CHARACTERIZATION OF BENZODIAZEPINE AND γ -AMINOBUTYRIC ACID RECOGNITION SITES AND THEIR ENDOGENOUS MODULATORS

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Abstract

The solubilization and partial purification from rat brain cortex homogenates of [3 H] γ -aminobutyric acid (GABA) and [3 H]diazepam recognition sites and of their endogenous modulators (GABA-modulin and an endogenous compound which displaces specifically bound [3 H]diazepam) are reported.

A high percentage of GABA binding sites (virtually free of benzodiazepine binding sites) was solubilized from homogenates of rat brain cortex incubated at 0°C with 1% Triton X-100 and a mixture of protease inhibitors. A large proportion of benzodiazepine binding sites was solubilized in the absence of apparent GABA binding capacity by incubating crude synaptic membrane preparations at 37°C with 0.05% Triton X-100. The characteristics of these two solubilized binding sites resemble those of the membrane-bound binding sites. However, unlike the membrane-bound sites, solubilized GABA and benzodiazepine recognition sites have lost the ability to cross-react. Hence, solubilized benzodiazepine binding sites are insensitive to GABA stimulation, while solubilized GABA binding sites are no longer protected by the benzodiazepines against heat inactivation. These results indicate that GABA and benzodiazepine recognition sites reside in two different molecules which, when bound to membranes, can interact reciprocally and modulate their binding affinity for specific ligands.

The recognition sites for benzodiazepines and γ -aminobutyric acid (GABA) are located in the same postsynaptic membrane (Mohler et al., 1980a) and are coupled into a functional unit. In fact, GABA receptors function as a supramolecular entity, which includes the GABA recognition site, the benzodiazepine recognition site, and the Cl^- ionophore, in addition to a number of other unidentified components (Guidotti et al., 1977; Costa and Guidotti, 1979; Baraldi et al., 1979; Tallman et al., 1978, 1980; Sieghart and Karobath, 1980). The benzodiazepine recognition site may be used physiologically by an endogenous agonist that participates in GABAergic transmission by modulating the affinity of the specific recognition sites for GABA (Guidotti et al., 1978; Skolnick et al., 1978; Asano and Spector, 1979; Colello et al., 1978; Davis and Cohen, 1980; Braestrup et al., 1980). Such a close functional association between GABA and benzodiazepine binding sites is underscored by recent histological evidence showing the presence of benzodiazepine recognition sites vis-à-vis axon terminals containing glutamic acid decarboxylase (Mohler et al., 1980b). This is in line with the observation made by Gavish and Snyder (1980) that GABA and benzodiazepine recognition sites can be solubilized as a functionally coupled unit. These results raise the question of whether GABA and benzodiazepine recognition sites are part of the same molecule

or whether they are located in two distinct molecules functioning as an integral part of the same receptor. Since Gavish and Snyder (1980) solubilized only a portion of the membrane-bound GABA and benzodiazepine recognition sites, one might surmise that there are a number of functional arrangements for GABA and benzodiazepine receptors located in postsynaptic membranes and that the tight coupling is one of the ways in which these receptors are arranged. An understanding of this variability is crucial to define the molecular mechanisms whereby benzodiazepine recognition sites participate in the fine tuning of GABA receptors. The present paper shows that it is possible to solubilize a major portion of GABA and benzodiazepine recognition sites as separate molecular entities. In addition, evidence is presented showing that each of the two sites is regulated selectively by an endogenous modulator protein.

Materials and Methods

Male Sprague-Dawley rats, 200 to 250 gm, were sacrificed by guillotine and the brain was removed immediately. The cortices were dissected rapidly according to the method of Glowinski and Iversen (1966), placed on ice, and used as a source for the isolation and purification of [3 H]benzodiazepine and [3 H]GABA recognition sites.

Solubilization of [³H]benzodiazepine recognition sites

The starting material was frozen-thawed crude synaptic membranes (CSM) (Enna and Snyder, 1977), which were incubated at 0°C for 30 min in 20 vol of 50 mM Tris/citrate buffer, pH 7.1, and then washed three times with the same buffer. This preparation was incubated at 37°C for 30 min in the presence of 0.05% Triton X-100. The suspension was then centrifuged at $10^5 \times g$ for 1 hr at 4°C. Aliquots of 2 ml of the supernatant were filtered rapidly on a Sephadex G-100 minicolumn (2 × 8 cm) by centrifugation according to the method of Penefsky (1977). The recovery of [³H]diazepam binding sites throughout the column purification procedure was $75 \pm 9\%$ ($n = 3$). The purified supernatants were pooled and lyophilized, and the number of solubilized high affinity binding sites for [³H]benzodiazepines was measured.

Solubilization of [³H]GABA recognition sites

The cortices were homogenized with an Ultra Turrax homogenizer for 30 sec in 20 vol (w/v) of 50 mM Tris/citrate buffer, pH 7.1, containing 1% Triton X-100 (3.6 mg/10 mg of tissue), 1 mM EGTA (ethylene glycol bis(β -aminoethyl ether)- N,N,N',N' -tetra-acetic acid), 1% β -mercaptoethanol, 100 BAEE units/mg of protein of soybean trypsin inhibitor, 20 mM N -ethylmaleimide, 0.3 mM N -2-tosyl-1-lysine chloromethyl ketone. The homogenate was stirred at 4°C for 30 min and then centrifuged at $10^5 \times g$ for 1 hr at 4°C. Aliquots of 2 ml of the $10^5 \times g$ supernatant were filtered rapidly in succession, once on a Sephadex G-100 and twice on a Sephadex G-25 minicolumn (2 × 8 cm), using the method of Penefsky (1977). In this supernatant, the recovery of [³H]GABA binding sites was approximately 70%. This purification procedure removes endogenous GABA, inhibitor of GABA binding, Triton X-100, protease inhibitors, and Tris/citrate. The final eluate, reconstituted in 50 mM Tris/citrate buffer, pH 7.1, was used in the binding studies. In this sample, [³H]GABA recognition sites were still in a soluble form because (a) they were not precipitated by centrifugation at $200,000 \times g$ for 2 hr and (b) no binding of [³H]muscimol was detected if the sample was filtered through a 0.45- μ m Millipore filter.

