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Isolation, characterization, and purification to homogeneity of a rat brain protein (GABA-modulin)

(benzodiazepines/transmitter receptors)

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ABSTRACT **γ-Aminobutyric acid (GABA)-modulin is a brain neuropeptide that appears to modulate specific high-affinity (20 nM) GABA recognition sites in brain. When added to crude synaptic membranes this peptide inhibits binding of [^{3}H]GABA to the high-affinity site and prevents facilitation of [^{3}H]diazepam binding elicited by GABA. GABA-modulin has been purified to homogeneity by ammonium sulfate precipitation, gel chromatography, and reverse-phase HPLC. Homogeneity was confirmed by a variety of means, including chromatography under four different HPLC conditions, two different polyacrylamide gel electrophoreses, and end group analysis. Purified GABA-modulin contains approximately 126 amino acids and has a molecular weight of 16,500. The GABA-modulin molecule contains an abundance of hydrophilic basic residues, and neither cysteine nor GABA is present. End group analyses of GABA-modulin showed that histidine is the free COOH terminus and the NH_2 terminus is blocked. GABA-modulin specifically blocked both [^{3}H]GABA binding to synaptic membranes (IC_{50}, 0.5 μM) and GABA-stimulated [^{3}H]diazepam binding; the binding of [^{3}H]GABA to low-affinity sites was not affected.**

The binding of γ-aminobutyric acid (GABA) to specific recognition sites on postsynaptic neuronal membranes is regulated by endogenous modulators (1–4). One of these (2, 5) is the protein termed "GABA-modulin" (6), which noncompetitively reduces the number of high-affinity GABA recognition sites (2, 5). This regulation involves an allosteric mechanism that can be modified by diazepam (5). GABA-modulin also prevents GABA-induced stimulation of [^{3}H]diazepam binding to brain synaptic membranes (7).

Previously GABA-modulin has been extracted from either rat brain (2, 7) or NB_{2A} cloned mouse neuroblastoma cells (6). However, purification and characterization of this peptide has been difficult because of the presence of various materials interfering with GABA binding, including GABA (8) itself, and because of enzymatic degradation of GABA-modulin. This report describes the isolation, purification to homogeneity, and amino acid composition of GABA-modulin isolated from rat brain.

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats (175–200 g) were obtained from Zivic–Miller (Allison Park, PA).

Materials. Sephadex G-100 and G-75 gels were from Pharmacia. Bio-Gel P-2 and the Bio-Sil ODS-10 (octadecylsilyl) reverse-phase HPLC column were from Bio-Rad. The Zorbax CN HPLC column was from Du Pont. The Synchropak AX 300 anion-exchange HPLC column was from Synchrom (Linden, IN). Trypsin (treated with tosyllysine chloromethyl ketone) and chymotrypsin were from Worthington. HPLC chromatography was carried out with a Spectraphysics system (Santa Clara, CA).

Protein Determination and NH_2- and COOH-Terminal Amino Acid Analysis. Proteins were detected colorimetrically by the Lowry method (9). Automated NH_2-terminal analysis was attempted on a Beckman sequencer, using 0.1 M Quadrol program no. 031281 without Polybrene. Released phenylthiohydantoins were identified by HPLC analysis (10). The COOH-terminal residue was identified by Akabori hydrazinolysis (11).

Polyacrylamide Slab Gel Electrophoresis. Acidic (pH 3.2) urea gels (2.8 mm thick) were prepared according to the procedure described by Panyim and Chalkley (12). $NaDodSO_4$ gels were run according to Laemmli and Favre (13) with minor modifications.

