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Astroglia-Released Factor with Negative Allosteric Modulatory Properties at the $GABA_A$ Receptor

EVIDENCE FROM BINDING STUDIES

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ABSTRACT. We have previously shown, using whole-cell patch-clamp techniques, that astrocytes release a negative allosteric modulator of the γ-aminobutyric acid type A receptor ($GABA_A$ receptor) with β-carboline-like properties, thus, likely to act at the benzodiazepine site. Here, using patch-clamp and binding techniques, we confirm that the low-molecular-weight fraction of astroglia-conditioned medium (ACM lmf) contains a factor(s) that negatively modulates $GABA_A$-receptor function. This factor, like β-carbolines, enhances the specific binding of [^{35}S]*t*-butyl bicyclophosphorothionate (TBPS) to adult rat cortical membranes in the presence of GABA. However, it fails to interact with various ligands of the benzodiazepine (BZD) site of the $GABA_A$ receptor ([^{3}H]flunitrazepam, [^{3}H]Ro 15-1788 and [^{3}H]Ro 15-4513). The question of the actual binding site of the astroglia-derived factor on the $GABA_A$ receptor, thus, remains open and can be addressed only after the purification of the active molecule(s) of ACM lmf has been completed, and a labeled form of the endogenous ligand becomes available. Taken together, however, the data suggest that type 1 astrocytes are able to modulate the effects of the main inhibitory neurotransmission in the central nervous system. BIOCHEM PHARMACOL 52;3:465–473, 1996.

KEY WORDS. $GABA_A$ receptor; negative allosteric modulator; astroglia; binding; patch-clamp

The GABAergic system is ubiquitous in the CNS because 20 to 30% of the synapses use GABA† as a transmitter [1, 2]. Most of this inhibitory transmission is mediated by the A type of GABA receptor. This receptor is a macromolecular complex of 5 subunits forming an anion channel. An increasing number of subunits have been described and classified in families of α, β, γ, δ, and ρ subunits [3–5]. Various combinations of these subunits endow the receptor with different pharmacological profiles [6–9]. Many modulatory sites on the $GABA_A$ receptor have been reported for BZD, barbiturates, neurosteroids, and convulsants [2, 10–13]. These allosteric sites could allow a fine tuning of GABAergic neurotransmission, at least in theory, because little is known about the endogenous ligands of these modulatory sites.

In a previous work [14], we reported that astrocytes released a low-molecular-weight factor that specifically inhibits GABA-induced whole-cell currents recorded in cultured CNS neurons. As this astroglia-derived factor-induced inhibition of GABA-gated currents was blocked by Ro 15-1788, a specific BZD antagonist, and was mimicked by β-carbolines, a class of BZD inverse agonists, we suggested that astroglia releases a yet-unidentified endogenous negative allosteric modulator at the BZD site of the $GABA_A$ receptor.

The aim of the present study is: 1. to confirm these results using an alternative technique (i.e. a ligand binding competition assay), and 2. to establish, for purification purposes, a method for assaying and quantifying the astroglia-derived factor that is more suitable and less time-consuming than patch-clamp recordings.

MATERIAL AND METHODS

Cell cultures

CEREBELLAR GRANULE CELL CULTURE. Rat cerebellar granule cells were obtained from 7-day-old rat pups using methods fully described previously [14, 15]. Briefly, cerebella were carefully dissected and freed of meninges. They were, then, cut into small fragments that were incubated in trypsin (0.25% in Ca^{2+} – Mg^{2+} – free salt solution) for 20 min at 37°C. They were, then, washed with high K^+-Minimum Essential Medium (MEM; GIBCO, Ghent, Belgium), where the K^+ concentration was increased to 25 mM

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† *Abbreviations:* BZD, benzodiazepine; DMCM, methyl 6,7-dimethyloxy-4-ethyl-β-carboline-3-carboxylate; FG 7142, *n*-methyl-β-carboline-3-carboxamide; GABA, γ-aminobutyric acid; GFAP, glial fibrillary acidic protein; TBPS, *t*-butyl bicyclophosphorothionate.

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(a concentration that allows long-term survival of cerebellar granule neurons [16]) and the Na^+ concentration decreased in equimolar amounts, supplemented with glucose (final concentration of 6 g/L), pyruvate (1 mM), bovine insulin (Sigma, St. Louis, MO, U.S.A.; 5 μg/mL) and Horse Serum (HS; Gibco; 10% v/v). Dissociation was obtained by up and down aspirations using flame-narrowed Pasteur pipettes (approximately 1 to 0.2 mm diameter). The resulting cell suspension was filtered through a 15 μm nylon sieve, cells were counted using a hematocytometer and diluted to a cell concentration of 2.5×10^6 cells per mL. Fifty microliters of this cell suspension were seeded in poly-ornithine (0.1 mg/mL in distilled water)-coated glass coverslips in 35 mm-diameter plastic Petri dishes (NUNC, Roskilde, Denmark) for patch-clamp experiments, and in poly-ornithine-coated 100 mm-diameter plastic Petri dishes (NUNC) for binding assays. Previous immunocytochemical studies have shown that more than 90% of the cells in these cultures are neurons [15]. The medium was renewed twice weekly and cells were used after 10–15 days *in vitro*.