Solubilization of GABA·benzodiazepine (BZD) recognition complex

This complex was prepared according to the procedure described by Gavish and Snyder (1980). Frozen-thawed CSM(s) incubated at 0°C in 50 mM Tris/citrate buffer (pH 7.1) for 30 min and then washed three times with the same buffer was used as a starting material for the preparation of the receptor complex. The final suspension was incubated at 0°C for 30 min in the presence of 1% Triton X-100 and then centrifuged at $10^5 \times g$ for 1 hr. The supernatant was filtered by the rapid centrifugation method of Penefsky (1977) on a Sephadex G-100 column (2 × 8 cm). The resulting supernatant, which was termed "solubilized GABA·BZD recognition complex," was used in the binding studies.

Extraction of "GABA-modulin" and of the endogenous inhibitor of specific [³H]benzodiazepine recognition sites

Frozen/thawed CSM(s) from cortex of rat brain was used as the starting material. Endogenous GABA and

other amino acids that inhibit [³H]GABA binding were removed by incubating the CSM(s) at 37°C for 30 min in the presence of 0.05% Triton X-100 (0.18 mg/mg of membrane protein) and then washing the CSM(s) three times with 50 mM Tris/citrate buffer, pH 7.1 (Massotti and Guidotti, 1980). GABA-modulin and the endogenous inhibitor of benzodiazepine binding were extracted by homogenizing these membranes in H₂O (1 mg of protein/ml of H₂O) containing 0.05% Triton X-100. The suspension was incubated at 0°C for 30 min, sonicated for 30 sec, and then centrifuged at $10^5 \times g$ for 60 min. The operation was repeated a second time. The two $10^5 \times g$ supernatants were lyophilized and, after reconstitution in H₂O, were termed "extract A" and used as a source for the preparation of either of the two inhibitors. Typically with this procedure, 12 μ g of protein inhibitors are extracted from 1 mg of membrane proteins.

The inhibitor of [³H]benzodiazepine binding was isolated by incubating "extract A" for 15 min at 95°C and by chromatographing the $10^5 \times g$ supernatant (20 min) with a Bio-Gel P-2 column (1 × 80 cm) and then with a Sephadex G-100 (1 × 80 cm) column equilibrated with 10 mM Tris/citrate buffer, pH 7.1.

GABA-modulin was isolated from the $10^5 \times g$ supernatant of fresh "extract A." One milliliter of this supernatant contained approximately 2.5 mg of protein, 2 mg of phospholipids, and 5 pmol of GABA. The sample was filtered five times by centrifugation on Bio-Gel P-2 (2 × 8 cm) (Mazzari et al., 1980) and then applied to a Sephadex G-50 column (1 × 80 cm) equilibrated with Tris/citrate buffer, pH 7.1.

Determination of GABA

The GABA content of various preparations was determined using high pressure liquid chromatography (HPLC). After 0.2 N perchloric acid extraction, the samples were processed according to Schmid et al. (1980). GABA also was determined according to Bertilsson and Costa (1976) by ion monitoring (m/e 233 and 394) using a LKB 9000 gas chromatograph and mass spectrometer (GC-MS). The sensitivity was approximately 10 pmol for HPLC and 5 pmol for GC-MS.

Determination of phospholipids

The sample was extracted with 5 vol of chloroform/methanol (2:1 by volume) according to Folch et al. (1957). The presence of phospholipids was examined on Silica Gel G thin layer chromatography using the method of Skipski and Barclay (1969). After developing, the plates were dried and the phospholipids were visualized by exposure to iodine.

Studies on the thermolability of [³H]GABA recognition sites

Suspensions of solubilized (300 to 400 μ g of protein/ml) or membrane-bound (800 to 1000 μ g of protein/ml) recognition sites for [³H]GABA were preincubated at 4°C with 10^{-5} M GABA (15 min), 10^{-6} M diazepam, or other benzodiazepines (1 hr). After incubation at 60°C for 1, 3, 6, 9, 12, and 24 min, the suspensions were cooled on ice.

In order to remove the GABA or the benzodiazepines from the solubilized recognition sites, these suspensions

were filtered twice by centrifugation (Penefsky, 1977) on a Sephadex G-25 gel minicolumn.

To remove GABA or benzodiazepines from the membranes, the suspensions were centrifuged at $48,000 \times g$ for 10 min and the pellets were washed twice with a double volume of 50 mM Tris buffer, pH 7.1.

Binding assay of [^3H]GABA, [^3H]muscimol, and [^3H]diazepam

The following procedures were used to assay the high affinity binding of the different ^3H ligands to soluble or membrane-bound specific recognition sites.

Solubilized GABA recognition sites. The reaction was initiated by adding 20 μl of the various [^3H]GABA or [^3H]muscimol solutions (to reach a final concentration of 0.3 to 240 nM in a total volume of 200 μl) to a test tube containing solubilized recognition sites (40 to 60 μg of protein). After 5 min of incubation at 0°C , the reaction was terminated by applying 50 μl of the incubation mixture on a minicolumn (0.5×6 cm) of Sephadex G-25 kept at 0°C . Bound [^3H]GABA was separated from the free [^3H]GABA by centrifuging the minicolumn at 1000 rpm for 2.5 min (Penefsky, 1977). The radioactivity present in the first 50 μl of filtrate corresponded to the amount bound to the receptor. In the absence of protein, less than 0.5% of the radioactivity emerged in the void volume. Specific binding was calculated as the difference between the total binding and the binding that occurred in the presence of 1 mM GABA or 1 μM muscimol.