GABA-Modulin Assay. This assay measures the ability of tissue extracts to inhibit the binding of [^{3}H]GABA to crude synaptic membranes treated with Triton X-100 (0.05%) or $AgNO_3$ (0.1 mM) (2, 14). The assay (final volume 1 ml) was carried out in 20 mM potassium phosphate buffer (pH 7.0). Materials were added to the reaction tube in the following order: suspension of synaptic membranes (0.3–0.4 mg of protein), GABA-modulin extract or control buffer (10–100 μl), [^{3}H]GABA or [^{3}H]muscimol (various concentrations in 50 μl of buffer). Nonspecific binding (usually 10% of the total binding) was determined by adding 1 mM GABA. After 10 min of incubation at 2–4°C the reaction was terminated by centrifugation (7). The same conditions were used to study the effect of GABA-modulin on GABA-stimulated [^{3}H]diazepam binding [for further details see Massotti *et al.* (7)]. The effect of GABA-modulin on [^{3}H]etorphine (15), [^{3}H]-adenosine (16), [^{3}H]imipramine (17), and [^{3}H]quinuclidinyl benzilate (18) binding was studied by published methods, with the concentration of the ligand in the K_d range.

GABA-Modulin Iodination. GABA-modulin (250 μg of protein), diluted in 100 μl of phosphate-buffered saline, was treated with approximately 2 mCi (1 Ci = 3.7×10^{10} becquerels) of Na^{125}I and 20 μl of chloramine-T (0.2 mg/ml) for 15 sec, followed by 20 μl of sodium metabisulfite (2 mg/ml). The sample was desalted with a Bio-Gel P-2 column equilibrated with 0.1 M acetic acid, and purity of iodinated material was tested by both $NaDodSO_4$/polyacrylamide gel electrophoresis and reverse-phase HPLC.

RESULTS

Isolation of GABA-Modulin. The steps employed to extract GABA-modulin from rat brain are reported in Fig. 1; typically, 20 rat brains were used for each preparation. After decapitation, each brain was rapidly (15–30 sec) removed and homogenized

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Abbreviation: GABA, γ-aminobutyric acid.

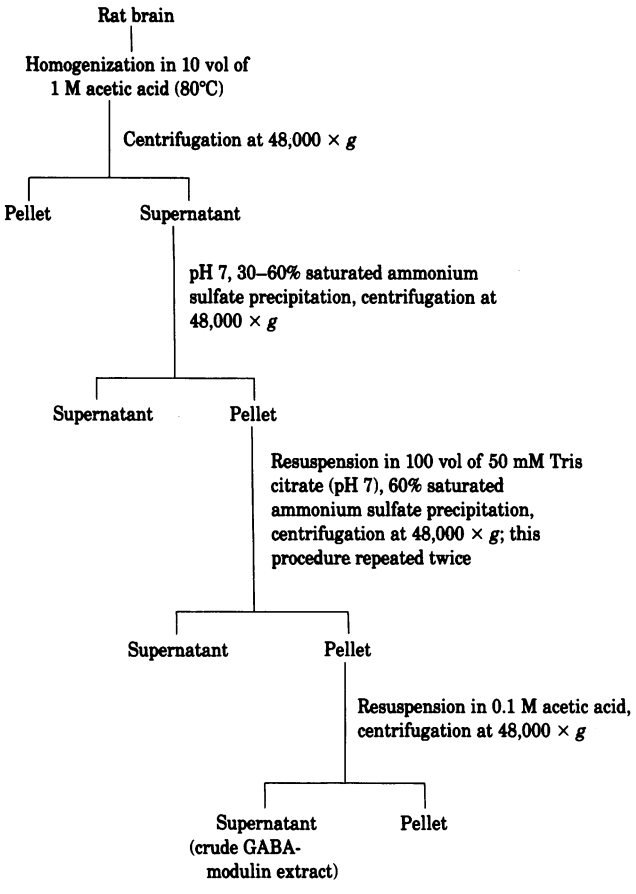

FIG. 1. Scheme for preparation of crude GABA-modulin extract.

in hot (80°C) 1 M acetic acid. After the ammonium sulfate precipitation steps (see Fig. 1) an aliquot of the crude GABA-modulin extract was chromatographed in succession on Sephadex G-100 and Sephadex G-75 columns, both equilibrated with 0.1 M acetic acid (Fig. 2), and finally on a Bio-Sil ODS-10 reverse-phase HPLC column equilibrated with 0.1 M $NaClO_4$/0.1% H_3PO_4, pH 2.5, eluted with 0–60% (vol/vol) acetonitrile gradient (Fig. 2).