ASTROGLIA CULTURE AND MEDIUM CONDITIONING. Astrocytes were isolated from newborn rat cerebral cortex [17]. Cerebral hemispheres were dissected, carefully freed of meninges, and dissociated by sieving through a nylon mesh (pore size = 225 μm). Cells were collected in MEM supplemented with 10% (v/v) FCS, bovine insulin (5 μg/mL), and glucose (6 g/L, final concentration) and filtered through a second nylon mesh (pore size = 25 μm). The cell suspension obtained from one animal was seeded into one plastic Petri dish (100 mm diameter). More than 93% of the cells in these cultures were immunopositive for glial fibrillary acidic protein [15], and less than 5% of the cells were immunopositive for A2B5. Hence, the preparation used was almost completely devoid of type 2 astrocytes. Cultures were kept at 37°C in a 95% air/5% CO_2 humidified atmosphere. The medium was renewed after 1 and 4 days.

For conditioning, the medium was discarded after 7 days of cultivation and cultures were incubated at 37°C in 7 mL of standard solution (see next section) supplemented with leucine (4×10^{-4} M). After 24 hr of conditioning, the medium was collected, centrifuged (3500 *g* for 15 min), ultrafiltrated in 2 steps, first through PM10 (cuff off = 10 kDa) and second through YM2 (cut off = 1 kDa) Amicon membranes. This low-molecular-weight fraction of astroglia-conditioned medium (ACM lmf) was kept frozen at −20°C until use.

Electrophysiology

SOLUTIONS, ELECTRODES AND DRUGS. For recording of membrane currents, cells were transferred to the stage of an inverted phase contrast microscope and maintained at room temperature (20–25°C) in a recording chamber that was continuously perfused, permitting application of drugs or conditioned media.

The standard solution contained (mM):NaCl 116.0; glucose 11.1; $NaHCO_3$ 26.2; KCl 5.4 and $CaCl_2.2H_2O$ 1.8 and was buffered at pH 7.2 with 5 mM HEPES. GABA was applied by iontophoresis (0.5 M, pH 3.2 in distilled water, 10 nA positive ejection current for 200 msec). FG 7142 and ACM lmf were applied by perfusion into the chamber.

Recording and iontophoresis pipettes were made from borosilicate capillaries (Hilgenberg, Malsfeld, Germany) with resistances ranging from 2 to 8 MΩ for recording pipettes and from 150 to 250 MΩ for iontophoresis pipettes using a BB-CH-PC microelectrode puller (Mecanex, Nyon, Switzerland). Recording pipettes were filled with a solution containing (mM): KCl 130.0; $CaCl_2.2H_2O$ 1.0; EGTA 10.0 and $(Na)_2$- or Mg-ATP 5.0 and was buffered at pH 7.4 with 10 mM HEPES.

DATA RECORDING AND PROCESSING. Voltage-clamp recordings were performed with a BioLogic (Claix, France) RK300 patch-clamp amplifier using the tight-seal whole-cell recording configuration [18]. Current traces were digitized and stored on an AT-compatible computer system. Data acquisition was controlled and data analysis performed using the CED1401 acquisition board and softwares (Cambridge, U.K.).

Binding Assay

MEMBRANE PREPARATION. All procedures for preparing membranes were carried out at 0–3°C.

Cortices from adult rats and cerebella from adult or 7-day-old rats were dissected. One gram of fresh or frozen (−70°C) tissue was homogenized in 50 mL of Tris HCl 50 mM buffer pH 7.3 with an Ultra-Turrax homogenizer for 45–60 sec. The homogenate was centrifuged for 10 min at 50,000 *g*. The supernatant was discarded, and the pellet resuspended by rehomogenization in 50 volumes buffer/g original tissue weight.

In the case of the *crude membrane preparation*, the resuspended pellet preparation was centrifuged for 10 min at 50,000 *g*. The resulting pellet was homogenized in 5 volumes buffer/g original tissue weight. Determination of protein content was, then, performed according to the method described by Bradford [19]. The homogenate was kept frozen at −70°C until use.

In the case of the *washed membrane preparation*, the initial resuspended pellet preparation was incubated in the buffer for 30 min at 4°C and, then, centrifuged for 10 min at 50,000 *g*. The pellet was washed twice with homogenization, as before, and centrifuged for 10 min at 50,000 *g*. The resulting pellet was resuspended in 50 volumes buffer/g original tissue weight and kept frozen at −20°C overnight. At that stage, determination of protein concentration was performed. The suspension was, then, quickly thawed and, after centrifugation for 10 min at 50,000 *g*, 2 additional washes of the pellet were performed as described above. The final pellet was resuspended in the appropriate volume of binding buffer (see below and Table 1).

In the case of *membranes prepared from cerebellar granule cell cultures*, the cultures were rinsed twice with 5 mL PBS.

TABLE 1. Binding assays: radiolabeled and cold ligands, membrane preparations, and incubation conditions

Total binding		Membrane preparation (protein concentration; mg/mL)	Incubation conditions				Nonspecific binding	
Radiolabeled ligand [specific activity (Ci/mmol)]	Final concentration (nM)		Buffer†	Volume (mL)	Time (min)	Temperature (°C)	Ligand	Final concentration (μM)
[^{3}H]flunitrazepam (NEN) [87]	1.5	crude (0.1)	#1	1	60	4	diazepam	10
[^{3}H]Ro 15-1788 (NEN) [76.8]	2.5	crude (0.1)	#1	1	60	4	diazepam	10
[^{3}H]Ro 15-4513 (NEN) [24]	2	crude (0.1)	#1	1	60	4	Ro 15-1788 diazepam*	10 10
[^{3}H]muscimol (Amersham) [12]	3	washed (≈0.5)	#2	2	30	4	GABA	1000
[^{35}S]TBPS (NEN) [157.7]	2	washed (≈0.5)	#2	2	45	21	picrotoxin	100

* Total binding is determined using Ro 15-1788, which binds both DS and DI sites; diazepam is used to determine DI binding, DS binding being the difference between total binding and DI binding.

