Coexistence of GABA Receptors and GABA-Modulin in Primary Cultures of Rat Cerebellar Granule Cells

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GABA-modulin (GM), a basic polypeptide purified from rat brain synaptosomes, which is an allosteric inhibitor of GABA recognition sites, has been detected in primary cultures of cerebellar interneurons enriched in granule cells by immunohistochemistry, using a specific antibody raised in rabbit injected with GM purified from rat brain synaptosomes. In these cultures, GM is expressed by the granule cells, which are postsynaptic to GABAergic interneurons, but not by glial cells. In rat cerebellar sections anti-GM antiserum intensely stains the granular cell layer and Purkinje cell dendrites and cell bodies. GM has been purified from the cerebellar granule cell cultures and appears to be identical under biochemical, immunological, and functional criteria to authentic GM purified from rat brain synaptosomes. Granule cell cultures devoid of GABAergic neurons contain the GABA/BZ/C1⁻ receptor complex; in fact, intact cell monolayers, incubated in physiological buffer at 25°C, express ³H-muscimol and ³H-flunitrazepam binding sites, which are comparable to the sites detected in cell membrane preparations and which modulate each other reciprocally. It is concluded that GM might participate in the supramolecular organization of the GABA receptor complex, perhaps functioning as a modulator of this receptor protein.

Transmitter receptor function can be modulated by regulatory influences either at the ligand-binding domain or at the signal-transduction domain; thus, receptors can become “decision-making devices” adjusting their signal-transduction efficiency continuously. In this capacity resides the receptor’s ability to optimize the interneuronal exchange of information in the face of changes in the synaptic microenvironment. Specific membrane-associated proteins participate in this modulation; for example, guanine nucleotide binding proteins, which are highly concentrated in neuronal membranes (Sternweis and Robishaw, 1984), not only are involved in the regulation of receptor-mediated activation of adenylate cyclase and polyphosphoinositide phosphodiesterase (Lefkowitz et al., 1982; Cockcroft and Gomperts, 1985), but also regulate the affinity of several recognition

sites, including that of beta-adrenergic receptors (Rodbell, 1980). Regulation by membrane-bound proteins is not unique to receptors coupled to enzymes: In the case of the chloride channel-coupled GABA recognition sites, a polypeptide has been isolated from synaptosomal membrane preparations, termed GABA-modulin (GM) (Guidotti et al., 1982; Vaccarino et al., 1985), which modifies the number of high-affinity GABA-recognition sites.

Structurally, GM is a basic polypeptide, which has been purified and characterized and appears to be located selectively in the synaptosomal fraction of rat brain (Vaccarino et al., 1985). It shares a number of characteristics with a well-known class of basic proteins that were isolated from myelin and, in particular, with the small myelin basic protein (SMBP) (Dunkley and Carnegie, 1974; Vaccarino et al., 1985). However, GM can be differentiated from SMBP because of its molecular weight, amino acid composition, tryptic peptide map, and lack of absolute homology in amino acid sequence (Vaccarino et al., 1985). It is conceivable that GM represents a new class of basic proteins contained in the synaptic environment that may modulate neurotransmitter recognition site characteristics. To test whether GM could be colocalized with the GABA/benzodiazepine (BZ)/C1⁻ receptor complex, we used primary cultures from neonatal rat cerebellum as a neuronal model (Balazs et al., 1982; Gallo et al., 1982, 1985). These cultures, which have been shown to differentiate *in vitro* both morphologically and functionally (Gallo et al., 1982; Levi et al., 1984), contain a predominance of granule cells (more than 90% of the total cells) that receive GABAergic innervation by a less abundant population of GABAergic interneurons (see Results).

The results presented here indicate that granule cells physiologically contain a high amount of GM and express GABA and BZ recognition sites in their membrane, in agreement with the idea that GM may be functionally associated with postsynaptic GABA receptors.

Materials and Methods

Granule cell culture

Primary cultures of cerebellar interneurons enriched in granule cells were obtained from 8-d-old rat cerebellum (Wilkin et al., 1976; Levi et al., 1984; Gallo et al., 1985). Cells were plated (2.5×10^6 cells/dish) onto 36 mm Falcon dishes coated with 5 μ g/ml poly(L-lysine) (MW = $70\text{--}150 \times 10^3$) and were cultured in the following medium: Basal Eagle’s medium (Flow Laboratories), 10% fetal calf serum (GIBCO, Chagrin Falls, Ohio), 25 mM KCl, 2 mM glutamine, and 100 μ g/ml gentamycin (GIBCO). Cytosine arabinofuranoside (Ara-C; 10 μ M) (Sigma Chemical Company, St. Louis) was added after 16 hr *in vitro* to inhibit the replication of non-neuronal cells. Both for histochemical and binding studies, 8-d *in vitro* cultures were used; however, for binding experiments, the incubation medium was removed and substituted with fresh medium after 7 d in culture. Using this procedure, GABAergic interneurons

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Figure 1. Saturation isotherms and Scatchard plots of ^3H -muscimol (*A*) and ^3H -flunitrazepam (*B*) binding to intact granule cell monolayers. Assays were performed in the presence of 2–160 nM ligand concentration range for ^3H -muscimol and 2.5–50 nM for ^3H -flunitrazepam. In both *A* and *B*, the points show the data of 1 representative experiment, which was repeated 3 times using different preparations of granule cell cultures. Bound = ^3H -muscimol or ^3H -flunitrazepam specifically bound, calculated by subtracting the radioligand bound in the presence of 10^{-3} M GABA or 10^{-6} M clonazepam, respectively, from the total binding.

disappear from the culture, whereas granule cells remain intact (Aloisi et al., 1985).