Details on the amount of protein, sample volume, recovery and purification are reported in Table 1. The recovery and degree of purification were estimated by adding 0.9 μg of ^{125}I-labeled GABA-modulin (specific activity 10,000 cpm/ng) to the initial homogenate. Usually 20 rat brains yielded 0.5 mg of purified GABA-modulin.

Different HPLC or acrylamide gel electrophoresis conditions and COOH-terminal amino acid analyses were used as criteria to establish the purity of GABA-modulin. As shown in Fig. 3, with 50–500 μg of protein, a single UV absorbing peak was observed in four different HPLC conditions.

Furthermore, the purity of GABA-modulin was assessed with three different polyacrylamide gel electrophoretic systems: (*i*) 12% $NaDodSO_4$ gel, (*ii*) 17% $NaDodSO_4$ gel, and (*iii*) 15% urea/acid pH gel. In all of these conditions, application of 10–200 μg of protein produced a single band staining with Coomassie blue (Fig. 4). COOH-terminal amino acid analysis using hydrazinolysis was adopted as third criteria for purity of GABA-modulin. This analysis showed that the COOH terminus was free and that histidine was the only detectable amino acid. NH_2-terminal amino acid analysis indicated that the NH_2-terminus was blocked. In fact, no sequence could be determined with the native preparation.

Characteristics of GABA-Modulin. The amino acid composition of purified GABA-modulin is shown in Table 2. It contains a relative abundance of hydrophilic basic residues and no cysteine. No GABA was detected by this analysis or by another means (sensitivity of the other method for GABA was 2.5 pmol) (19). When purified GABA-modulin was incubated for 30 min at 37°C with trypsin or chymotrypsin, the inhibitory activity on [^{3}H]GABA binding was lost. Injection of the digested material on reverse-phase HPLC showed that the peak of protein as-