† Buffer #1 = Tris HCl 50 mM; pH 7.4; Buffer #2 = Tris HCl 50 mM, NaCl 120 mM, KCl 5 mM, $CaCl_2$ $2H_2O$ 2 nM and $MgCl_2$ $6H_2O$; pH 7.3

They were, then, scraped and the resulting cell suspension was centrifuged for 10 min at 2000 *g*. The pellet was resuspended in 50 mM Tris HCl pH 7.3 buffer (1 mL for 5 100 mm-diameter Petri dishes) and treated as dissected tissues.

BINDING ASSAYS. All experimental points were done in triplicate. Table 1 gives, for each ligand tested, the specific activity and final concentration of the ligand, the membrane preparation employed (crude or washed), the incubation buffer, volume, temperature and time, and the ligands used for determination of nonspecific binding.

After addition of the radiolabeled ligand, the membrane suspension, and the tested products to the incubation tube, the samples were thoroughly mixed and incubated at the appropriate temperature for a fixed time. After incubation, the samples were poured directly onto Whatman GF/B glass fiber filters with vacuum applied and immediately washed with 3 × 4 mL buffer. Radioactivity on the filters was determined by conventional liquid scintillation counting using a Beckman LS3801 counter.

Chromatographic Procedures

SEPHADEX LH20 CHROMATOGRAPHY. Ultrafiltered astrocyte-conditioned medium was fractioned on a Sephadex LH20 gel in the presence of methanol. ACM lmf (20 mL) were applied to a Sephadex LH20-containing column (2.5 × 80 cm) that had been pre-equilibrated with 25% methanol in water. [^{3}H]GABA (NEN) at 27 pM was added to ACM lmf as an external standard. The absorbance of the eluate was monitored at 226 nm. After elution of the void volume, 40 fractions of 2 mL each were collected. Aliquots of each fraction (0.2 mL) were directly counted for their [^{3}H]GABA content. The remaining 1.8 mL were dried in a SpeedVac concentrator (Savant), reconstituted in 0.6 mL of binding buffer (see Table 1) and assayed for modulatory activity of [^{3}H]muscimol binding, as described.

DANSYLATION AND HPLC PROCEDURES. ACM lmf (100 μL, 10-fold concentrated after Sephadex LH20 chromatography) were added to 10 μL $LiCO_3$ (80 mM, pH 8.5) before adding 10 μL of dansyl chloride (1.5 mg/mL in acetonitrile). This mixture was incubated for 35 min at 25°C in the dark. The reaction was stopped by adding 10 μL of ethylamine 2%. A RoSiL C18 HE (5 μm, 150 × 4.6 mm, Bio-Rad) column heated to 50°C was equilibrated in 85% of solvent A (tetrahydrofurane 3%, acetic acid 0.57%, and triethylamine 0.088% in distilled water) and 15% of solvent B (tetrahydrofurane 3%, acetic acid 0.57%, triethylamine 0.088%, and methanol 70% in water). After injection of 20 μL of the sample, amino acids were eluted by means of a linear gradient (flow rate of 1.5 mL/min), the percentage of solvent B being increased to reach 100% after 40 min. Initial conditions were restored after 2 min. The next sample was injected after a 5-min re-equilibration period. Fluorescence was measured with a Perkin-Elmer LS-4 spectrometer set at 334 nm for excitation and at 522 nm for emission. Aqueous solutions of amino acids (0.1 mM) were used as standards and processed as the samples.

Reagents

GABA, bicucullin methiodide, and taurine were from Sigma. Flurazepam, diazepam, and flumazenil (Ro 15-1788) were from Hoffmann-LaRoche (Basel, Switzerland). DMCM and FG 7142 were from RBI (Natick, MA, U.S.A.). All other products utilized were of analytical grade.

RESULTS

ACM lmf Does Not Interact With the BZD Site of the $GABA_A$ Receptor

We have previously shown that ACM lmf-induced inhibition of GABA-gated currents recorded in whole-cell patch-clamped cerebellar granule neurons in culture is reversibly

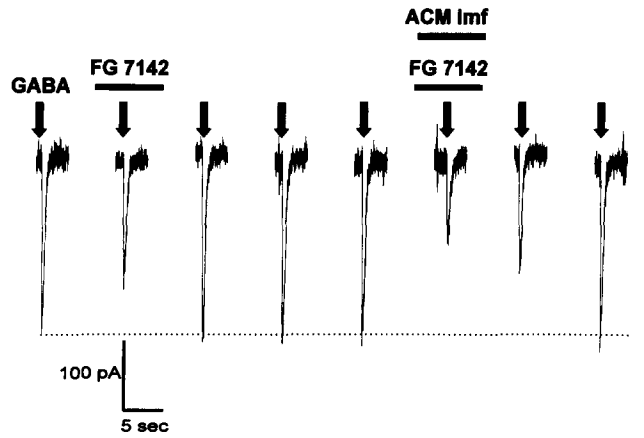

FIG. 1. Additive effects of FG 7142 and ACM lmf on the GABA-induced current of cerebellar granule cells in primary cultures. GABA (arrows) was applied by iontophoresis during whole-cell recording from a granule cell. The GABA responses were compared in the presence of 10^{-4} M FG 7142 alone or together with ACM lmf applied to the bathing perfusion (bars; 15 sec application, of which 12 sec was before triggering GABA iontophoresis). The recordings were obtained from a single representative cell and separated by 60 sec. The horizontal dashed line represents the level of the GABA response under control conditions (test current). Similar recordings were obtained from 6 different cells (see text).

blocked by Ro 15-1788 (5 μM), a specific antagonist of the BZD site of the $GABA_A$ receptor, and is mimicked by the β-carbolines [14]. From these experiments, we concluded that ACM lmf behaves like a negative allosteric modulator (inverse agonist) at the BZD site of the $GABA_A$ receptor. If this conclusion is valid, ACM lmf should be expected: 1. not to induce an additional inhibition of GABA-gated current in neurons treated with a saturating concentration of a β-carboline; and 2. to compete with the binding of radiolabeled ligands of the BZD site of the $GABA_A$ receptor.

