Biochemical Pharmacology, Vol. 32, No. 6, pp. 1093–1096, 1983.
Printed in Great Britain.

0006-2952/83/061093-04 $03.00/0

4-AMINOBUTYRIC ACID METHYL ESTER HYDROCHLORIDE, A PRECURSOR OF 4-AMINOBUTYRIC ACID

MARIA BIANCHI, RENZO DEANA, GIUSEPPE QUADRO, GILLES MOURIER and LAURO GALZIGNA*

Institute of Biological Chemistry, University of Padova, MEDEA Researches, Milano, Italy; and Centre de Recherches INSERM, rue de Navacelles 60, Montpellier, France

(*Received* 18 *June* 1982; *accepted* 25 *October* 1982)

Abstract—4-Aminobutyric methyl ester hydrochloride (GME) is able to cross the blood–brain barrier after intracardiac administration to the rat. GME has an LD_{50} of 1300 mg/kg in mice and 950 mg/kg in rats, exhibits an antiaggressive effect and is able to decrease isoniazid-induced convulsions in the rat. GME is hydrolyzed to 4-aminobutyric acid (GABA) by brain homogenates, acts as an inhibitor of GABA binding to crude synaptic plasma membranes, activates the release and inhibits the uptake of GABA by rat synaptosomes and acts as a competitive inhibitor of the so-called GABAse system *in vitro*.

A role for central 4-aminobutyric acid (GABA)† in neurological conditions has been advocated [1], but GABA cannot be used to control clinical states related to abnormal cortical excitation because it is unable to cross the blood–brain barrier.

On the contrary, different drugs with affinity for GABA receptors and capable of overcoming the blood–brain barrier can be used as anticonvulsants. These are either heterocyclic GABA analogues [2] or simple hydrophobic GABA derivatives [3] acting as inhibitors of GABA binding to crude synaptic membranes [4].

Although doubts have been cast upon the use of simple GABA derivatives as GABA mimetics [1], the use of GABA-like molecules with no conformational restriction acting as GABA precursors can be a way of implementing the recently advocated soft-drug approach [5].

The mechanism of action of such GABA precursors in controlling experimental conditions of cerebral GABA deficiency is related to their power to be converted back to GABA after crossing the blood–brain barrier.

A simple hydrophobic GABA derivative is its methyl ester hydrochloride (GME) which proved capable of protecting experimental animals against electroshock convulsions [6].

The present paper illustrates the biotransformation of GME together with its action on isolated synaptosomes and inhibition of the purified GABAse system *in vitro*. The crossing of the blood–brain barrier, the measurement of acute toxicity, antiaggressive effect and anticonvulsant action on isoniazid-induced convulsions are also described.

MATERIAL AND METHODS

Preparation and properties of GME. The chemical synthesis of GME was carried out according to the procedure described by Spath *et al.* [7] by using GABA (BDH, Poole, U.K.) and acid methanol (Merck, Darmstadt, F.R.G.) as starting reagents.

The product obtained is a white hygroscopic powder, soluble in water and ethanol, fairly soluble in ethylene and propylene glycol, and insoluble in chloroform. The compound is stable and remains chromatographically pure when stored at 4° as a dried powder. Its m.p. is 118–121°.

Acute toxicity. Twenty Swiss male mice (20 g) and 20 Wistar male rats (150 g) intraperitoneally injected with solutions of GME in saline were used for toxicity tests.

Behavioural effects. Groups of 20 Wistar male rats (150 g) were used to evaluate the effect of GME on the righting, auricular and grasping reflexes, muscular tone and sedation. The antiaggressive action was evaluated by scoring the fighting behaviour after electrical stimulations (50 msec, 0.6 A) with a Li-12100 Letica Stimulator. Doses of 500 and 750 mg/kg GME were intraperitoneally injected and the effects were observed after time intervals ranging from 10 min to 48 hr.

Anticonvulsant effect. Randomized groups of 16 Sprague–Dawley male rats (130 g) were subcutaneously injected with 160 mg/kg isoniazid (Fluka, Buchs, Switzerland). Solutions of 100 mg/kg GME in saline were injected through the caudal vein (i.v.) 10 min before isoniazid and the types of convulsion, their latency and the survival of rats were observed.