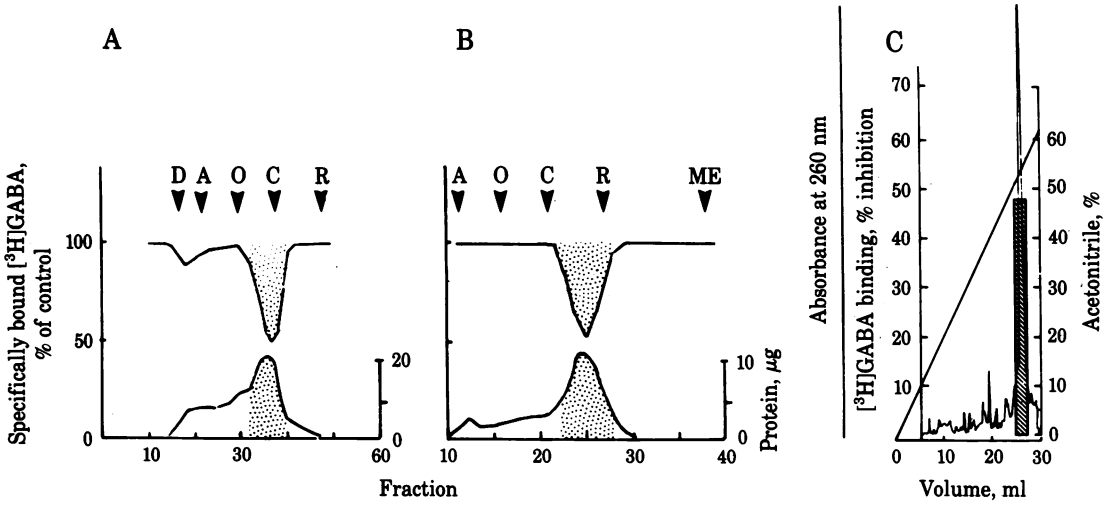

FIG. 2. Purification procedure for GABA-modulin. The crude GABA-modulin extract (Fig. 1), derived from 10 rat brains, was carefully resuspended in 5 ml of 0.1 M acetic acid and, after centrifugation at 48,000 × *g* for 10 min, the clear supernatant was chromatographed successively on 2.5 × 70 cm Sephadex G-100 (*A*) and Sephadex G-75 (*B*) columns equilibrated with 0.1 M acetic acid. The columns were developed with 0.1 M acetic acid with a flow rate of 0.2 ml/min, and 5-ml fractions were collected. The upper traces and the left ordinates indicate inhibition of [^{3}H]GABA binding; the lower trace and the right ordinates indicate protein measured in 200 μl with the Lowry method. Shaded areas represent the fractions with [^{3}H]GABA binding inhibitory activity. Elution markers: D, blue dextran; A, albumin; O, ovalbumin; C, chymotrypsinogen; R, ribonuclease; ME, 2-mercaptoethanol. Inhibitory activity for [^{3}H]GABA binding was determined by using 100 μl of each fraction after it was lyophilized and resuspended in 1 ml of 20 mM potassium phosphate, pH 7. (*C*) Reverse-phase HPLC of 250 μg of partially purified GABA-modulin from Sephadex G-100 and G-75. The conditions were Bio-Sil ODS-10 column (2 × 250 mm), flow rate 1 ml/min, temperature 20°C. After sample application, the column was washed for 30 min with the starting buffer (0.1 M $NaClO_4$/0.1% H_3PO_4). The peptides were eluted with a gradient from 100% starting buffer to 70% acetonitrile/30% starting buffer. Fractions (1 ml) were collected, neutralized, lyophilized, and analyzed for [^{3}H]GABA binding inhibitory activity. GABA-modulin activity (approximately 80%) emerges in a 1-ml fraction (hatched bar) corresponding to 53% acetonitrile.

Table 1. Purification of GABA-modulin from 20 rat brains

Step	Vol, ml	Protein, mg	^{125}I-Labeled GABA-modulin, cpm × 10^{-6}	Recovery, %	Purification, -fold
1. Homogenate	400	5,200	18	100	—
2. 48,000 × *g* supernatant	305	1,000	16.6	92	4.6
3. 48,000 × *g* supernatant after 30% $(NH_4)_2SO_4$ fractionation	1,000	600	14.8	82	7.1
4. 30–60% $(NH_4)_2SO_4$ precipitate resuspended in buffer	1,000	400	13.2	73	9.5
5. 60% $(NH_4)_2SO_4$ precipitate resuspended in buffer	1,000	180	12	66	19
6. 60% $(NH_4)_2SO_4$ precipitate resuspended in 0.1 M acetic acid	100	26	11	61	120
7. Sephadex G-100 filtrate	40	5	7.2	38	400
8. Sephadex G-75 filtrate	30	1.5	2.7	15	510
9. HPLC eluate	6	0.5	1.4	8	810

Fold purification was calculated by comparing the specific activity of GABA-modulin at the different stages of purification with the specific activity of the initial homogenate.

sociated with GABA-modulin had disappeared and a number of other peaks were formed. However, none of these peaks maintained inhibitory activity for [^{3}H]GABA binding. The molecular weight of GABA-modulin was estimated to be approximately 16,000 on the basis of amino acid composition, and 17,000 on the basis of $NaDodSO_4$ gel electrophoresis (Fig. 4).

Purified GABA-modulin noncompetitively obliterated the brain membrane sites that bind [^{3}H]GABA or [^{3}H]muscimol with high affinity (Fig. 5), whereas it failed to change the low-affinity [^{3}H]GABA binding. In addition (Fig. 5 *A* and *B*), when the ^{3}H label was equally distributed between high- and low-affinity GABA binding sites, the maximal decrease of [^{3}H]GABA binding elicited by GABA-modulin was approximately 50% of the total binding. The concentration of GABA-modulin that inhibits 50% (IC_{50}) of the high-affinity binding of [^{3}H]GABA is approximately 0.5 μM. Similar results were obtained when GABA-modulin was added to solubilized [^{3}H]GABA recognition sites (data not shown). At this, and at 10 times higher concentration, GABA-modulin does not bind [^{3}H]GABA. In fact, when 100 μg of GABA-modulin was incubated with [^{3}H]GABA and then chromatographed on a small Sephadex G-25 column [see Massotti *et al.* (7) for details] no [^{3}H]GABA eluted in the void volume. We have compared the ability of GABA-modulin to inhibit [^{3}H]GABA binding with that of other peptides or proteins. Albumin (up to 0.5 mg/ml), lysozyme (up to 100 μg/ml), histone (Sigma type II A, up to 100 μg/ml), and small myelin basic protein from rat (kindly provided by M. Kies, National Institute of Mental Health) (see Fig. 5*A*) failed to reduce the binding of [^{3}H]GABA to crude synaptic cortical membranes. In other experiments, purified GABA-modulin in concentrations up to 2.5 or 5 μM failed to change the binding of [^{3}H]diazepam, [^{3}H]etorphine, [^{3}H]quinuclidinyl benzilate, [^{3}H]imipramine, and [^{3}H]adenosine to crude synaptic membranes. However, GABA-modulin is a potent inhibitor of GABA-stimulated [^{3}H]diazepam binding. The IC_{50} for this effect is approximately 0.5 μM (see Fig. 6*A*). In the presence of GABA-modulin, the K_a