Solubilized benzodiazepine recognition sites. The reaction was initiated by adding 20 μl of the various [^3H]diazepam solutions (to reach final concentrations of 0.3 to 48 nM in a total volume of 200 μl) to a test tube containing 40 to 60 μg of solubilized proteins. After 1 hr of incubation at 0°C , the reaction was terminated by adding 200 μl of 0.1% γ -globulin and 200 μl of 24% polyethylene glycol to precipitate the solubilized receptors as described by Yousufi et al. (1979). The suspension was filtered immediately under a vacuum on a GF/B filter. The filter was washed with 9 ml of 50 mM Tris/citrate buffer, pH 7.1, containing 8% polyethylene glycol. In some experiments, the results obtained with the method of Yousufi et al. (1979) were repeated by terminating the reaction by filtration of the suspension on a Sephadex G-25 column (0.5×6 cm) according to Penefsky (1977). No significant difference in the number of specific binding sites was found between the two methods used to terminate the reaction. Specific binding was calculated as the difference between the total binding and the binding that occurred in the presence of 10^{-6} M diazepam or clonazepam.

Solubilized GABA-BZD recognition complex. [^3H]GABA or [^3H]diazepam binding assays were performed as previously reported for the solubilized receptors.

Binding of [^3H]GABA, [^3H]muscimol, or [^3H]diazepam to the membrane-bound receptors. This binding was carried out with minor modifications of the method previously described (Baraldi et al., 1979).

Statistical analysis

The characteristics of [^3H]GABA and [^3H]diazepam binding were determined by using a Scatchard plot analysis of the saturation curves. Specifically, the Scatchard plots of the [^3H]GABA binding data were prepared ac-

cording to the graphic method of Rosenthal (1967) to determine the binding parameters (number of equivalent binding sites and the corresponding dissociation constants) in a complex system consisting of one ligand and two or more populations of binding sites. In each experiment, the values of the K_D and B_{max} were between 5 and 10% of the mean K_D and B_{max} values. The statistical significance of the results was determined by the Students t test (two tailed). Unbiased estimates of the standard error of the mean were computed and the means were compared as unpaired observations with equal variance.

Protein assay

The protein concentration was measured by the method of Lowry et al. (1951).

Results

Characterization of the endogenous modulator of [^3H]GABA and [^3H]diazepam binding. The inhibitor of [^3H]GABA binding used in these experiments was isolated by Bio-Gel P-2 and Sephadex G-50 chromatography (see "Materials and Methods"). After Sephadex G-50, one major peak of inhibitory activity was obtained and this peak was eluted by a buffer volume similar to that of ribonuclease A. No significant inhibitory activity of [^3H]GABA binding was eluted in the position in which [^3H]GABA emerged. In a typical preparation, 100 μl of inhibitory peak (containing 10 μg of protein) produced a 50% inhibition of [^3H]GABA binding. The characteristics of this inhibitor (GABA-modulin) are summarized in Table I.

A preliminary purification of the endogenous inhibitor of [^3H]diazepam binding was carried out by Bio-Gel P-2 column chromatography of a heat-treated brain extract (see "Materials and Methods"). This inhibitor emerged from this column with the void volume (see Fig. 1, upper). When the effluent from the Bio-Gel P-2 column that contained this inhibitor was chromatographed with a Sephadex G-100 column, the major peak of inhibition emerged after the void volume and before ovalbumin (Fig. 1, lower). The characteristics of the material endowed with the capacity to inhibit [^3H]diazepam binding are summarized in Table I.

Solubilized preparation of benzodiazepine binding sites. The recognition sites for [^3H]benzodiazepines which are located on CSM can be solubilized free from [^3H]GABA binding capacity by treating frozen-thawed CSM (see "Materials and Methods") with 0.05% Triton X-100 at 37°C for 30 min. When this procedure is repeated three times and each time is followed by a CSM washing with Tris buffer, the number of [^3H]diazepam recognition sites that remain bound to the CSM decreases. In contrast, there is no apparent decrease in the number of Na^+ -independent, high or low affinity recognition sites for [^3H]GABA (see Table II). After each treatment, the decrease in the number of [^3H]benzodiazepine binding sites located in CSM was paralleled by the appearance of an almost equal number of [^3H]benzodiazepine recognition sites in the $10^5 \times g$ supernatant (Table III). The solubilization of [^3H]benzodiazepine binding sites occurred during the first and second incubation of the CSM with 0.05% Triton X-100; the third

TABLE I

Characteristics of membrane-bound molecules that interfere with binding of [3 H]GABA and [3 H]diazepam

The characteristics of GABA-modulin were: (1) GABA-modulin elution pattern from a G-50 or G-100 column was similar to that of ribonuclease A (a marker of $M_r = 14,000$). (2) When the supernatant of extract A (see "Materials and Methods" for details) was heat treated (95°C for 15 min), the inhibitory activity eluted from the Sephadex G-50 column was reduced by approximately 70%. (3 and 4) When GABA-modulin preparations were incubated at 37°C for 4 hr in the presence of pronase or trypsin (100 μ g/ml), their inhibitory activity decreased and the activity present was due to inhibitory material of small molecular weight (500 to 800). (5 and 7) GABA-modulin inhibited noncompetitively the binding of GABA to high affinity sites located on Triton X-100-treated CSM(s); GABA-modulin failed to change the [3 H]GABA binding to low affinity sites located on the same membranes (see also Toffano et al., 1978). (6 and 8) GABA-modulin did not inhibit [3 H]diazepam binding when tested on Triton X-100-treated CSM (Table V). However, GABA-modulin (up to 25 μ g of protein) inhibited the GABA-induced (10^{-7} M) stimulation of [3 H]diazepam (1.5 nM) binding in proportion to the amount of GABA-modulin added. (9 and 10) When a preparation of GABA-modulin (100 μ g of protein in 1 ml) was incubated with 20 pmol of [3 H]GABA or 6 pmol of [3 H]diazepam and then the mixture was applied to a Sephadex G-25 minicolumn (Penefsky, 1977), more than 99.9% of the label was eluted without the GABA-modulin. (11) GABA-modulin can be precipitated almost completely by treating the preparation with ammonium sulfate (30 to 60%). The protein that precipitated with less than 30% or more than 60% ammonium sulfate failed to show inhibitory activity. (12) In a GABA-modulin preparation containing 100 μ g of protein, the phospholipid content extracted with chloroform/methanol is undetectable (<25 μ g). (13) The GABA content in a GABA-modulin preparation containing 100 μ g of protein was below the sensitivity of our HPLC or GC-MS method (5 pmol for GC-MS and 10 pmol for HPLC). In addition, formic acid extracts of this GABA-modulin preparation failed to inhibit [3 H]GABA binding. (14) GABA-modulin preparations (20 μ g of protein in 100 μ l) or GABA (1 nmol in 100 μ l) were incubated at 26°C for 50 min with GABASE (0.05 unit) in a medium identical to that used for the assay of GABA in tissue extracts (Okada et al., 1971). In the presence of heat-inactivated GABASE, GABA-modulin produced a 75% and GABA a 96% inhibition of specifically bound [3 H]GABA, respectively (4064 cpm, control; 1456 cpm, GABA-modulin; and 200 cpm, GABA). When the same samples were incubated in the presence of active GABASE, the amount of [3 H]GABA binding was 2058 cpm and 4130 cpm for GABA-modulin and GABA, respectively. (15) GABA-modulin preparations dialyzed 12 hr at 0°C versus 4000 vol of Tris buffer lose only 20% of their original inhibitory activity.

