDTA. The receptor was retained by the column under these conditions and eluted with a 60 ml linear gradient of 0–1.2 M KCl in the same buffer at a rate of 1 ml/min. Aliquots of alternate 1-ml fractions were assayed directly for [^{3}H]flunitrazepam or [^{3}H]muscimol binding. Fractions containing $GABA_A$ receptors were pooled as either early (0.3–0.6 M KCl) or late (0.7–1.0 M KCl) eluting peaks of ligand-binding activity. These were dialyzed against dialysis buffer and either reapplied to the anion-exchange column or assayed for ligand-binding properties.

2.4. *Ligand-binding assays*

Receptor fractions were incubated in polypropylene tubes on ice for 45 min with either 8–10 nM [^{3}H]flunitrazepam (spec. act. 79–90 Ci/mmol, NEN) or 20 nM [^{3}H]muscimol (spec. act. 6–20 Ci/mmol, NEN) in 50 mM Tris-citrate (pH 7.2). Incubations were conducted with and without 100 M unlabeled flunitrazepam or 1 mM GABA. Samples were filtered through polyethylenimine-treated glass-fiber (Whatman GF/B) filters as described by Bruns et al. [25] for trapping of detergent-solubilized proteins. For estimates of receptor-ligand affinity, assays were performed in triplicate with 8 concentrations of [^{3}H]flunitrazepam between 0.5 and 100 nM or 7 concentrations of [^{3}H]muscimol between 3 and 200 nM. Specific binding was estimated as the difference between assays in the presence and absence of excess unlabeled flunitrazepam or GABA.

For competition assays, 7 concentrations of unlabeled CL 218872 from 10 nM to 10 μM were included in equilibrium binding assays as described above in which the concentration of [^{3}H]flunitrazepam was either 2 or 4 nM.

Protein values for Triton X-100-solubilized samples were obtained using the bicinchoninic acid method [26] with commercial reagents (Pierce) and bovine serum albumin as standard.

Radioligand-binding data were analyzed using EBDA and LIGAND computer programs, as adapted for the IBM PC by McPherson [27]. The EBDA program provided initial estimates of binding parameters for different binding isotherm models, while the LIGAND programs' non-linear curve-fitting subroutines were used to identify the curves which most closely approximated the data.

3. RESULTS

The profile of [^{3}H]flunitrazepam binding which eluted from the anion-exchange HPLC column with increasing salt concentration is presented in fig.1. The larger peak of binding sites typically eluted with 0.3–0.6 M KCl, while a smaller peak eluted later with 0.7–1.0 M KCl. The relative quantity of benzodiazepine binding in these peaks varied between preparations with the later eluting fraction always containing fewer binding sites. In seven HPLC analyses of affinity-purified receptor, the average ratio of [^{3}H]flunitrazepam in peak 1 vs peak 2 was 2.4:1 (2.4 ± 0.6 SD). The distribution of [^{3}H]muscimol-binding sites mirrored that observed for [^{3}H]flunitrazepam binding (fig.2) in the KCl eluate from this column.

When the receptor which eluted between 0.3 and 0.6 M KCl was collected, dialyzed and rechromatographed under the same HPLC conditions, the ligand binding remained as a single peak, retaining the original elution characteristics with respect to the salt concentration (fig.3). Similarly, the collection, dialysis and rechromatography of the later eluting $GABA_A$ receptor produced a single large peak which eluted with the higher salt concentration, and a much smaller peak corresponding to the early eluting form (fig.4).

The two ionic peaks obtained from anion-exchange chromatography were assayed for both [^{3}H]flunitrazepam and [^{3}H]muscimol binding and the receptor/ligand affinity data obtained from each were similar. Estimated single-site K_d values were 3.0–5.0 nM for benzodiazepine binding and 7.0–7.3 nM for muscimol binding. The binding isotherms for the affinity column and HPLC eluates could also be resolved by the LIGAND program into two affinity states for both the