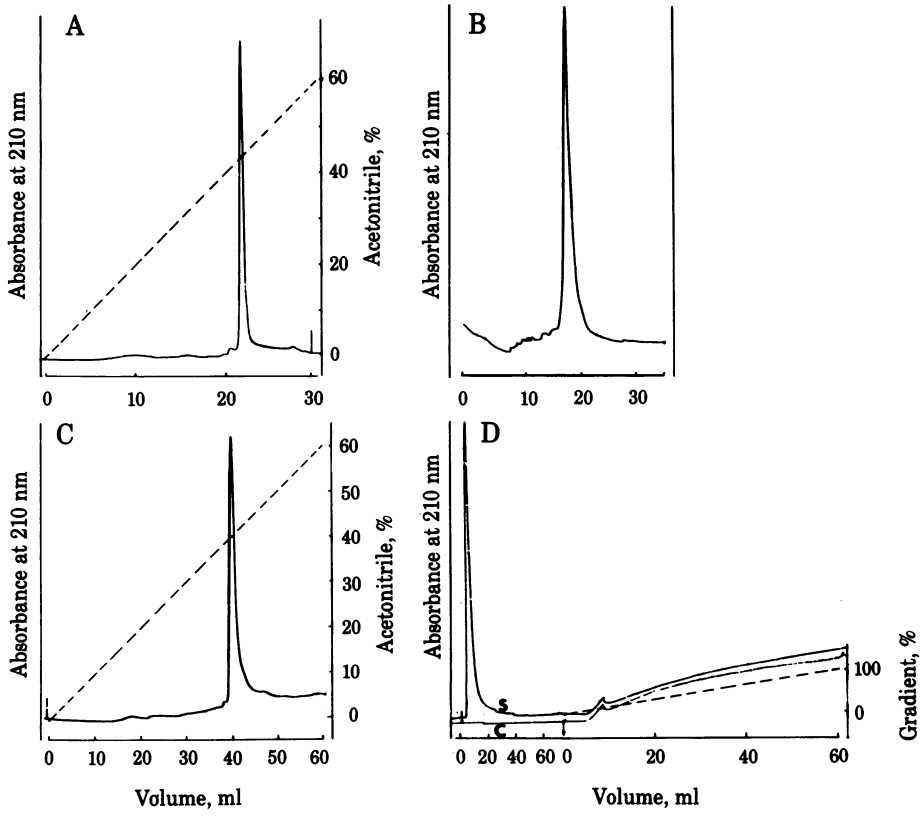

FIG. 3. HPLC analyses of purified GABA-modulin. GABA-modulin obtained from reverse-phase HPLC of Fig. 2 was tested for purity under four different HPLC conditions: (*A*) Bio-Sil ODS-10 column equilibrated with 0.1 M NaH_2PO_4/0.2% H_3PO_4, pH 2.5. The peptide was eluted with a 30-min linear gradient of acetonitrile from 0% to 60% (----). GABA-modulin was eluted with 44% acetonitrile. One hundred micrograms of protein was applied to the column. (*B*) Bio-Sil ODS-10 column developed with 70% (0.1 M NaH_2PO_4/0.2% H_3PO_4) + 30% acetonitrile under isocratic conditions; flow 1 ml/min. GABA-modulin was eluted in 18 min and 45 sec. Fifty micrograms of protein was applied to the column. (*C*) Zorbax CN equilibrated with 0.1 M NaH_2PO_4/0.2% H_3PO_4, pH 2.5. Elution was with a 60-min linear gradient of acetonitrile from 0% to 60%. GABA-modulin was eluted with 40% acetonitrile. One hundred micrograms of protein was applied to the column. (*D*) Synchropak AX-300 oxiranes anion exchange column equilibrated with 0.02 M Na_2HPO_4, pH 8.3; flow 1 ml/min. GABA-modulin elutes with the void volume. No other proteins were eluted when the column (at the arrow) was eluted with a linear gradient from 0.02 M Na_2HPO_4 to 0.2 M Na_2HPO_4/1 M $NaClO_4$, pH 8.3. ----, The gradient. S, Absorption profile after application of 500 μg of GABA-modulin; C, absorption profile in control conditions.

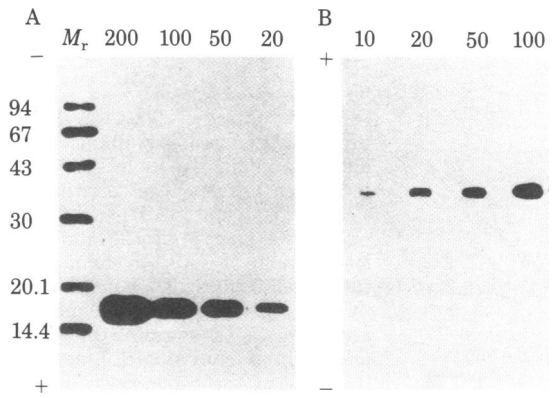

FIG. 4. Polyacrylamide gel electrophoresis. GABA-modulin obtained from reverse-phase HPLC of Fig. 2 was desalted on a 0.5 × 30 cm Bio-Gel P-2 column equilibrated with 0.1 M acetic acid. The amount of protein in each lane is given in μg. (*A*) $NaDodSO_4$/12% polyacrylamide gel. The molecular weight markers (M_r, labeled × 10^{-3}) used were phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400). Proteins move toward the anode. (*B*) Urea/15% polyacrylamide gel (pH 3.2). Proteins move toward the cathode. The concentration of urea was 6.25 M. Coomassie brilliant blue G was used to stain the protein.

(activation constant) for GABA stimulation of [^{3}H]diazepam binding increases from 0.1 μM to 1.2 μM (Fig. 6*B*). The modification by GABA-modulin of the GABA-elicited facilitation of [^{3}H]diazepam binding appears to be competitive.