GABA determination

Granule cell cultures were incubated at 25°C in 0.9 ml of Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO_3 , 2.3 mM CaCl_2 , 1 mM MgCl_2 , 5.6 mM glucose, 5 mM HEPES, pH 7.4). The cell monolayer and an aliquot of the incubation medium were extracted with 0.4 N HClO_4 containing 5 nmol/ml citrulline as standard. After centrifugation, the 33,000 $\times$ g supernatant was injected into a high-pressure liquid chromatography (HPLC) system equipped with a 3.1 mm $\times$ 25 cm Bio-Rad Aminex A9 cation-exchange resin column maintained at 55°C. Amino acids were eluted from the column by 0.25 M sodium acetate buffer (pH 4.7) and quantified fluorimetrically as described by Schmid (1980).

^3H -muscimol and ^3H -flunitrazepam binding studies

Intact cells. The binding of various ligands was studied at equilibrium as described by Gallo et al. (1985). Briefly, granule cell monolayers were washed once with Locke's solution and then incubated at 25°C with 1 ml of Locke's solution containing ^3H -muscimol or ^3H -flunitrazepam (both from New England Nuclear, Boston) plus drugs to be studied. Since for ^3H -muscimol binding equilibrium is reached in 10 min, this ligand was incubated for 15 min. ^3H -flunitrazepam was incubated for 30 min. To stop the incubation, the monolayer was rapidly washed with

Locke's solution, the cells were resuspended in 1 ml of 0.1 N NaOH, and the NaOH suspension was used for counting the radioactivity and for protein determination (Lowry et al., 1951). The specific binding was calculated by subtracting the nonspecific binding (radiolabel bound in the presence of 10^{-3} M nonradioactive GABA or 10^{-6} M nonradioactive clonazepam, respectively) from the total ^3H -muscimol or ^3H -flunitrazepam binding. The radiolabel bound to the plastic dishes was subtracted from the value of all samples. Using concentrations lower than 50 nM for ^3H -muscimol or 30 nM for ^3H -flunitrazepam, 70% of the total binding was specific.

Crude membranes. Crude rat brain synaptic membranes, prepared according to Enna and Snyder (1977), were frozen-thawed and treated with 0.05% Triton-X 100 just before ^3H -muscimol binding (Guidotti et al., 1982). Membranes from granule cells were prepared by harvesting the cultures in ice-cold H_2O followed by homogenization with Polytron and freezing. The day of the experiment, the membranes were washed once with 50 mM Tris-HCl, pH 7.4, resuspended in Locke's solution and incubated (100 μg /protein tube) at 25°C with ^3H -muscimol or ^3H -flunitrazepam for 10 or 30 min, respectively. ^3H -flunitrazepam assay was terminated by filtration through Whatman GF/C filters, while ^3H -muscimol assay was terminated by centrifugation (Massotti et al., 1981).

Preparation of the anti-GM antiserum

Purified GM, 500 μg (Vaccarino et al., 1985), emulsified in Freund's complete adjuvant enriched with 4.5 mg/ml *Mycobacterium butyricum*

Table 1. Inhibitory potency of benzodiazepines and β -carboline derivatives on ^3H -flunitrazepam binding to granule cell monolayers and granule cell membranes

Ligand	K_i (nM)	
	Cell monolayers	Cell membranes
Flunitrazepam	6.5 $\pm$ 0.31	4.4 $\pm$ 0.23
Clonazepam	6.5 $\pm$ 0.21	
Diazepam	23 $\pm$ 1.9	18 $\pm$ 0.91
R015-1788	3.9 $\pm$ 0.3	
DMCM	3.0 $\pm$ 0.52	3.3 $\pm$ 0.13
β -CCM	7.7 $\pm$ 0.55	
β -CCE	3.9 $\pm$ 0.41	

^3H -flunitrazepam binding to granule cell monolayers and granule cell membranes was performed as described in Materials and Methods. The mean inhibitory concentration (IC_{50}) was obtained from a semilogarithmic plot of the dose-response curve derived from the ligand inhibition of ^3H -flunitrazepam binding. The inhibition constant (K_i) was calculated with the equation: $K_i = \text{IC}_{50}/1 + [\text{L}]/K_d$, where $[\text{L}]$ is the radioligand concentration (3.0 nM for cell monolayers and 1 nM for cell membranes) and K_d is the dissociation constant of the ligand. The K_d values were obtained from the Scatchard plots (Fig. 1B and Results). The values represent the means $\pm$ SEM of 3 separate experiments. Abbreviations: R015-1788, ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4-H-imidazo [1-5a]-[1,4] benzodiazepine-3-carboxylate; DMCM, methyl-6,7-dimethoxy-4-ethyl- β -carboline-3-carboxylate; β -CCM, methyl- β -carboline-3-carboxylate; β -CCE, ethyl- β -carboline-3-carboxylate.