Figure 1 shows the membrane inward currents evoked by iontophoretically applied GABA in a representative cerebellar granule neuron in culture that was voltage-clamped at −70 mV. As can be seen, the inhibition of GABA-evoked currents induced by application of FG 7142 together with ACM lmf was larger than that induced by 10^{-4} M FG 7142 alone (51% and 76% of the test current, respectively). This concentration of β-carboline was 100-fold greater than that that totally inhibited [^{3}H]flunitrazepam binding (see below) and 10-fold greater than the concentration above which the inhibitory effect of FG 7142 on GABA-induced currents was maximum. Using the same set of cultures and the same batch of ACM, measured mean currents (in percentage of test currents ± SEM, $n = 6$) were 56.8 ± 4.4% for ACM, 70.8 ± 4.6% for FG 7142, and 41.1 ± 5.5% for ACM together with FG 7142. The last value was not different ($P > 0.05$; ANOVA) from calculated additive effects (40.2%). These observed additive inhibitions of FG 7142 and ACM lmf on GABA-induced currents argue against the existence of a common site for both modulators.

We, then, attempted to determine an interaction between ACM lmf and the BZD site of the $GABA_A$ receptor using classical binding competition techniques. On an unwashed membrane suspension obtained from adult rat cortices, FG 7142 and flurazepam (positive allosteric modulator or agonist at the BZD site of the $GABA_A$ receptor) inhibited the specific binding of 1.5 nM [^{3}H]flunitrazepam in a dose-dependent fashion (Fig. 2, left panel; IC_{50} for FG 7142 and flurazepam: 4.5×10^{-7} M and 1.0×10^{-8} M, respectively. In contrast, ACM lmf, up to a concentration of 60% of the incubation volume, had no effect on [^{3}H]flunitrazepam binding (however, see next section concerning

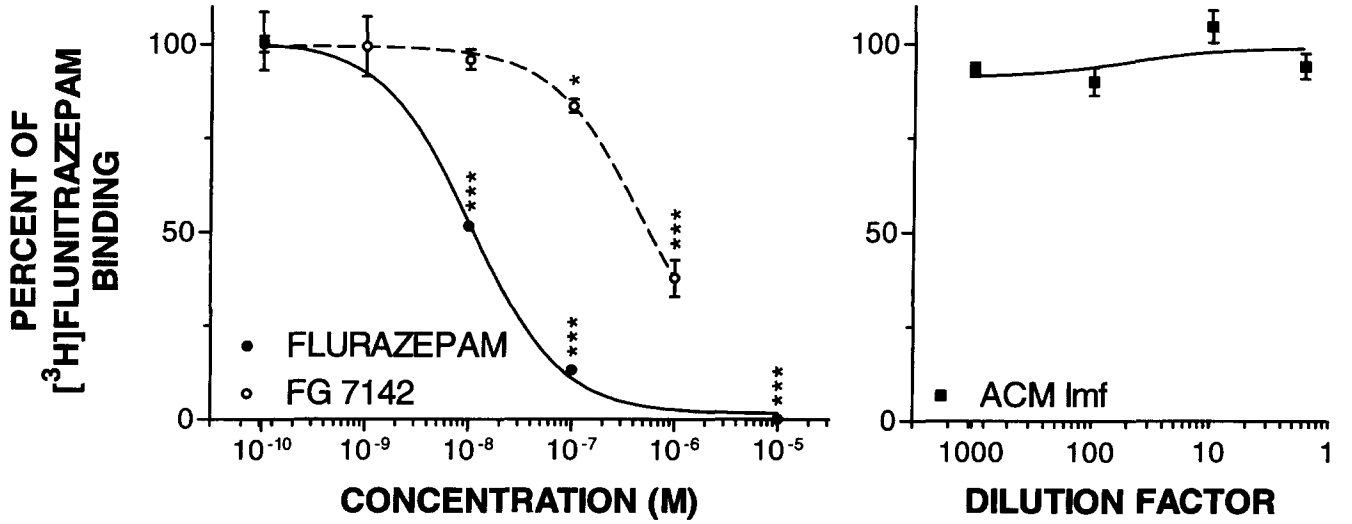

FIG. 2. Effects of increasing concentrations of flurazepam (●), FG 7142 (○) [left panel], and ACM lmf (■) [right panel] on [^{3}H]flunitrazepam (1.5 nM) specific binding to cortical membranes from adult rats. Results (expressed in percent of [^{3}H]flunitrazepam specific binding in control buffer) are mean and standard deviation ($n = 3$) for each condition. The same results were obtained in at least 2 separate experiments. ANOVA variance analysis: *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$.