The characteristics of [3 H]diazepam binding inhibition (DBI) were as follows: (1) The molecular weight of DBI was greater than 1800 (it emerged with the void volume from a Bio-Gel P-2 column) and smaller than 100,000 (it emerged after the void volume in a Sephadex G-100 column) (Fig. 1). (2) DBI was stable (100%) after treatment at 95°C for 15 min. (4) Pronase (100 μ g/ml for 2 hr at 37°C destroyed approximately 50% of DBI activity (Massotti et al., 1980). (5) DBI failed to inhibit the high affinity [3 H]GABA binding (see also Table V). (6) DBI inhibited competitively the binding of [3 H]diazepam to high affinity sites located on Triton X-100-treated CSM (Fig. 2). (9 and 10) In an experimental condition similar to that used for GABA-modulin, DBI (up to 200 μ g of protein) bound less than 0.2% of [3 H]diazepam or [3 H]GABA. (15) Samples of the inhibitor dialyzed for 24 hr versus 4000 vol of Tris buffer lost less than 10% of its original inhibitory activity.

Characteristics	GABA-modulin ^a	Endogenous Inhibitor of [3 H]Diazepam Binding ^b
1. Molecular weight	~15,000	>1,800
2. Thermostability	No	Yes
3. Tryptic digestion	Yes	— ^c
4. Pronase digestion	Yes	Yes
5. Inhibition of [3 H]GABA binding	Yes	No
6. Inhibition of [3 H]diazepam binding	No	Yes
7. Mechanism of inhibition	Noncompetitive	Competitive
8. Inhibition of GABA-stimulated [3 H]diazepam binding	Yes	—
9. Binds [3 H]GABA	No	No
10. Binds [3 H]diazepam	No	No
11. Precipitated by 30 to 60% ammonium sulfate	Yes	No
12. Phospholipid content	<25 μ g/100 μ l	—
13. GABA content	<1 pmol/100 μ l	—
14. GABASE resistant	Yes	—
15. Dialyzable	No	No

^a In the assay, 100 μ l of GABA-modulin containing 10 μ g of protein was used.

^b In the assay, 100 μ l of inhibitor of [3 H]diazepam binding containing 50 μ g of protein was used.

^c —, not tested.

incubation failed to solubilize additional [3 H]diazepam binding sites (Table II). As shown in Tables II and III, two Triton X-100 washes solubilized more than 50% of the [3 H]benzodiazepine binding sites, while they failed to extract the high or the low affinity binding sites for [3 H]GABA. The absence of apparent GABA binding sites was not due to the presence of contaminating GABA in the solubilized preparation because the content of GABA measured by HPLC and GC-MS techniques was below 1 pmol/assay tube (see also Table III). Higher concentrations (up to 2%) of Triton X-100, Lubrol (0.5%, 2 mg/mg of protein), or deoxycholate (2%, 10 mg/mg of protein) failed to solubilize the [3 H]benzodiazepine binding sites that remained bound to CSM after two 0.05% Triton X-100 treatments. However, each of these treatments produced the solubilization of a significant amount of GABA binding sites. When the solubilized material was applied to a Sephadex G-100 column (1 $\times$ 80 cm), approximately 90% of the solubilized recognition sites for [3 H]benzodiazepine emerged from the column with the bulk of the

proteins. This preliminary finding suggested to us that a filtration of the solubilized binding sites through a Sephadex G-100 minicolumn (see "Materials and Methods") could give us a rapid and partial purification useful for measuring the density and other characteristics of [3 H]benzodiazepine binding sites.

The solubilized and partially purified [3 H]benzodiazepine binding sites bind [3 H]diazepam with a K_D of approximately 2.0 nM and a B_{max} of about 5.8 pmol/mg of protein (Fig. 3), whereas these recognition sites, when they are bound to the membrane, like those that cannot be solubilized, have a K_D of 8 nM and a B_{max} of 1.4 pmol/mg of protein. The solubilized binding sites are saturable (Fig. 3), stereospecific (Fig. 4), and have a Hill coefficient of 0.92. Moreover, the [3 H]diazepam bound to solubilized recognition sites was displaced by various benzodiazepines with a rank order of potency similar to that displayed for [3 H]diazepam bound to recognition sites located on CSM (see Fig. 4). The only exception was flunitrazepam which was more potent in displacing

Figure 1. Bio-Gel P-2 and Sephadex G-100 chromatography of the endogenous ligand of [^3H]diazepam binding. The inhibitor was extracted from rat brain cortex as described under "Materials and Methods." Two milligrams of protein was applied to the Bio-Gel P-2 column. Fractions 11 to 18 were pooled, together lyophilized and resuspended in 1 ml of 50 mM Tris/citrate, and applied to the Sephadex G-100 column equilibrated with the same buffer and 100 mM NaCl. Two hundred microliters of each fraction was used to measure the inhibitory activity of [^3H]diazepam (1.5 nM) binding. Blue Dextran (DB), ovalbumin (OVA), and β -mercaptoethanol (βME) were used as markers of the elution volume. S.B., specifically bound; O.D., optical density.