powder (Difco) was injected subcutaneously into a rabbit. The animal was boosted 4 weeks later with GM (250 μg) emulsified in incomplete Freund's adjuvant (Sigma) and after another week the rabbit was bled. The anti-GM antiserum showed a weak cross-reaction with myelin basic proteins (MBPs). To eliminate this cross-reactivity, the antiserum was routinely preabsorbed with MBPs using the method of Calabrese (1984). Briefly, an excess of SMBP and large myelin basic protein (LMBP) was absorbed in the wells of a 96 well microtitration plate (Flow Laboratories, McLean, VA). After blocking the unspecific sites with 10% fetal calf serum in PBS containing 0.5% BSA (PBS/FCS), the wells were filled with 200 μl of anti-GM antiserum diluted 1:100 in PBS/FCS. After 1 hr reaction at 25°C, the antiserum solution was transferred to other wells (coated with MBPs and treated with PBS/FCS as above) and incubated for an additional hour. This operation was repeated once more for a total of 3 hr incubation.

Radioimmunoassay

Radioimmunoassay (RIA) was performed as follows. Samples to be tested were diluted in PBS plus 5% BSA (PA buffer) containing 2 mg/ml histone type II A (Sigma; PAH buffer) and assayed in the following system: 50 μl of diluted sample, 100 μl of MBPs-preabsorbed anti-GM antiserum diluted 1:1000 in PA buffer, 50 μl of ^{125}I -GM diluted in PAH buffer to have approximately 20,000 cpm/0.5 ng/assay. After incubating the samples overnight at 4°C, bound and free antigen were separated by adding 300 μl of a suspension of protein A (Sigma; 0.25% in PBS) and incubating for 1 hr at 4°C. After centrifugation (20 min at 3000 $\times$ g), the supernatants were aspirated, the pellets washed once with PBS, and then counted for ^{125}I in an automatic gamma counter.

Preparation of iodinated basic proteins

GM or SMBP (10 μg , diluted in 100 μl PBS) was treated with approximately 2 mCi of ^{125}I -iodine and 3 iodo-beads (Pierce Chemical Company, Rockford, IL) for 15 min at 25°C. The reaction was stopped by adding 700 μl of 0.1 M acetic acid, and the sample was desalted with a Bio-Gel P-2 column eluted with 0.1 M acetic acid containing 1% BSA. The purity of the iodinated material was tested by reverse-phase HPLC (see below).

Purification of antibodies

IgG immunoglobulins were purified by chromatography on protein A-Sepharose (Pharmacia) (Lindmark et al., 1983). Briefly, the antiserum was diluted 1:5 with 0.1 M citrate buffer (pH 6.2) and applied to a 7 ml column of protein A-Sepharose previously equilibrated with the same

Table 2. Inhibitory potency of GABA receptor ligands ^3H -muscimol binding to intact granule cell monolayers

Ligand	K_i (nM)
Muscimol	7.1 $\pm$ 0.5
GABA	140 $\pm$ 12
Bicuculline	1400 $\pm$ 90
α -Baclofen	$> 2 \times 10^{-4}$
Nipecotic acid	$> 1 \times 10^{-4}$

^3H -muscimol binding to granule cell monolayers was performed as described in the Materials and Methods. The mean inhibitory concentration (IC_{50}) was obtained from a semilogarithmic plot of the dose-response curve derived from the ligand inhibition of ^3H -muscimol binding. The inhibition constant (K_i) was calculated by the equation: $K_i = \text{IC}_{50}/1 + [\text{L}]/K_d$, where $[\text{L}]$ is the radioligand concentration (5 nM for muscimol, 40 nM for GABA and bicuculline inhibition) and K_d is the dissociation constant of the ligand, obtained from the Scatchard plot (Fig. 1A). The values represent the means $\pm$ SEM of 3 separate experiments.

buffer. After extensive washing, the immunoglobulins were eluted with 0.1 M citrate buffer, pH 3.

Immunohistochemistry

After 8 d in culture, the dishes containing primary cultures of cerebellar interneurons were washed with 0.1 M PBS (pH 7.4) and then fixed with 4% paraformaldehyde in PBS for 10 min at 25°C. The cells were first incubated for 20 min with a 1:10 dilution of normal goat serum (NGS) and then incubated with the specific antisera in PBS containing 0.1% NGS at 4°C for 24 hr. In order to block axonal transport, some dishes were treated with colchicine (10^{-6} M) at 37°C for 12 hr prior to the staining. The following antisera were used:

1. the polyclonal anti-GM antiserum;
2. the monoclonal antibody (mouse hybrid cells, clone G-A-5) to glial fibrillary acidic protein (GFAP; Boehringer Mannheim, Indianapolis, IN), which cross-reacts with 55 K intermediate filaments in astrocytes and Bergman glia but is not reactive with neurofilaments (Debus et al., 1983);
3. the monoclonal antibody (mouse hybrid cells, clone V-9) anti-vimentin (AV; Boehringer Mannheim), which cross-reacts with 57 K intermediate filaments in connective tissue, endothelial cells and astrocytes but is not reactive with nerve processes (Osborn et al., 1984);
4. the monoclonal mouse antibody against neurofilaments (NF) (Sternberger-Meyer, Jarrettsville), which cross-reacts with 150 and 200 K neurofilaments in neurons but does not react with filaments in astrocytes (Debus et al., 1983); and
5. the polyclonal anti-glutamic acid decarboxylase (GAD, E.C. 4.1.1.15), a generous gift from Dr. Kopin, National Institutes of Health, Bethesda, MD (Oertel et al., 1980).

For optimal staining, saponin (0.1%) was present in the incubation medium in the case of GFAP, NF, and GAD antibodies; GM immunostaining was not modified by saponin treatment. The anti-GM antiserum was diluted 1:800, GFAP 1:200, AV 1:160, GAD 1:200, and NF 1:1000. For double immunofluorescence staining the cells were incubated with a mixture of anti-GM, plus GFAP or AV or GAD or NF antibodies. The incubation was terminated by several washings followed by incubation with the secondary antibodies (1:150) in PBS plus 0.1% NGS for 15 min. Secondary antibodies were affinity-purified goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC; Cappel) and goat anti-rabbit IgG conjugated with rhodamine isothiocyanate (RITC; Jackson Immunoresearch), or swine anti-rabbit IgG conjugated with rhodamine (Cappel) or swine anti-goat IgG conjugated with fluorescein (Boehringer Mannheim). After washing, the cells were covered with buffered glycerol and viewed by fluorescence microscopy (Leitz, Ortholux II). The specimens were photographed on Tri-X or Ektachrome film (Kodak, 400 ASA).

Control dishes were incubated with preimmune serum (1:200) or with GM-antiserum preabsorbed with different amounts of GM or SMBP. Preabsorption was carried out by adding 5–20 $\mu\text{g}/\text{ml}$ of the peptide to the GM-antiserum diluted 1:800 and incubated in PBS at 4°C overnight. Omission of either the link antiserum or the specific antiserum completely abolished the staining.

For immunohistochemistry of cerebellar slices, the rat brains were perfused with 4% paraformaldehyde solution for 20 min and further

Figure 2. Reciprocal modulation of GABA (*A*) and BZ (*B*) binding sites in granule cell monolayers. ^3H -muscimol (*A*) and ^3H -flunitrazepam (*B*) specific binding was performed in intact cells as described, using 20 nM ^3H -muscimol and 2.6 nM ^3H -flunitrazepam, in the presence of different concentrations of unlabeled flunitrazepam and GABA, respectively. Control binding was 400 ± 48 fmol/mg protein for ^3H -muscimol and 94 ± 5.0 fmol/mg protein for ^3H -flunitrazepam.

processed for peroxidase-antiperoxidase immunostaining according to Sternberger (1979). The optimal dilution for GM antiserum was 1:1200, and the immunolabeling was visualized with 3,4-diaminobenzidine reaction (Sternberger, 1979).

Purification of GM from cultured granule cells

Primary granule cell cultures at 8 d of *in vitro* growth were extracted with 1 M acetic acid, homogenized with Polyttron, boiled for 5 min, and centrifuged. The crude extract was applied on a Sephadex G-75 column (1×80 cm) eluted with 1 M acetic acid, the fractions containing the anti-GM immunoreactivity were pooled, lyophilized, and applied on a μ Bondapak C_{18} preparative column (7.8 mm $\times$ 30 cm; Waters Associates, Milford, MA) equilibrated with 0.1% TFA in water and eluted with a nonlinear gradient of acetonitrile containing 0.1% TFA (from 0 to 20% in 5 min, then from 20 to 40% in 100 min; flow rate, 2 ml/min). GM purified from granule cells as well as from rat brain synaptosomes eluted with a retention time of 47 min. In some cases, a partial purification of GM was achieved by absorption of the crude extract on a C_{18} Sep-Pak cartridge (Waters) eluted with 3 ml of 60% 2-propanol containing 0.1% TFA.

Polyacrylamide gel electrophoresis

One-dimensional SDS gels (0.75 mm thick) were prepared according to Laemmli (1970); proteins were stained with silver nitrate according to Merrill et al. (1981).

Amino acid analysis

Hydrolysis of proteins and amino acid analysis were carried out as previously described (Vaccarino et al., 1985) using a Hitachi 835 high-speed automatic amino acid analyzer equipped with a data processor unit (Hitachi, Tokyo).

Results

Characterization of the granule cell cultures

Immunocytochemical analysis revealed that 8 d *in vitro* primary cultures of dissociated cells prepared from newborn rat cere-

bellum contain approximately 92% small neurons, as detected by anti-neurofilaments antibody (NF), morphologically identifiable as granule cells; 3% GFAP-positive or AV-positive astroglial or connective tissue cells (Fig. 4, *B*, *D*); 4–5% GAD-positive interneurons (Fig. 6). These results are in agreement with previous reports (Kingsbury et al., 1985; Nicoletti et al., 1986).