TABLE 2. Effects of DMCM, diazepam, and ACM lmf on [^{3}H]Ro 15-1788 (2.5 nM) specific binding to membranes prepared from different brain structures

Structure	K_i (nM) DMCM	K_i (nM) Diazepam	Effect of 1/10 ACM lmf (% of specific binding ± SD)
Cortex (adult)	13.7	8.2	97.8 ± 2.9
Cerebellum (adult)	12.9	6.8	104.0 ± 1.7
Cerebellum (7-day-old)	ND	13.6	96.3 ± 4.4
Cultured cerebellar granule neurons (7 days *in vitro*)	6.8	10.0	96.8 ± 0.8

The effect of ACM lmf (1:10 dilution) on [^{3}H]Ro 15-1788 specific binding is expressed as percentage of binding in control buffer. Results are the mean of 2–3 separate experiments, each consisting of triplicate conditions of different concentrations of drugs.

TABLE 3. Effect of ACM lmf on [^{3}H]Ro 15-4513 (2 nM) specific binding to diazepam-insensitive (DI) sites of membranes prepared from rat cerebella

Structure	Percent of DI sites	Effect of 1/10 ACM lmf (% of DI specific binding)
Cerebellum (adult)	26.0	105.0 ± 4
Cerebellar granule neurons (culture)	31.5	102.2 ± 8

Percent of DI sites corresponds to the ratio of [^{3}H]Ro 15-4513 specific binding in the presence of 10 μM diazepam to the same binding in control buffer. The effect of ACM lmf (1:10 dilution) is expressed as percentage of binding in control buffer containing 10 μM diazepam. Results are the mean of 2–3 separate experiments, each consisting of triplicate conditions.

the effect on the binding of washed preparations). The same concentration of that batch of ACM lmf inhibited 40% of the GABA-induced current in patch-clamp experiments. Similar results were obtained using [^{3}H]Ro 15-1788 as the radioligand (not shown). It must be pointed out that there are differences in the biological material used in binding studies compared to that employed in patch-clamp studies (adult vs developing; cortex vs cerebellum; *ex vivo* vs *in vitro*). At the level of the $GABA_A$ receptor, this can mean qualitative as well as quantitative differences in the expression of the different subunits of the receptor [20–23], which could, in turn, modify the affinity of BZD ligands for their binding site and, hence, of the putative endogenous modulator present in ACM lmf. Therefore, we tested the interaction between ACM lmf and the specific binding of 2.5 nM [^{3}H]Ro 15-1788 on different unwashed membrane preparations, obtained from adult and 7-day old rat cerebella or from cerebellar granule cell cultures. Data are summarized in Table 2: no effect of ACM lmf on [^{3}H]Ro 15-1788 binding could be observed with any of these membrane preparations.

Because the cerebellum is enriched in a subpopulation of $GABA_A$ receptors that are insensitive to diazepam (DI sites) [24–26], we also measured the interaction between ACM lmf and the specific binding of 2 nM [^{3}H]Ro 15-4513 in the presence of 10 μM diazepam on unwashed membranes prepared from adult rat cerebella and from cerebellar granule cell cultures (Table 3). These results demonstrate that ACM lmf does not interfere with the binding of [^{3}H]Ro 15-4513 to the DS and DI BZD sites of the cerebellum.

ACM lmf Interacts With the GABA Site of the $GABA_A$ Receptor. This interaction can be attributed to GABA released by astrocytes.

Because the negative allosteric properties of ACM lmf at the $GABA_A$ receptor could not be attributed to an interaction with the BZD site, we decided to test other binding sites of the receptor, including the GABA site itself.

Figure 3 (left panel) shows the dose-dependent effects of an agonist (GABA; $IC_{50} = 6 \times 10^{-8}$ M), a partial agonist (taurine; $IC_{50} = 6.8 \times 10^{-5}$ M), and an antagonist (bicuculline, BCC; $IC_{50} = 4 \times 10^{-6}$ M) on the specific binding of 3 nM [^{3}H]muscimol to washed membranes prepared from adult rat cortices. Under the same conditions, ACM lmf, at concentrations as low as 1% of the incubation volume, also inhibited [^{3}H]muscimol binding. The inhibiting concentration of ACM lmf, however, varied from batch to batch and was usually observed at 10% of the incubation volume.

Because astrocytes are known to release GABA into their surrounding medium [27, 28], the possibility that GABA, itself, could be responsible for ACM lmf-induced inhibition of [^{3}H]muscimol binding cannot be rule out. In such a case, expected concentrations of GABA in ACM lmf (calculated from comparisons between dose-response curves of both GABA and ACM lmf) should range from 0.1 to 1×10^{-6} M. To test this hypothesis, we performed several sets of experiments, 2 of which are illustrated in Fig. 4. In a first set of experiments, ACM lmf was chromatographed on a column containing Sephadex LH20 and the effects of the resulting fractions on the specific binding of [^{3}H]muscimol were tested (Fig. 4, upper panel). Because [^{3}H]GABA was added to ACM lmf at a concentration of 27 pM before chromatography, direct counting of the [^{3}H]GABA content of the fractions was, thereby, possible. A perfect match between the elution profiles of the ACM lmf inhibitory activity of [^{3}H]muscimol binding and [^{3}H]GABA is clearly apparent.