DISCUSSION

Although several reports (1–8) have described the presence of endogenous inhibitors of the specific binding of [^{3}H]GABA to its recognition sites in crude synaptic membranes or in membranes from cloned cell cultures (NB_{2A}), identification of the molecular nature of these inhibitors has been at best partial. GABA-modulin is one such inhibitor located in purified synaptic membranes (6, 7). The present report describes a method for the purification to homogeneity of GABA-modulin from rat brain. This endogenous inhibitor of [^{3}H]GABA binding is a peptide with a molecular weight of approximately 16,500. During its preparation, GABA-modulin tended to be sticky, adsorbing to other molecules and eluting from Sephadex columns with them. This coelution may explain the different positions of GABA-modulin in comparison to that of the corresponding molecular weight markers in Sephadex G-100 and G-75 columns. Completely homogenous preparations of GABA-modulin were obtained when the Sephadex eluate was applied to reverse-phase HPLC. By using the HPLC technique, the material was purified to homogeneity in a single (30-min) chromatographic run. HPLC with different columns and buffer conditions, polyacrylamide gel electrophoresis at different pH values, analysis of amino acid composition, and COOH-terminal amino acid analysis concurred to support the view that GABA-modulin was purified to homogeneity. The major peak of protein eluting from the reverse-phase HPLC with 50% acetonitrile inhibited both binding of [^{3}H]GABA to the high-affinity site and GABA-induced stimulation of [^{3}H]diazepam binding with an IC_{50} of about 0.5 μM. This concentration was within the range of GABA-modulin concentration present in brain. In

Table 2. Amino acid composition of GABA-modulin purified from rat brain

Residue	Relative no. of residues
Asx	9
Thr	8
Ser	13
Glx	10
Pro	11
Gly	14
Ala	7
Cys	0
Val	4
Met	2
Ile	3
Leu	6
Tyr	2
Phe	6
His	7
Lys	8
Arg	15
(Trp)	*
	125

The analysis was repeated two times. A Durrum D500 automatic amino acid analyzer was used.
* Tryptophan was destroyed during analysis.