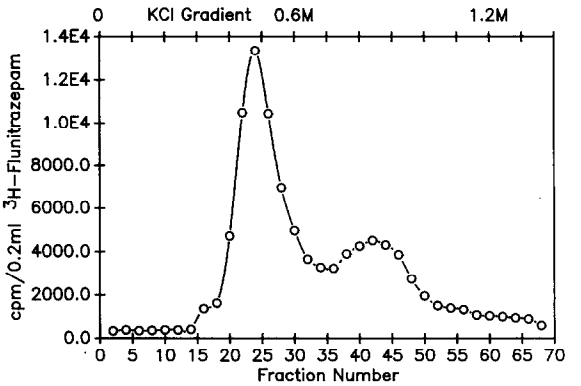

Fig.1. [^{3}H]Flunitrazepam binding to $GABA_A$ receptors resolved on anion-exchange HPLC is presented. The receptors, solubilized from P1 membranes, were purified on a 1012-S affinity column, eluted with NaSCN, applied to HPLC in 50 mM Tris-citrate (pH 7.4) containing 10% sucrose and 0.1% Triton. The column was washed with the same buffer for 20 min and eluted with a gradient of KCl from 0 to 1.2 M over 60 min. The flow rate throughout the procedure was 1 ml/min. Aliquots (0.2 ml) of alternate 1-ml fractions were assayed for benzodiazepine binding using 10 nM [^{3}H]flunitrazepam. Similar elution profiles were obtained from 7 experiments.

benzodiazepine-binding (K_d ranges 0.2–1.6 and 52–88 nM) and muscimol-binding site (K_d ranges 1.6–2.4 and 32–68 nM). The receptors solubilized with detergent from P1 membranes exhibited

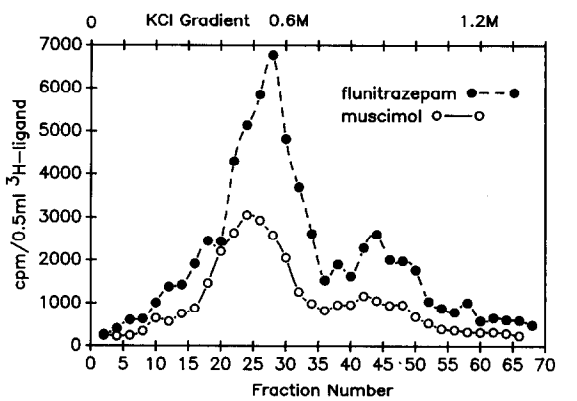

Fig.2. [^{3}H]Flunitrazepam (●---●) and [^{3}H]muscimol (○—○) binding to $GABA_A$ receptors resolved on anion-exchange HPLC is presented. The receptor, solubilized from P2 membranes, purified on a 1012-S affinity column, eluted with NaSCN and chromatographed on DEAE anion-exchange HPLC, as described in fig.1. Aliquots (0.5 ml) of alternate 1-ml fractions were assayed with 10 nM [^{3}H]flunitrazepam and 20 nM [^{3}H]muscimol. Similar results were obtained from two experiments.

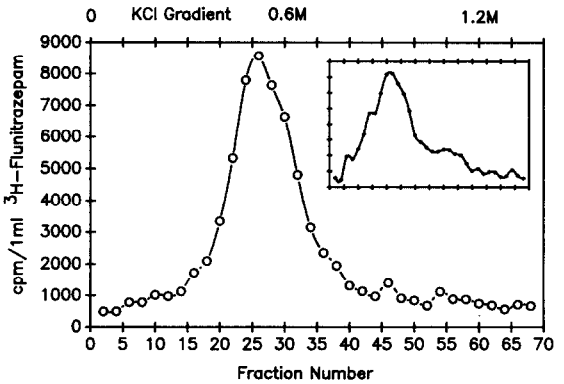

Fig.3. [^{3}H]Flunitrazepam binding to the early eluting $GABA_A$ receptor population which eluted with 0.3–0.6 M KCl on an initial DEAE anion-exchange HPLC column (inset), was dialyzed, concentrated and rechromatographed under the same conditions. Chromatography was as described in fig.1. Aliquots (0.25 ml) of alternate 1-ml fractions were assayed from the first elution and the entire 1 ml fraction was assayed from the second elution. Similar data were obtained in two analyses of the early eluting population of $GABA_A$ receptors.

single affinity binding sites for [^{3}H]flunitrazepam of 6.1 nM (Hill coefficient, 1.0) and for [^{3}H]muscimol of 18 nM (Hill coefficient, 1.02).