[^3H]diazepam from solubilized than from membrane-bound recognition sites (Fig. 4). Table IV shows that the binding of [^3H]diazepam to solubilized recognition sites is no longer increased by the addition of GABA nor blocked by the addition of 10^{-4} M bicuculline. This finding contrasts with the GABA-induced stimulation of [^3H]benzodiazepine binding to membrane-bound recognition sites or to solubilized GABA·BZD recognition complex (see Table IV). The endogenous compound that inhibits [^3H]diazepam binding to specific recognition sites is equally potent in inhibiting the binding of [^3H]benzodiazepines to membrane-bound recognition sites, to solubilized recognition sites complexed with GABA recognition sites, or to recognition sites that are solubilized without GABA binding sites (Table V). The solubilized [^3H]diazepam binding sites are less stable than those that are membrane bound. They have a half-life of 2 days when the solution containing [^3H]diazepam solubilized recognition sites is kept at 0 to 4°C .

Preparation of solubilized [^3H]GABA recognition sites. [^3H]GABA recognition sites were purified and solubilized using as a starting material a homogenate of rat brain cortex. The [^3H]GABA recognition sites were sol-

ubilized by homogenizing the tissue in a solution containing 1% Triton X-100 and a number of protease inhibitors (see "Materials and Methods"). The final concentration of GABA in this homogenate was about 5×10^{-5} M.

This extraction procedure solubilizes 72% of the GABA binding sites but only 8% of the benzodiazepine binding sites (see Table VI). Concentrations of Triton X-100 higher than 1% failed to increase the extent of GABA binding site solubilization. Scatchard analysis of the

Figure 2. Lineweaver-Burk plot of [^3H]diazepam binding to crude synaptic membranes in the absence (A) and presence (B) of the endogenous ligand for benzodiazepines. The assay was carried out in membranes washed three times with Triton X-100 (see "Materials and Methods" for details). The membranes were preincubated 1 hr at 0°C with Tris (A) or with 50 μg of protein fraction containing the inhibitor (B). The apparent dissociation constant (K_D) is 6.5 nM in A and 12 nM in B. S.B., specifically bound.

TABLE II

Effect of 0.05% Triton X-100 treatment on the maximal density of [^3H]GABA and [^3H]diazepam binding sites in CSM prepared from rat brain cortex

Frozen-thawed CSM(s) prepared according to Gavish and Snyder (1980) were incubated at 37°C for 30 min in the absence (none) or presence of 0.05% Triton X-100 (0.18 mg/mg of protein) (1st Triton) and then washed twice with 50 mM Tris/citrate buffer, pH 7.1. Aliquots (300 to 400 μg of protein) were used for the assay. The residual aliquot was stored at -20°C for 12 hr. On these membranes, incubation at 37°C for 30 min in the presence of Triton X-100 was repeated a second (2nd Triton) and a third (3rd Triton) time. Each value is the mean $\pm$ SEM of at least five experiments performed in triplicate.

Membrane Treatment	B_{max}		
	S.B. ^a [^3H]GABA		S.B. [^3H]Diazepam
	High Affinity	Low Affinity	High Affinity
	pmol/gm tissue		
None	25 $\pm$ 2	190 $\pm$ 21	160 $\pm$ 11
1st Triton	37 $\pm$ 2 ^b	200 $\pm$ 18	110 $\pm$ 14 ^c
2nd Triton	55 $\pm$ 4 ^c	212 $\pm$ 10	67 $\pm$ 4 ^c
3rd Triton	90 $\pm$ 5 ^c	225 $\pm$ 22	60 $\pm$ 6 ^c

^a S.B., specifically bound.

^b $p < 0.05$ when compared with the control group (none) according to the Student's t test.

^c $p < 0.01$ when compared with the control group (none) according to the Student's t test.

TABLE III
Selective solubilization of benzodiazepine recognition sites from CSM

Pooled $10^5 \times g$ supernatants (200 to 300 μ g of protein/ml) and final pellets (400 to 600 μ g of protein/ml) from crude synaptic membranes treated twice at 37°C for 30 min with (0.05% Triton X-100) or without (none) Triton X-100 were used for [3 H]muscimol (10 nM) and [3 H]diazepam (24 nM) binding assay. Each value is the mean $\pm$ SEM of three experiments performed in triplicate. The specific activity of [3 H]muscimol and [3 H]diazepam bound to the solubilized recognition site was calculated based on the content of membrane proteins. The values of the pooled supernatants were not corrected for the loss (see "Materials and Methods") encountered during the G-100 and/or G-25 chromatography purification procedure. The content of GABA in the solubilized preparation was less than 1 pmol/assay tube.

Treatment	S.B. ^a [3 H]Muscimol			S.B. [3 H]Diazepam		
	Residual Pellet	Pooled Supernatants		Residual Pellet	Pooled Supernatants	
	pmol/mg protein		% solub.	pmol/mg protein		% solub.
None	5.0 $\pm$ 0.7	nd ^b		3.3 $\pm$ 0.04	0.2 $\pm$ 0.01	8
0.05% Triton X-100 ($\times$ 2)	6.5 $\pm$ 0.4	0.2 $\pm$ 0.3	0.3	1.4 $\pm$ 0.10 ^c	1.5 $\pm$ 0.08 ^c	51

^a S.B., specifically bound.

^b nd, not determined.

^c $p < 0.01$ when compared with control (none) according to the Student's t test.