Granule cells-enriched cerebellar cultures contain 6–7 nmol GABA/mg protein and release approximately 0.02 nmol of GABA in 1 ml of Locke's solution after 20 min incubation at 25°C. The amount of GABA in the extracellular medium, although small, could interfere in the determination of the binding characteristics of ^3H -muscimol and ^3H -flunitrazepam. It has been reported (Aloisi et al., 1985) that a change of the culture medium at day 7 results in a selective degeneration of GABAergic cells. Therefore, for the binding experiments, the GABAergic interneurons were eliminated from the culture using the above procedure. We confirmed that after this change in the medium the immunohistochemical signs of GAD-positive interneurons virtually disappeared from the cultures (data not shown) and GABA was undetectable in the medium (<0.01 nmol).

Location of GABA and BZ recognition sites in granule cell cultures

Using granule cell intact monolayers free of GABAergic neurons, the binding of ^3H -muscimol or ^3H -flunitrazepam was measured at 25°C in Locke's solution to keep osmolarity and ionic conditions in the physiological range (see Materials and Methods). ^3H -flunitrazepam saturation data are consistent with a single binding component having a K_d of 11 nM and a B_{max} of 0.45 pmol/mg protein (Fig. 1*B*). ^3H -muscimol saturation data

Figure 3. Immunoreactivity of the anti-GM antiserum against basic proteins and granule cell extract. The MBPs—preabsorbed antiserum raised against GM was assayed by RIA as described in Materials and Methods with standard GM purified from synaptosomes (●); SMBP (■) and LMBP (○) purified from rat brain myelin, and crude granule cell extract (◇).

indicate the presence of a single population of recognition sites having a K_d of 12.5 nM and a B_{max} of 0.5 pmol/mg protein (Fig. 1A). Using membranes prepared from granule cells and suspended in Locke's solution, the Scatchard plots were linear for both ligands; the K_d for ^3H -flunitrazepam binding was 3.2 nM and the K_d for ^3H -muscimol binding was 5 nM. The B_{max} was 1.2 and 0.9 pmol/mg protein for ^3H -muscimol and ^3H -flunitrazepam, respectively.

Table 1 shows the K_i values of several BZ and beta-carboline derivatives for the displacement of ^3H -flunitrazepam binding from intact granule cells; the order of potency of these ligands is comparable to that obtained in granule cell membranes. ^3H -muscimol binding to intact monolayers was displaced by GABA agonists and antagonists with a rank order of potency similar to that reported for rat brain membranes: muscimol > GABA > bicuculline (Table 2). Neither 10^{-4} M nipecotic acid, an inhibitor of neuronal GABA uptake (Johnston et al., 1976), nor 2×10^{-4} M baclofen, a GABA_B receptor antagonist (Bowery et al., 1984), modified ^3H -muscimol binding (Table 2).

In addition to high-affinity binding sites, intact cerebellar granule cells express low-affinity binding sites for GABA and BZ, which reciprocally modulate each other. As shown in Figure 2, ^3H -muscimol and ^3H -flunitrazepam binding increased by the addition of micromolar concentrations of unlabeled flunitrazepam and GABA, respectively: 50% stimulation of ^3H -muscimol binding was obtained with 2.5×10^{-6} M flunitrazepam, while 50% stimulation of ^3H -flunitrazepam binding was achieved with 10^{-5} M GABA. The effect of flunitrazepam on ^3H -muscimol binding was blocked by the BZ antagonist RO15-1788 (control values: 280 ± 20 fmol/mg protein; 5×10^{-6} M flunitrazepam: 380 ± 9 fmol/mg protein; 5×10^{-6} M flunitrazepam plus 10^{-5} M RO15-1788: 290 ± 27 fmol/mg protein; $n = 3$).

Location of GM in granule cells

An antiserum was raised against GM purified from rat brain synaptosomes (Vaccarino et al., 1985) in order to establish whether granule cells contain GM in addition to GABA, BZ recognition sites and Cl^- channels regulated by GABA (Guidotti

et al., 1985). This antiserum originally showed a weak cross-reactivity with MBPs, which was virtually eliminated by preabsorption of the antiserum with MBPs (see Materials and Methods). After preabsorption, the antiserum still retained a high affinity for GM (50% displacement of the radioactive tracer with 2 pmol of protein) but showed a 4000-fold specificity for GM with respect to MBPs as assayed by RIA (Fig. 3). Thus, in all the subsequent experiments the anti-GM antiserum was routinely preabsorbed with MBPs.