Biotransformation in brain and blood after in vivo *administration.* Twenty-one Wistar male rats (180 g)

* Please address all correspondence to: L. Galzigna, Professor of Biochemistry, Institute of Biological Chemistry, University of Padova, via Marzolo 3, 35100 Padova, Italy.

† Abbreviations: GABA, 4-aminobutyric acid; GME, 4-aminobutyric acid methyl ester hydrochloride; MFG, mass fragmentography.

were intracardiacally injected with 1.5 mg of GME dissolved in saline, killed by decapitation after different time intervals and the amount of GABA was measured in blood and brain.

Binding of [^{3}H]-*GABA to receptors of crude synaptic plasma membranes.* Sixteen Sprague–Dawley male rats (130 g) were used and the crude synaptic plasma membranes were prepared by treating brain cortex homogenates with 0.03% Triton X-100 (v/v) as previously described [4]. [^{3}H]-GABA (NEN, Dreieichenhain, F.R.G.) binding was followed in an Na-free medium according to Enna and Snyder [8] and the radioactivity was measured with a Packard Tri-Carb 3375 liquid scintillation spectrometer with Insta-gel as the scintillant liquid. Freshly prepared GME free of chromatographically detectable GABA was used.

In vitro *biotransformation.* 2.5 mg of GME dissolved in 1 ml of saline were incubated with either 8 ml of rat serum, 1.5 mg of synaptosomes or a total homogenate obtained from one brain hemisphere, the other being used as a control. The amount of GABA liberated after incubation for 15 min at 37° was measured after extracting the samples according to Aldermann and Shellenberger [9].

Crossing of the blood–brain barrier. This was measured after intracardically injecting 1.5 mg of GME dissolved in 0.3 ml of saline. The rats were decapitated 5 min after the injection and the brain dissected and put in liquid nitrogen within 2 min. The dissected brain was homogenized with an equal vol. of 4% (v/v) perchloric acid, centrifuged for 3 min at 500 *g* and the collected supernatant was neutralized with 5 N KOH, freeze/thawed and recentrifuged at 2000 *g* for 3 min. After acidification with a final concentration of 0.32 M HCl, 1 ml of the supernatant was passed through an acid Dowex W × 4 (Sigma, St. Louis, MO) column (1 × 10 cm). A total of 27 male Wistar rats (140 g) were used for the experiments.

Effect of GME on the uptake and release of [^{14}C]-*GABA by rat synaptosomes.* Synaptosomes were prepared according to Nicholls [10] by centrifuging rat brain homogenates in an isolation medium of 320 mM sucrose, 5 mM 2-[(2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino-1-propanesulphonic acid and 0.5 mM EDTA in Sorvall SW27. A discontinuous gradient with 12% (w/v), 9% (w/v) and 6% (w/v) Ficoll allowed the separation of the synaptosomes. The protein concn of synaptosomes was measured with the Biorad (Bio-Rad Lab., Richmond, CA) procedure according to Bradford [11]. Synaptosomes were also used for an experiment on the biotransformation of GME after incubating a total amount of 150 μg of GME with 1.5 mg of synaptosome protein. Release and uptake of [^{14}C]-GABA were studied by following the conditions of Bradford [12] in a medium composed of 124 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $MgSO_4$, 20 mM NaH_2PO_4 and 0.5 EDTA at pH 7.4 in a final vol. of 1 ml with 1–1.5 mg of synaptosome protein. For the uptake the samples were incubated at 25° for 5 min before adding the GME. After 5 min [^{14}C]-GABA (Radiochemical Centre, Amersham, U.K.) (10,000 dpm) was added. After 1 min the samples were centrifuged at 3200 *g*, the pellet was washed with the incubation medium and then 0.5 ml of 10% (w/v) trichloroacetic acid were added. After 1 hr in ice the samples were centrifuged at 3200 *g* for 1 min and the pellet was used for the radioactivity measurement. For the release the same medium was used. The samples were pre-incubated with the synaptosomes for 5 min, pre-loaded with [^{14}C]-GABA and, after 5 min, GME was added. After 5 min incubation the samples were centrifuged at 3200 *g* for 3 min and 100 μl of the supernatant were taken for counting. In both uptake and release experiments the blanks were samples without GME.