Figure 3. Binding of [3 H]GABA (panel A) and [3 H]diazepam (panel B) to solubilized and partially purified GABA and diazepam recognition sites. The saturation curves (shown in the insets) were analyzed by Scatchard plot and Rosenthal (Rosenthal, 1967) plot analysis. The values represent the mean of three separate experiments. B/F, bound/free.

saturation curve for [3 H]GABA binding to solubilized GABA recognition sites revealed two apparent binding sites for GABA (see Fig. 3). The K_D of these two binding sites were similar to those described for [3 H]GABA recognition sites located in CSM (Enna and Snyder 1977; Toffano et al., 1978). The binding of [3 H]GABA to solubilized recognition sites had the following characteristics:

1. It was inhibited by bicuculline with an IC_{50} of approximately 5×10^{-5} M.
2. It was inhibited by GABA-modulin with a potency similar to that observed in CSM or in solubilized GABA·BZD recognition complex (Table V).
3. The thermoinactivation characteristics of solubilized [3 H]GABA binding sites were identical to those of the recognition sites bound to CSM (see Fig. 5).
4. The rate of heat (60°C) inactivation of the solubilized GABA recognition sites was reduced when 10^{-5} M GABA was present in the incubation mixture (Fig. 5).
5. Diazepam, as well as other active benzodiazepines (flunitrazepam, clonazepam, RO 11 3128), but not the inactive RO 11 3624, prevented the heat inactivation.

Figure 4. Inhibition of [3 H]diazepam binding to solubilized and membrane-bound recognition sites by various benzodiazepines. The reaction was initiated by adding 1.5 nM [3 H]diazepam to the incubation mixture. Each point represents the K_I ($K_I = IC_{50}/1 + (C/K_D)$) obtained from at least three series of experiments performed in triplicate. The various benzodiazepines were added directly in the incubation mixture in concentrations (C) ranging from 10^{-6} to 10^{-10} M. The dissociation constant used to calculate K_I was 2.6 nM and 8.0 nM for soluble and membrane-bound recognition sites, respectively. The SEM was less than 10% of the mean values.

TABLE IV
GABA-induced stimulation of [3 H]diazepam binding to solubilized or CSM-bound recognition sites

The CSMs treated with 0.05% Triton X-100 twice were prepared as reported in Table I. GABA was added to the incubation mixture together with 1.5 nM [3 H]diazepam 60 min before assay. Each value is the mean $\pm$ SEM of at least three experiments performed in triplicate.

Type of Preparation	S.B. ^a [3 H]Diazepam		Change
	-GABA	+GABA 10^{-6} M	
	pmol/mg protein		%
CSM treated with 0.05% Triton X-100	0.27 $\pm$ 0.01	0.40 $\pm$ 0.01	+48 ^b
Solubilized GABA·BZD recognition complex	0.41 $\pm$ 0.02	0.75 $\pm$ 0.08	+85 ^b
Solubilized benzodiazepine recognition sites	0.39 $\pm$ 0.01	0.35 $\pm$ 0.02	-10

^a S.B., specifically bound.

^b $p < 0.01$ when the group incubated in the presence of GABA is compared with the corresponding group incubated without GABA.

TABLE V

Effects of endogenous inhibitors of [3 H]GABA and [3 H]diazepam binding to different types of preparations of specific recognition sites

The data are expressed as specifically bound 3 H ligand in picomoles per mg of protein. An aliquot of "GABA-modulin" (10 μ g of protein) or of the endogenous inhibitor of benzodiazepines (50 μ g of proteins) was added directly into the incubation mixture containing 40 to 60 μ g of solubilized recognition sites or 300 to 400 μ g of membrane proteins. The binding assay was performed in the presence of 1.5 nM and 20 nM [3 H]diazepam and [3 H]GABA, respectively. Each value is the mean $\pm$ SEM of at least three experiments performed in triplicate.

Additions	[3 H]GABA Recognition Sites			[3 H]Diazepam Recognition Sites		
	Solubilized	GABA·BDZ Complex	Membrane Bound	Solubilized	GABA·BDZ Complex	Membrane Bound
Control	1.20 $\pm$ 0.09	0.70 $\pm$ 0.06	0.72 $\pm$ 0.07	0.55 $\pm$ 0.08	0.51 $\pm$ 0.05	0.30 $\pm$ 0.02
GABA-modulin	0.52 $\pm$ 0.03 ^a 45%	0.44 $\pm$ 0.04 ^b 52%	0.55 $\pm$ 0.04 ^b 76%	0.65 $\pm$ 0.10 118%	0.60 $\pm$ 0.05 117%	0.35 $\pm$ 0.01 116%
Endogenous inhibitor of benzodiazepine binding	1.0 $\pm$ 0.09 87%	0.60 $\pm$ 0.07 86%	0.65 $\pm$ 0.07 90%	0.28 $\pm$ 0.03 ^a 51%	0.32 $\pm$ 0.04 ^b 63%	0.23 $\pm$ 0.04 ^a 76%

^a $p < 0.01$ when compared to the control values according to the Student's t test.

^b $p < 0.05$ when compared to the control values according to the Student's t test.

TABLE VI

Distribution of [3 H]GABA and [3 H]diazepam recognition sites in the supernatant and pellet of brain cortex homogenates incubated with Triton X-100

The homogenate was prepared from fresh brain cortex with 20 vol of 50 mM Tris/citrate buffer, pH 7.1, containing 1% Triton X-100 plus the cocktail of protease inhibitors as described under "Materials and Methods." The homogenate was incubated for 30 min at 4°C and centrifuged for 1 hr at $10^5 \times g$. The supernatant was passed on G-100 and twice on G-25 minicolumns in order to remove endogenous GABA. Binding was measured using 80 nM [3 H]GABA and 24 nM [3 H]diazepam. Each value is the mean $\pm$ SEM of three experiments performed in triplicate. The values are not correct for the loss during the G-100 and G-25 column chromatography. The [3 H]diazepam bound to the original homogenate was 128 $\pm$ 10 pmol/gm of cortex. To measure the number of membrane-bound GABA recognition sites, the $10^5 \times g$ pellets were washed three times in 20 vol of Tris buffer.