HPLC chromatography on C18-derived phase after dansylation is a semiquantitative routine procedure to detect amine groups and, hence, amino acids such as GABA. In a second set of experiments, a standard solution containing 10^{-6} M GABA was processed in parallel with an ACM preparation (10-fold concentrated LH20 peak of [^{3}H]muscimol binding inhibitory activity). A peak of dansylated product(s) can be seen on the elution profile of the ACM preparation at the same position as GABA (Fig. 4, lower

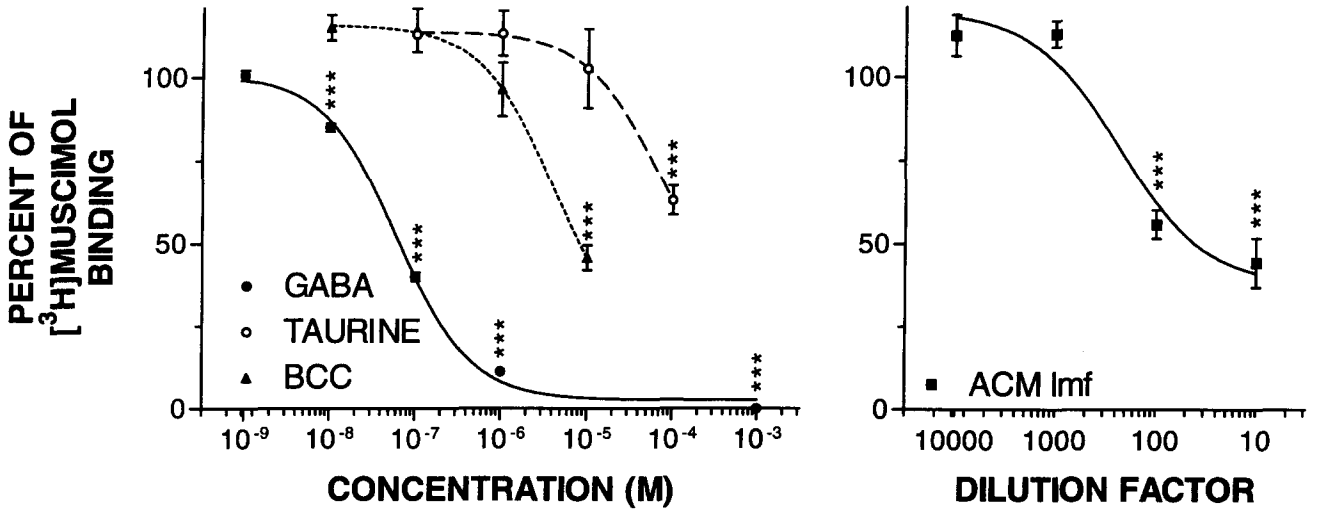

FIG. 3. Effects of increasing concentrations of GABA (●), taurine (○), bicucullin (BCC; △) [left panel], and ACM lmf (■) [right panel] on [^{3}H]muscimol (3 nM) specific binding to cortical membranes from adult rats. Results are expressed as in Fig. 2.

panel). The estimated concentration of GABA in ACM lmf based on HPLC analysis was ~0.2–0.4 μM, which fits the estimated concentration based on [^{3}H]muscimol binding inhibitory activity (~0.5–1.5 μM).

Two other sets of experimental results confirm that GABA is the factor responsible for the [^{3}H]muscimol binding inhibitory activity of ACM lmf; 1. similar enhancement of [^{3}H]flunitrazepam binding to washed membranes (i.e. devoid of endogenous GABA) induced by GABA and ACM lmf (estimated GABA concentration based upon this binding assay is ~0.2 μM), and 2. absence of extraction by isobutanol of both the GABA and ACM lmf inhibitory activity of [^{3}H]muscimol binding as opposed to the persistence of ACM lmf-induced inhibition of GABA-gated whole-cell currents in the isobutanol extract (data not shown).

In the Presence of 5 μM GABA, ACM lmf Potentiates [^{35}S]TBPS Binding and, Thus, Behaves Like a Negative Modulator of the GABA$_A$ Receptor

To confirm the negative modulatory property of ACM lmf on the GABA$_A$ receptor function, we, next, measured its effect on the binding of 2 nM [^{35}S]TBPS to washed membranes prepared from adult rat cortices. TBPS binds to the so-called convulsant site of the GABA$_A$ receptor, which is close to the chloride channel. This binding assay has been widely used to test and classify modulators of the GABA$_A$ receptor, especially benzodiazepines [29, 30]. Because the binding kinetic of [^{35}S]TBPS is slow (equilibrium reached after 2–3 hours), the assay is usually performed under non-equilibrium conditions and, hence, potentiating and inhibiting effects should be understood as acceleration or slowing down of the association of [^{35}S]TBPS to its site, without implicating an effect on maximum binding.

Because we demonstrated in the previous section that GABA was present in ACM lmf, we performed the binding assay of [^{35}S]TBPS in the presence of 5×10^{-6} M GABA (i.e. a concentration at least 5 times greater than the suspected concentration of GABA in ACM lmf). Under such conditions, flurazepam, a positive allosteric modulator at the BZD site, decreased or slowed down the binding of [^{35}S]TBPS ($EC_{50} = 1.3 \times 10^{-8}$ M), and FG 7142, a negative modulator at the same site, enhanced or accelerated it ($EC_{50} = 1.3 \times 10^{-7}$ M) (Fig. 5, left panel). In the same experiment, ACM lmf also enhanced [^{35}S]TBPS binding (EC_{50} = 1.8% of incubation volume) (Fig. 5, right panel) and, thus, behaves as a prototypic negative allosteric modulator of the GABA$_A$ receptor.

DISCUSSION

In this paper, we confirm that cultured astrocytes release a negative allosteric modulator of the GABA$_A$ receptor. Indeed, ACM lmf enhances the specific binding of [^{35}S]TBPS in the presence of GABA, behaving in this assay like β-carbolines, and in an opposite manner to positive allosteric modulators such as flurazepam. However, this negative modulatory activity of ACM lmf on GABA$_A$-receptor function cannot, as for β-carbolines, be attributed to a direct interaction of the astroglia-derived factor with the BZD site because ACM lmf does not displace various ligands specific to that site. Finally, we have demonstrated that the ACM lmf-induced inhibition of [^{3}H]muscimol binding is a consequence of an astrocytic release of GABA.