Expression of GM-like immunoreactivity in granule cells

Immunohistochemically, the anti-GM antiserum brightly stained a uniform population of small neurons, morphologically iden-

Table 3. Immunofluorescent staining in primary cultures of cerebellar granule cells with anti-GM antiserum preabsorbed with different basic proteins

Antiserum preabsorption	Immunofluorescent intensity	
	Granule cells	Astrocytes
GM ($\mu\text{g/ml}$)		
0	+++	0
5	++	0
10	+	0
20	0	0
SMBP		
0	+++	0
5	+++	0
10	+++	0
20	++	0

At left is shown the amount of protein used in the preabsorption of the anti-GM antiserum. Immunohistochemical staining intensity was graded by comparison with dishes stained with preimmune serum: 0, no reactivity (equal to preimmune); +, weak scattered fluorescence; ++, moderate fluorescence; +++, intense fluorescence.

Granule cells were identified by their reaction with NF antibody and morphology, and astrocytes by their reaction with GFAP antibody, both performed by double-staining with anti-GM antiserum as described in the Materials and Methods.

Figure 4. Fluorescence photomicrograph of cerebellar neuronal cultures enriched in granule cells, double-stained with anti-GM antiserum (*A*, *C*) and either with anti-vimentin (VM) antiserum (*B*) or with anti-glial fibrillary acidic protein (GFAP) antiserum (*D*). In *A*, granule cells (arrow) and their fibers are stained with anti-GM antiserum; in *B*, the same field double-stained with VM antiserum shows a large epithelioid cell (*n*) with a branch (arrowheads) that is not stained in *A* with anti-GM (*n*, arrowheads). In *C*, granular cells (arrow) are stained with anti-GM; in *D*, the same field double-stained with GFAP shows an astrocyte (*n*) and branches (arrowheads) that are not stained in *C* with anti-GM (*n*, arrowheads). Bar, 30 μ m (all micrographs).

tifiable as the granule cells (Fig. 4, *A*, *C*), which were also immunoreactive for neurofilaments by double-immunostaining (data not shown). Astrocytes or other non-neuronal cells did not contain any GM-like immunoreactivity (Fig. 4, *B*, *D*). When the cells in an identical incubation were treated with preimmune antiserum (1:200), only a faint fluorescence was observed (data not shown). Preabsorption of the anti-GM antiserum with 5 μ g/ml of GM diminished the stain, while 15 μ g/ml of GM abolished it; however, 15 μ g/ml of SMBP failed to diminish the staining (Table 3, Fig. 5). The immunoreactivity associated with the anti-GM antiserum was absent in the nucleus and evident in the

granule cell bodies, as well as along the cell processes (Fig. 5), and this pattern was unchanged with colchicine pretreatment (Fig. 6, *B*, *D*).

After incubation of the cultures with the anti-GAD antiserum, numerous GAD-positive fibers were detected (Fig. 6*A*). If the cultures were pretreated for 12 hr with 10^{-6} M colchicine, only GAD-positive neuronal cell bodies (3–4% of the total neuronal population; Fig. 6, *B*, *C*) were demonstrated; GAD-positive fibers and perikarya contained only traces of GM immunoreactivity, as demonstrated by double immunostaining (Fig. 6, *A–D*). Routine histochemical studies were performed on cul-

Figure 5. Fluorescence photomicrographs of cerebellar neuronal cultures enriched in granule cells. In *A*, cells stained with anti-GM antiserum; in *B*, corresponding field after preabsorption of the GM antiserum with 15 μ g GM, showing an almost complete abolition of staining. In *C*, the antiserum was preabsorbed with 15 μ g SMBP, showing that the staining was not considerably diminished. Bar 15 μ m.

tures maintained *in vitro* without medium change for 8 d; however, a medium change on day 7 (Aloisi et al., 1985), which results in the disappearance of the GAD staining, did not affect GM immunostaining in the granule cells (data not shown). Immunocytochemical studies were performed in rat cerebellar slices to address the issue of GM expression in neurons other than granule cells. As shown in Figure 7, a high density of GM immunoreactivity was present in the granule cell layer and a lower density in the molecular layer, where GM-like immunoreactivity was concentrated in the dendritic tree and cell body of the Purkinje cells (Fig. 7, *A, B*). The immunoreactivity was virtually abolished by preabsorption of the antiserum with 15 μ g/ml of GM.

Biochemical and immunological characteristics of GM extracted from granule cells and from rat brain synaptosomes

The amount of GM-like immunoreactive material present in the granule cells was determined by RIA using the MBPs-preabsorbed anti-GM antiserum, either on a crude granule cell extract or after partial purification through Sep-Pak cartridges (see Materials and Methods). In both cases, the concentration of GM-like immunoreactivity in granule cells was comparable. The concentration estimated after Sep-Pak purification was 1.2 ± 0.09 μ g/mg protein (average of 4 determinations). Identical aliquots of granule cell extract were assayed in parallel with an antibody raised against MBPs (a generous gift of Dr. G. Hashim, St. Luke's Hospital Center, New York, NY), which has a 10-fold specificity for MBPs with respect to GM and does not stain granule cells by immunocytochemistry (data not shown). The anti-MBPs antibody gave values below the detection level.