Preparation	[3 H]GABA	[3 H]Diazepam
	pmol/gm cortex	
$10^5 \times g$ supernatant	68 $\pm$ 6	7 $\pm$ 0.9
$10^5 \times g$ pellet	20 $\pm$ 2	9 $\pm$ 8

vation of [3 H]GABA binding sites in CSM; however, these compounds failed to protect solubilized [3 H]GABA recognition sites from heat inactivation (see Fig. 5).

- The half-life of the solubilized GABA recognition sites was approximately 10 hr when the sample was maintained at 0 to 2°C.

Discussion

Previous reports in the literature have described the solubilization of GABA or benzodiazepine binding sites (Greenlee and Olsen, 1979; Yousufi et al., 1979; Asano and Ogasawara, 1980a, b; Gavish et al., 1979; Chude, 1979; Lang et al., 1979). However, most of these studies failed to clarify whether the benzodiazepines and GABA receptors were extracted as a complex or as two separate subunits. In addition, in two studies (Yousufi et al., 1979 and Gavish and Snyder, 1980) in which the question of whether GABA and BZD binding sites can be solubilized as independent molecules has been addressed, the results reported appear to be discrepant. Yousufi et al. (1979) reported that the solubilization of benzodiazepine binding sites yields a preparation that is insensitive to GABA stimulation and, presumably, does not include GABA recognition sites; in contrast, Gavish and Snyder (1980)

Figure 5. Thermolability of solubilized or membrane-bound GABA recognition sites; effect of 10^{-5} M GABA, 10^{-6} M diazepam (DIAZ), and 10^{-6} M RO 11-3624. The recognition sites were incubated at 60°C for different times (see "Materials and Methods" for details). Bindings were determined in the presence of 80 nM [3 H]GABA. The basal values were 0.29 $\pm$ 0.04 pmol/mg of protein and 0.41 pmol/mg of protein for membrane-bound and solubilized receptor sites, respectively. Each point is the mean of at least three experiments. The SEM was less than 10% of the mean values. S.B., specifically bound.

reported that GABA and benzodiazepine binding sites are solubilized as a complex because the addition of GABA increased the binding affinity of [3 H]flunitrazepam by an extent similar to that observed when both recognition sites were bound to CSM.

We have been able to duplicate Gavish and Snyder's findings that GABA and benzodiazepine binding proteins can be solubilized as a complex (Guidotti et al., 1980; see also Table IV). However, by modifying the extraction procedure, we can solubilize either benzodiazepine or GABA recognition sites practically devoid of the other binding site. The rationale for setting up the modification of Gavish and Snyder's procedure evolved from our studies on GABA and benzodiazepine binding using CSM.

When CSMs were incubated with 0.05% Triton X-100 at 37°C, the number of membrane-bound GABA recognition sites increased, while that of benzodiazepine binding sites was reduced (see also Table II). Conversely, a treatment of CSM with 1 or 2% Triton X-100 significantly reduced the number of [3 H]GABA binding sites present

in CSM. Thus, the binding proteins for [3 H]benzodiazepines, but not those for [3 H]GABA, were solubilized by incubating at 37°C with 0.05% Triton X-100, frozen-thawed, and repeatedly washed CSM prepared from rat brain cortex. The absence of [3 H]GABA binding sites in this preparation of solubilized CSM proteins was proved both by the lack of high affinity binding sites for [3 H]-muscimol and by the absence of [3 H]diazepam binding stimulation following the addition of GABA. We proved that, in this preparation, the lack of [3 H]muscimol binding and that of GABA-induced stimulation of [3 H]diazepam binding was not due to the presence of GABA contamination. It is possible to suggest that the GABA receptors were extracted from CSM, but they were inactivated. We do not favor this hypothesis because we still find [3 H]GABA binding sites in the membranes and when CSMs are extracted with higher concentrations of Triton X-100 (0.5 to 1%), the number of [3 H]GABA binding sites solubilized is equivalent to the number of binding sites lost from CSM. A maximal number of benzodiazepine recognition sites (approximately 50% of those located in CSM) was solubilized by incubating the CSM with 0.05% Triton X-100 for two successive times (Table III). A third treatment of these CSMs with 0.05% or with 0.2, 0.5, 1, or 2% Triton X-100 failed to produce the solubilization of additional [3 H]benzodiazepine binding sites.

The characteristics of the solubilized [3 H]benzodiazepine binding sites were similar to those of the sites located in CSM. They were stereospecific and the rank order of the ability of a series of benzodiazepines to displace [3 H]diazepam bound to these sites was similar to that to displace the ligand bound to recognition sites located in CSM. Actually, the affinity of the solubilized sites for [3 H]diazepam is 2 to 3 times higher than that of sites located in the intact CSM. A similar difference has been reported for binding sites where GABA·BZD recognition sites are solubilized as a functional complex (Guidotti et al., 1980).

GABA binding sites virtually free of benzodiazepine binding sites were solubilized by incubating homogenates of rat brain cortex at 0°C with 1% Triton X-100. From Table VI, it can be calculated that approximately 70% of the GABA recognition sites can be extracted using this procedure. The advantage of using crude brain homogenate as a starting material to prepare soluble GABA recognition sites is that of utilizing the protective action of large concentrations of endogenous GABA against the rapid degradation (peptidases?) of the specific binding site for the amino acid. The recovery of solubilized GABA recognition sites can be improved by supplementing the extraction buffer with a mixture of peptidase inhibitors to prevent the rapid degradation of the solubilized GABA recognition sites.

The solubilized GABA recognition sites, similar to those bound to CSM, include two populations of sites: a high ($K_D = 30$ nM) and a low ($K_D = 280$ nM) affinity site for [3 H]GABA. The binding of GABA to these sites is inhibited by bicuculline. However, unlike the GABA recognition sites located in CSM (Fig. 5), the addition of benzodiazepines fails to protect the solubilized GABA

recognition sites from heat inactivation. These results indicate that the protection exerted by benzodiazepines against the heat inactivation of GABA recognition sites is mediated by the binding of benzodiazepines to specific recognition sites.