In order to assess the possibility that the two peaks of binding activity identified by anion exchange represented type I and II receptors, the sensitivity of their [^{3}H]flunitrazepam binding to

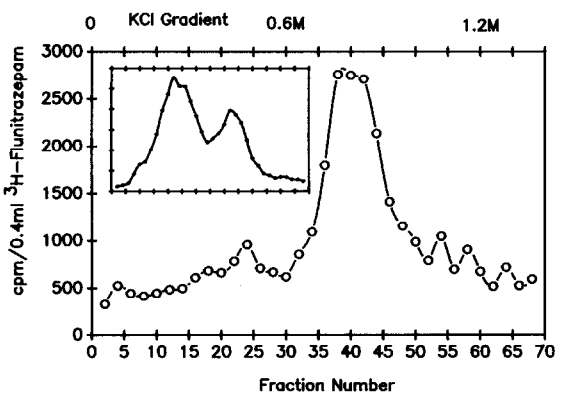

Fig.4. [^{3}H]Flunitrazepam binding to late eluting $GABA_A$ receptor population which eluted with 0.7–1.0 M KCl on an initial DEAE anion-exchange HPLC column (inset), was dialyzed, concentrated and rechromatographed under the same conditions. Chromatography was as described in fig.1. Aliquots (0.2 ml) of alternate 1-ml fractions were assayed from the first elution and 0.4-ml aliquots of 1-ml fractions from the second elution were assayed.

displacement with CL 218872 was assayed. The CL 218872-binding affinity was estimated from Hofstee [28] transformations of the data. The first HPLC peak exhibited CL 218872 affinities of 36 nM and 1.8 μM (at 2 nM [^{3}H]flunitrazepam) and 47 nM and 2 μM (at 4 nM [^{3}H]flunitrazepam), while the later eluting receptor exhibited affinities of 47 nM/2.7 μM and 73 nM/3 μM. Analysis of these data in Hofstee transformation plots (% radioligand displaced vs % displaced/CL 218872 concentration) produced very similar curves for the two ionic peaks, a result which suggests that the proportion of the CL 218872-defined type I and II receptors in the two peaks is similar.

4. DISCUSSION

Two populations of $GABA_A$ receptor have been resolved by anion-exchange HPLC. Differential elution of these populations with a salt gradient suggests that they possess differing densities of anionic groups by which they interact with the high-performance column. Despite this apparent chemical difference, these populations appear to be similar in terms of their affinity for flunitrazepam, muscimol and CL 218872.

Differences which have been reported between presumptive subclasses of $GABA_A$ receptors include differential solubility in detergent [13,14], multiple [^{3}H]flunitrazepam-photolabeled receptor subunits [16], and differential sensitivity to glycosidase treatment [19,21]. Additionally, subclasses of $GABA_A$ receptors possess differing affinities for CL 218872 and β-carbolines [7–12]. Consistent with studies on other neurotransmitter receptors, these data suggest that there may be considerable structural diversity in the $GABA_A$ receptor.

It is possible that these ionic populations have been produced as a consequence of the affinity chromatography or concentration of the sample prior to HPLC. To address this possibility, a Triton-solubilized brain membrane preparation was chromatographed on HPLC without any purification step or sample concentration. Due to the greatly increased protein concentration in this sample, the peaks of benzodiazepine binding were slightly less well articulated, however, both were clearly resolved at the appropriate point in the KCl elution gradient. The failure of the early and late

eluting receptor isolates to exhibit interconversion between sequential HPLC analyses militates against the view that they are in a dynamic equilibrium in vitro. Additionally, while there was variation in the relative amounts of binding activity in the ionic peaks between preparations, we detected no correlation between this variation and the time in vitro before HPLC.

Transmitter receptor and ion channel heterogeneity is emerging as a consistent theme in neurobiology. Variation in subunit composition, glycosylation and phosphorylation may each contribute to this diversity and may reflect differences in cellular localization, functional state or metabolic condition of the receptor complex. If the differences described here in $GABA_A$ receptor mobility during ion-exchange chromatography are physiologically relevant, this HPLC-based resolution of receptor subtype will be useful in obtaining structure/function correlates, which complement subunit cDNA sequence information.

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