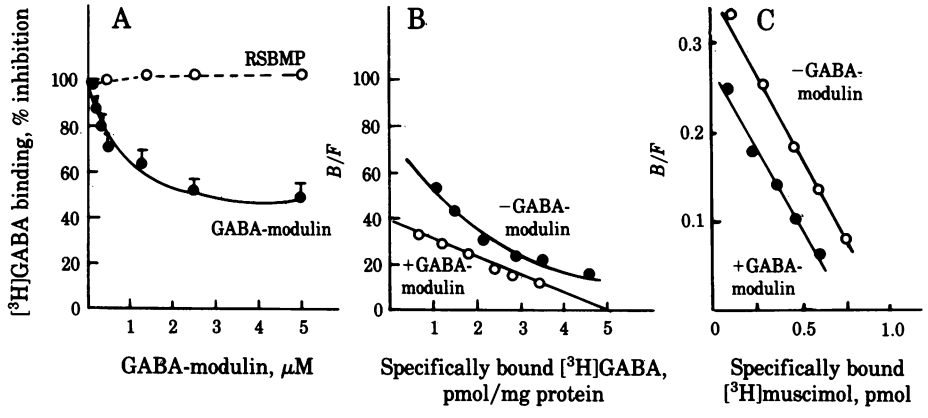

FIG. 5. Effect of GABA-modulin on [^{3}H]GABA binding to crude synaptic membrane preparation from rat cerebral cortex. (*A*) Different concentrations of GABA-modulin or small basic myelin proteins from rat (RSBMP) (obtained from M. Kies, National Institute of Mental Health) were incubated (10 min) at 0°C with 300 μg of Triton X-100-treated membrane proteins; the binding was measured with 20 nM [^{3}H]GABA. Each value is the mean ± SEM of three experiments run in triplicate. The 100% value is 0.6 pmol of specifically bound [^{3}H]GABA per mg of membrane protein. (*B*) Scatchard plot analysis of the binding of [^{3}H]GABA (10–240 nM) to Triton X-100-treated membranes in presence of 2.5 μM GABA-modulin (○) or buffer alone (●). *B/F*, bound (fmol/mg of protein)/free (pmol) [^{3}H]GABA. (*C*) Scatchard plot analysis of the binding of [^{3}H]muscimol (1–20 nM) to $AgNO_3$-treated membranes in presence of 1 μM GABA-modulin (●) or buffer (○). Specifically bound [^{3}H]muscimol is expressed in pmol per assay.