It is interesting that the procedure adopted for the solubilization of [3 H]GABA binding sites solubilizes a very small percentage (8%) of the total [3 H]benzodiazepine binding proteins (see Table VI). Since this extraction was carried out at 0 to 2°C, comparison of the data of Tables III and VI suggests that the solubilization of the [3 H]benzodiazepine binding sites elicited by Triton X-100 is a temperature-dependent process.

Several reports on the solubilized binding sites of [3 H]GABA or [3 H]benzodiazepines fail to take into consideration that the process requires the removal of endogenous modulators or ligands for these recognition sites. Because we considered this possibility as a critical point, our purification procedure includes as a last step a procedure to free the solubilized recognition sites from endogenous materials (modulator peptides, amino acids, ions) that might interfere with the binding assay. This was achieved by using the minicolumn gel filtration procedure described by Penefsky (1977). With this gel filtration technique, it was possible to remove effectively the endogenous GABA from the solubilized recognition sites even when the starting material was fresh brain cortex homogenate. In addition, since this filtration technique is rapid, the degradation of the recognition sites during this purification step is minimal. Solubilization, purification, and subsequent recombination of GABA and benzodiazepine recognition sites with purified modulatory factors extracted from brain membranes has been the final goal of our present research. We have reported in the past that it is possible to extract from CSM a material (GABA-modulin) that inhibits [3 H]GABA and [3 H]benzodiazepine binding to CSM (Guidotti et al., 1978). The present experiments shed new light, suggesting that the inhibitor of GABA binding (GABA-modulin) is different from the compound that displaces [3 H]diazepam from its high affinity binding sites. Table I shows that the characteristics of the two modulators are different. In particular, GABA-modulin is relatively thermolabile, while the inhibitor of [3 H]benzodiazepine binding is highly thermoresistant. Thus, by using a thermoinactivation procedure (see "Materials and Methods") and different column chromatography techniques, it has been possible to separate GABA-modulin from the inhibitor of [3 H]benzodiazepine binding.

In addition to GABA-modulin, other endogenous compounds, such as GABA (Napias et al., 1980), phospholipids (Johnston and Kennedy, 1978; Lloyd and Davidson, 1979), and a number of small molecular weight materials (Yoneda and Kuriyama, 1980; Mazzari et al., 1980), interfere with the high affinity [3 H]GABA binding. We have sought, therefore, to establish whether, in our GABA-modulin preparation, sufficient quantities of GABA and phospholipids were present to inhibit the high affinity binding. The data summarized in Table I, indicate that a contamination by GABA is unlikely to play a role in the inhibition of high affinity GABA binding

by our GABA-modulin preparation because (1) in our chromatographic purification procedure, the position of GABA-modulin is different from that of GABA; (2) an amount of GABA-modulin preparation that inhibited the high affinity [^3H]GABA binding by more than 50% did not contain a measurable amount of GABA (less than 1 pmol); in these assay conditions, 2.5 pmol of authentic GABA produced 10% inhibition of [^3H]GABA binding; (3) GABA produces a competitive inhibition of [^3H]GABA binding, whereas GABA-modulin produces a non-competitive inhibition; (4) GABA is rapidly dialyzable, whereas GABA-modulin is not; (5) GABA produces stimulation of [^3H]diazepam binding, while GABA-modulin blocks the GABA-induced stimulation of [^3H]diazepam binding; (6) the inhibition elicited by GABA in concentrations up to 1 μM is completely destroyed by GABASE (see Napias et al., 1980), whereas the inhibition by GABA-modulin remains practically unchanged when incubated with GABASE; (7) the content of phospholipids in our preparation was below the amount that produced a significant decrease of [^3H]GABA binding (Toffano et al., 1978, 1980).

Several reports have suggested that the benzodiazepine recognition site may be the physiological site of action for endogenous ligands (Guidotti et al., 1978; Skolnick et al., 1978; Asano and Spector, 1979; Colello et al., 1978; Mohler et al., 1979; Davis and Cohen, 1980; Braestrup et al., 1980). The inhibitor of [^3H]diazepam binding that we have used in these experiments appears to be a protein with a molecular weight greater than 1800. These two characteristics clearly differentiate this material from the small molecular weight endogenous ligands of [^3H]benzodiazepine binding sites described in other reports (Skolnick et al., 1978; Asano and Spector, 1979; Braestrup et al., 1980; Davis and Cohen, 1980; Mohler et al., 1979). It differs from the inhibitor of [^3H]diazepam binding recently described by Paul et al. (1980) because of its higher molecular weight and because it does not block the GABA-induced stimulation of [^3H]diazepam binding. Preliminary experiments have indicated that the inhibitor of [^3H]benzodiazepine binding is unevenly distributed in various brain structures. From 100 mg of brain cortex, an amount of the inhibitor could be extracted that has the capacity to displace 1 pmol of [^3H]diazepam from its specific binding site (Massotti et al., 1980).

Both GABA-modulin and the endogenous inhibitor of [^3H]diazepam binding sites are more potent in inhibiting the binding of a specific ligand when tested with solubilized receptors. These data give credence to the view that the regulation of [^3H]GABA and [^3H]benzodiazepine recognition sites by the two modulators does not require the integrity of the membrane structure because it may occur even when the phospholipidic support for the recognition site is eliminated by extraction and solubilization of the binding sites with Triton X-100.

In conclusion, our data indicate that the recognition sites for [^3H]GABA and [^3H]benzodiazepines and the endogenous inhibitors of [^3H]GABA (GABA-modulin) and [^3H]diazepam binding can be extracted from the CSM and solubilized as separate units. Since these membrane components are functionally associated in the su-

pramolecular organization of GABA receptors, the "GABA receptor unit," the biochemical dissection of these components is the first step in understanding the mechanisms whereby these various molecules are operative in GABA receptor function.

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