Table 4. Amino acid composition of GABA-modulin (GM) purified from primary cultures of cerebellar granule cells or rat brain synaptosomes

Amino acid residue	Granule cell GM (mol %)	Synaptosomal GM (mol %)
Asp	7.5	7.8
Thr	4.4	5.5
Ser	8.6	8.6
Glx	11.2	10.7
Pro	7.0	8.2
Gly	13.2	11.6
Ala	9.0	8.1
Cys	—	—
Val	5.5	4.2
Met	1.4	1.5
Ile	3.3	3.0
Leu	5.8	5.4
Tyr	1.9	1.6
Phe	3.1	3.7
Lys	7.4	8.3
His	3.2	3.7
Arg	7.9	7.6
Trp	—	—
Total	100.4	99.5

GM extracted from granule cells was purified as described in Materials and Methods; GM from rat brain synaptosomes was purified as described by Vaccarino et al. (1985). The values represent moles of amino acid per 100 mol of total amino acid recovered after 22 hr of hydrolysis. Trp was destroyed during the analysis. Data for granule cell-derived GM represent the average analyses of 2 different preparations; data for GM are the average of 10 preparations.

Figure 6. Fluorescence photomicrographs of cerebellar neuronal cultures enriched in granule cells, double-stained with GM (red) and with glutamic acid decarboxylase (GAD, green) antisera. In *A*, a double-stained/double-exposed field showing that the majority of the cells (granule cells) and fibers are stained red with the GM antiserum, while some fibers (arrows) are stained green with the GAD antiserum only. Note that when the structures containing the 2 antigens are superimposed, the color turns to yellow (short arrows). In *B*, a double-stained/double-exposed colchicine-treated culture showing several GM-positive cells (short arrow) and fibers, and 1 GAD-positive cell body (arrow) containing only a faint GM-staining. *C* and *D*, Double staining with single exposure: *C*, GAD staining in colchicine-treated culture demonstrating 1 GAD-positive cell body

Figure 7. GM immunostaining in rat cerebellum. *A*, Low-magnification view demonstrating that the immunoreactivity is localized mainly in granular layer ($\times 15$). *B*, High-magnification view showing that some Purkinje cells (arrow) and dendritic trees (arrowhead) are also stained ($\times 450$).

The identity between the immunoreactive material extracted from granule cells and authentic GM purified from rat brain synaptosomes was established in 3 parallel ways.

1. Immunologically. A crude acetic acid extract from granule cells yielded a single immunoreactive peak on reverse-phase HPLC (Fig. 8), and this peak exactly coeluted with purified GM from rat brain synaptosomes. RIA of different dilutions of the extract with the anti-GM antiserum yielded a displacement curve with a slope identical to that given by increasing concentrations of a GM standard (Fig. 3).

2. Biochemically. After purification to homogeneity (see Materials and Methods), the GM-like immunoreactive material extracted from granule cell cultures had an identical retention time on reverse-phase HPLC and a similar amino acid composition to synaptosomal GM (Table 4). Moreover, SDS-PAGE produced a single protein band comigrating with synaptosomal GM and corresponding to an apparent molecular weight of about 17,000 (Fig. 9).

3. Functionally. GM extracted and purified from granule cells as described in (2) inhibited the binding of ^3H -muscimol to Triton-X-100-treated rat brain membranes (Fig. 10) and to membranes prepared from granule cell cultures (data not shown) with a potency comparable to synaptosomal GM ($\text{IC}_{50} = 0.5 \mu\text{M}$). Preincubation of GM with the anti-GM antibody purified with protein A-Sepharose did not abolish the inhibitory activity, suggesting that the functionally active site of GM is different from the immunoreactive epitope.

Discussion

The main finding emerging from this study is that intact cerebellar granule cells in primary culture, which express GABA and BZ recognition sites on the cell surface, contain high amounts of GM, an endogenous basic polypeptide that modulates the number of GABA binding sites.

The pharmacological properties of ^3H -muscimol and ^3H -flunitrazepam binding sites measured on intact granule cell mono-

Figure 8. HPLC chromatography of crude granule cells extract. A crude acetic acid extract from granule cell cultures was directly applied on a HPLC system equipped with a μ -Bondapak reverse-phase column (see Materials and Methods). Each fraction (2 ml) was lyophilized and radioimmunoassayed. The retention time of authentic GM purified from rat brain synaptosomes is indicated by the arrow.

(arrow, n = nucleus); *D*, the same field double-stained with GM antiserum, showing that the GAD-positive cell body (arrow) is not stained with GM antiserum (short arrow indicates the same cell in *C* and *D*). Bar, 20 μm (all micrographs).

SDS-PAGE

Figure 9. SDS-PAGE of purified basic proteins. GM purified from rat brain synaptosomes (*Syn-GM*, 4 μ g) and small myelin basic protein from myelin (*SMBP*, 2 μ g), were analyzed by SDS/15% polyacrylamide gel. The gel was stained with silver nitrate. The molecular-weight markers (*Mr*) used were BSA (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α -lactalbumin (14,400). Proteins migrated towards the anode.

layers are similar to those previously found in brain membranes preparations (Tables 1 and 2); for ^3H -flunitrazepam, our data confirm previous results (Allen and Dutton, 1984). Moreover, GABA and BZ recognition sites reciprocally modulate each other in granule cell monolayers (Fig. 2), as already observed for brain membranes (Tallman et al., 1978; Costa and Guidotti, 1979).