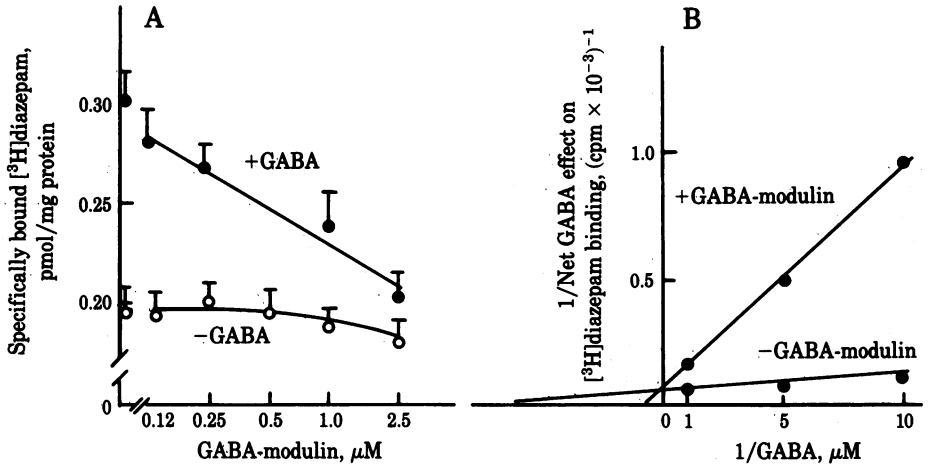

FIG. 6. Effect of GABA-modulin on the GABA-induced stimulation of [^{3}H]-diazepam binding. GABA was added to the incubation mixture together with 1 nM [^{3}H]diazepam and GABA-modulin. The reaction was stopped after 30 min. (*A*) Various amounts of GABA-modulin were incubated with the synaptic membranes in the presence or absence of 0.1 μM GABA. Each value is the mean of three experiments run in triplicate. (*B*) Lineweaver–Burk plot of the net increase in [^{3}H]diazepam binding after treatment with GABA (0.01–1 μM) in presence or absence of 2.5 μM GABA-modulin.

fact, it can be calculated from the recovery studies with ^{125}I-labeled GABA-modulin (Table 1) that the concentration of GABA-modulin in rat brain is approximately 6 μM. The action of GABA-modulin is specific for GABA binding, because at doses up to 5 μM it failed to influence the binding of several other ^{3}H-labeled ligands we tested. In addition, the specificity of GABA-modulin action is confirmed by the lack of effect by other proteins of similar molecular weight and charge on [^{3}H]GABA binding.

Amino acid composition, anion-exchange chromatography, and acrylamide gel electrophoresis with urea at acidic pH revealed that the protein is basic. We have therefore compared GABA-modulin structure and activity with those of the most well-known basic proteins extracted from brain. Histones, which have relatively smaller amounts of arginine and lysine, are eluted from a Bio-Sil ODS column with a higher percent of acetonitrile (higher than 60%), and have no effect on [^{3}H]GABA binding. Two myelin basic proteins are extracted from rat brain: a high molecular weight protein (approximately 170 amino acid residues) and a low molecular weight protein (approximately 126 amino acid residues) (20, 21). The high molecular weight form differs from GABA-modulin in the ratio of arginine to lysine, has a lower mobility on acidic urea and $NaDodSO_4$/polyacrylamide gels, and has a retention time longer than that of GABA-modulin when eluted from a Bio-Sil ODS column with 70% 0.1 M NaH_2PO_4/30% acetonitrile. The small rat myelin basic protein has some striking similarities to GABA-modulin when considered from the point of view of the amino acid composition (compare data of Table 1 with data of ref. 20 and 21). However, the presence of a histidine at the COOH terminus instead of arginine indicates that GABA-modulin is also different from the small myelin basic protein extracted from rat brain. In addition, the small rat myelin basic protein (5 μM) failed to inhibit the binding of [^{3}H]GABA or [^{3}H]muscimol to crude synaptic membranes (Fig. 5*A*).

Although the activity of GABA-modulin was destroyed by trypsin or chymotrypsin digestion, we could not exclude the possibility that the GABA-modulin we had purified was the precursor of the endogenous modulator; in fact, we have not yet thoroughly studied the activity of the GABA-modulin fragments produced by more limited cleavage.

An important question that remains open is whether GABA-modulin facilitates or inhibits the biological activity of GABA released by nerve stimulation. Preliminary experiments show that when GABA-modulin is injected into the cerebral ventricles it exacerbates the convulsions induced by isoniazid, suggesting that the increase in free GABA-modulin decreases the action of GABA released by nerve impulses.

Finally, the question of the mechanism by which GABA-modulin inhibits the binding of [^{3}H]GABA remains to be explored in detail. Because GABA-modulin inhibits the high-affinity GABA binding in an apparently noncompetitive fashion, it is proposed that the mechanism is primarily allosteric. In this regard, the observation that GABA-modulin is a good substrate for protein kinase may be of interest. It is therefore possible to study whether endogenous Ca^{2+}, cGMP-, or cAMP-dependent phosphorylation of GABA-modulin is operative in regulating the affinity of GABA recognition sites for their endogenous effector.

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