This release of GABA by astrocytes raised the question of whether or not GABA, itself, could be responsible for the observed effects of ACM lmf. Indeed, prolonged exposure of neurons to GABA could lead to desensitization of their receptors [31, 32] being interpreted as an inhibition. However, this possibility can be rule out because a perfusion

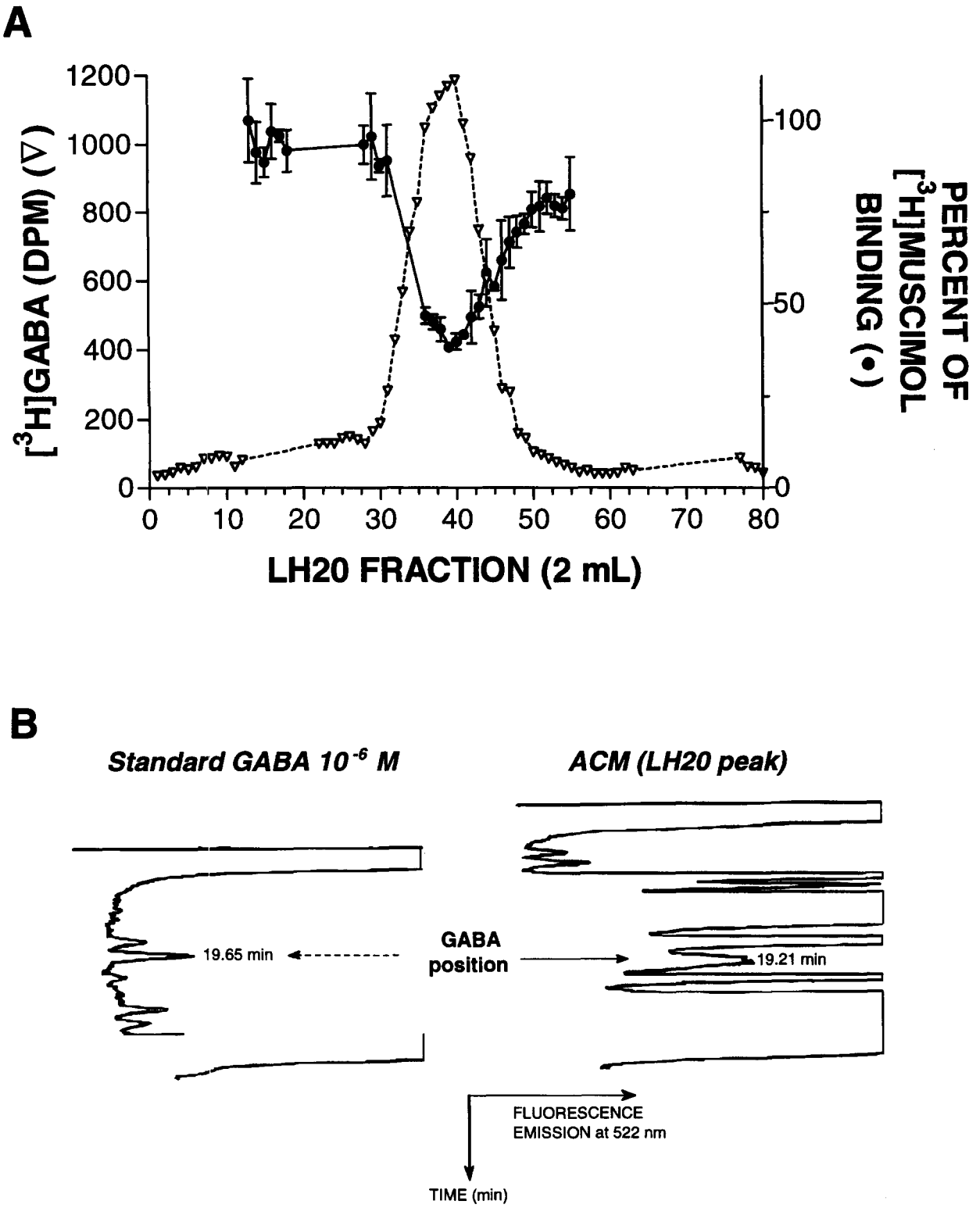

FIG. 4. Two lines of evidence for the presence of GABA in ACM lmf. (A): ACM lmf was chromatographed on Sephadex LH20 in the presence of 27 pM [^{3}H]GABA. Each 2-mL LH20 fraction was treated as follows: 5 mL of scintillation liquid was added to 0.2 mL of a fraction and counted for its [^{3}H]GABA content (△), and the remaining 1.8 mL were dried and, then, reconstituted in enriched Tris buffer (1:3 final dilution as compared to original ACM lmf) to measure its inhibition of [^{3}H]muscimol (3 nM) specific binding to cortical membranes from adult rats. Results of binding are expressed as in Fig. 2. (B): HPLC chromatography profiles after dansylation (see Material and Methods) of a 10^{-6} M GABA solution [left] and of ACM [right]. ACM corresponds to those LH20 fractions that inhibit [^{3}H]muscimol specific binding. Active fractions were dried and reconstituted in appropriate buffer (10:1 final concentration compared to original ACM lmf). Fluorescence emission scales are identical for GABA and ACM (LH20 peak).