Thus, the cerebellar granule cell preparation, both in terms of the homogeneity of the neuronal population and the virtual absence of GABA, provides a new and reliable model with which to correlate binding properties and functional parameters of the GABA/BZ/ Cl^- ionophore complex. In addition to GABA and BZ binding sites, granule cells express, in the presence of GABA, ^{35}S -TBPS binding sites, which are associated with the Cl^- channel (Gallo et al., 1985); these sites are probably located inside the Cl^- ionophore and become available for binding at the cell membrane surface only when GABA opens the Cl^- channels; the facilitatory action of GABA is potentiated by the benzodiazepines (Gallo et al., 1985).

Electrophysiologically, Cl^- currents evoked by GABA have

been measured in granule cell cultures under voltage-clamp conditions; moreover, BZ increase and beta-carbolines decrease GABA-evoked Cl^- conductance (Guidotti et al., 1985; Vicini et al., 1986).

Taken together, these data support the notion that, under assay conditions that preserve the integrity of the cell membrane and the microenvironment of the receptors, the components of the GABA/BZ/ Cl^- receptor-ionophore complex influence each other allosterically.

The allosteric regulation between the different recognition sites could involve additional modulators which are part of the supramolecular domain of the GABA receptor complex. Our data demonstrate that GM, a polypeptide with regulatory properties on ^3H -muscimol and ^{35}S -TBPS binding (Guidotti et al., 1982; Seifert and Casida, 1986) coexist with the GABA/BZ/ Cl^- receptor-ionophore complex in the granule cells. Moreover, we have demonstrated that the GM present in the granule cells is identical immunologically, biochemically, and functionally to GM purified from rat brain synaptosomes.

In cerebellar primary cultures, GM is selectively expressed

Figure 10. Inhibition of ^3H -muscimol binding by GM extracted from synaptosomes or cerebellar granule cells. Crude rat brain membranes, prepared according to Enna and Snyder (1977), were treated with Triton X-100 (0.05%) for 30 min at 37°C , washed 3 times in Tris buffer, and incubated with ^3H -muscimol for 5 min at $0-4^\circ\text{C}$ in the presence of different concentrations of GM. The concentration of membranes was 150–200 μg per assay, and the concentration of ^3H -muscimol was 10 nM.

by granule cells; in fact, GM is not detectable in glial or other contaminating cells (Fig. 4, *A–D*), and only a trace amount is visualized in the cell bodies or fibers of GAD-positive interneurons—presumably Golgi, basket, or stellate cells (Fig. 6, *A–D*). Interestingly, the pattern of immunostaining of the cultures by the GM antiserum was unchanged by colchicine pretreatment (Fig. 6, *B, D*). These data provide the first evidence that GM is selectively located in a population of neurons that functionally receive a strong GABA input and contain GABA receptors.

To address the question of whether the coexistence of GM with the GABA/BZ/ Cl^- ionophore–receptor complex observed in granule cells can be extended to other GABA-receptor-containing neurons, we performed immunocytochemical studies in rat cerebellar sections. This tissue was selected in order to visualize histochemically the density of GM-like immunoreactivity in granular, molecular, and Purkinje cell layers, in which the expression of GABA receptors has been extensively studied using monoclonal antibodies (Schoch et al., 1985) and autoradiography (Hosli et al., 1980; Schoch et al., 1985). The distribution of GM-like immunoreactivity in cerebellar sections is similar to the reported immunocytochemical localization of GABA receptors; interestingly, Purkinje cell bodies, which are known to be rich in GABA/BZ binding sites (Hosli et al., 1980), contain a high density of GM-like immunoreactivity.

The relatively high concentration of GM in granule cells (1.2 $\mu\text{g}/\text{mg}$ protein) should not be surprising in view of its proposed modulatory role on GABA receptors (Guidotti et al., 1982); in fact, G_i and G_o , 2 other regulatory proteins that bind GTP, account for more than 1.5% of the total membrane protein (Sternweis and Robishaw, 1984).

Given the known similarity in amino acid composition and peptide mapping between GM and SMBP (Vaccharino et al., 1985), we determined whether GM is the only basic protein present in granule cells, using antibodies specific for GM and/or MBPs. With RIA and immunocytochemistry, we detected GM, but not MBPs, in granule cells in primary culture. Thus, it seems that basic proteins in the brain comprise a family of

proteins localized in different cell types and with cell-specific functions. The ability to detect GM in neurons with a specific antiserum permits us to investigate the mechanism of its GABA binding inhibitory activity in detail. It is known that GM can be phosphorylated *in vitro* (Wise et al., 1983), and this phosphorylation abolishes its capacity to inhibit GABA binding. Studies utilizing granule cell cultures could investigate the physiological stimuli that trigger the phosphorylation of GM in the cells. A theoretical framework could be provided to explain the regulation of GABA receptor by GM and phenomena such as super- or desensitization of GABA receptors.

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