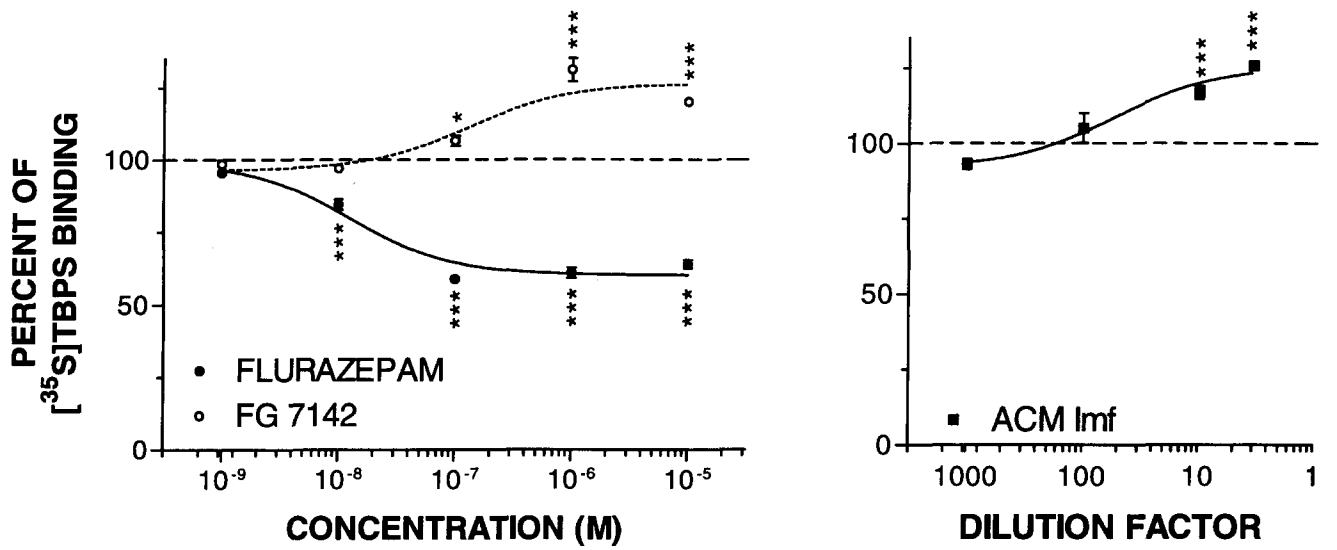

FIG. 5. Effects of increasing concentrations of flurazepam (●), FG 7142 (○) [left panel], and ACM lmf (■) [right panel] on [^{35}S]TBPS (2 nM) specific binding to cortical membranes of adult rats. [^{35}S]TBPS binding was measured under nonequilibrium conditions and in the presence of 5 μM GABA (see text). Results are expressed as in Fig. 2.

of 0.5×10^{-6} M GABA only marginally (5 to 10%) reduces currents activated by iontophoretically applied GABA. A more conclusive damning argument lies in the measured ACM lmf-induced enhancement of [^{35}S]TBPS binding done in the presence of 5×10^{-6} M GABA.

Because ACM lmf inhibits GABA-gated currents in a β-carboline-like fashion, and because this effect can be reversed by Ro 15-1788, the absence of interaction between ACM lmf and BZD specific binding is very surprising. To explain such an apparent discrepancy, two hypotheses can be put forth. First, ACM lmf could interact with the BZD site, but the experimental conditions used (incubation, temperature, etc.) did not permit this interaction to be observed. It is known that the binding of nonBZD ligands to the BZD site has its maximum efficacy at a higher temperature (approximately 20°C) than that commonly used (4°C) for BZD ligands [33, 34]. Moreover, the effect of some modulators of BZD binding, such as the effect of taurine, is only seen at room temperature [35]. This seems unlikely, however, because the specific binding of [^{3}H]flunitrazepam measured at room temperature is not displaced by ACM lmf (data not shown). According to the second, more speculative, hypothesis, ACM lmf would bind to a site different from the "classical" BZD site, one that could be either closely associated with or completely unrelated to the BZD site. Some authors have, indeed, reported the existence of a low-affinity site for BZD on the $GABA_A$ receptor [36] that could be an alternative site for β-carbolines, thereby accounting for the biphasic effects of some of these β-carbolines on the GABA-gated currents recorded from cells transfected with various subunit combinations [37]. Another site, one that binds Ro 5-4864, a ligand of the so-called "peripheral" BZD receptor, has also been reported [2, 38, 39]. It is important to point out that these two sites on the $GABA_A$ receptor are relatively poorly documented and that their existence, itself, remains, at present, a controversial issue. If, indeed, ACM lmf were to interact with one of these poorly defined binding sites, the reversal by Ro 15-1788 of ACM lmf-induced inhibition of GABA-gated currents could be explained by the partial agonist properties that have been reported under certain conditions [40].

We, therefore, believe that the molecular characterization of the factor present in ACM lmf that inhibits the GABA-gated currents is a prerequisite to a better understanding of its mechanism and site of action and of its (patho)physiological meaning.

The data reported here, and in the previous paper, were obtained on cultured astroglia and extrapolation from cultured cells to their normal *in vivo* counterparts must be done with caution. However, our data suggest that GFAP-positive, A2B5-negative type 1 astrocytes are able to modulate the main inhibitory transmission system in the CNS, further pointing out the fine tuning of GABAergic transmission. How and under which conditions that regulation is recruited is an important issue that can only be addressed after the factor(s) present in ACM lmf have been characterized at the molecular level.

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