Na^+ and K^+ were measured by atomic absorption (Perkin–Elmer Atomic Absorption Spectrometer) in the supernatant of the synaptosomes after the release and uptake experiments.

Determination of GABA by TLC, an enzymatic method and MFG. The TLC determination of GABA was carried out on Silica gel plates 60 F 254 (Merck, Darmstadt, F.R.G.) after purifying the samples according to Aldermann and Shellenberger [9]. A chromatographic check of GME showed that, under the conditions used, its R_f value was 0.1 while that of GABA was 0.5 on silica gel plates with acetone/water (1 : 1) as a solvent system; a calibration curve with increasing GABA concns allowed us to quantitate the results.

The so-called GABAse system from *Pseudomonas fluorescens* (Sigma, St. Louis, MO) containing GABA transaminase (EC 2.6.1.19) and succinate-semialdehyde dehydrogenase (EC 1.2.1.16) was used for the enzymatic detection of GABA. A quantitative determination was possible after calibration and the GABAse system was also utilized to study the effect of GME added *in vitro* on the two enzyme activities. The reaction medium contained 0.1 units of GABAse and 0.5 mM GABA in a final vol. of 3 ml of 0.1 M pyrophosphate buffer (pH 8.6). The MFG determination was carried out as described by Cattabeni *et al.* [13] after converting GABA to its trimethisilyl derivative.

RESULTS

The LD_{50} values calculated by the log–probit analysis were 1300 mg/kg (1350/1260 confidence limits) in mice and 950 mg/kg (1040/880 confidence limits) in rats.

The main behavioural effect observed after GME administration was an antiaggressive action in rats: this was noted with 500 mg/kg. The effect was evident 10 min after administration and lasted more than 4 hr with a maximum intensity between 20 min and 2 hr consisting of a complete abolishment of the aggressiveness. At 750 mg/kg GME abolised the aggressiveness for 4 hr and kept it at a minimum level for 24 hr. Only after 32 hr did the treated animals show the same aggressiveness as the controls.

GME reduces by 50% the isoniazid-induced convulsions, by 67% the tonic convulsions and by 34% the number of deceased rats. The latency of the convulsions was not affected by GME since mean ± S.D. values of 3267 ± 205 sec were observed in controls and 3331 ± 111 sec in the GME-treated rats.

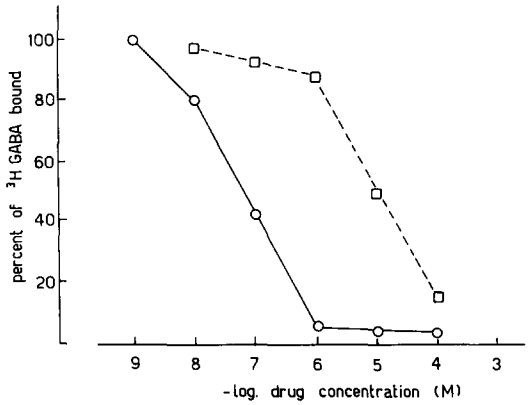

Fig. 1. Per cent of tritiated GABA bound to crude synaptic plasma membrane receptors as a function of the concn of added GABA (○——○) or GME (□---□). Experiments in triplicate.

Observation time was 30 min and a total of 40 observations per group were carried out.

Fig. 1 shows the inhibitory effect of GME on the binding of [^{3}H]-GABA to crude synaptic membrane receptors; the approximate IC_{50} value for GME is 10^{-5} M. Fig. 2 shows the amounts of GABA in rat brain and blood after the intracardiac administration of GME. The half-life of GME is similar in brain and blood, i.e. between 10 and 15 min.

The crossing of the blood–brain barrier of GME is compared and contrasted with that of GABA in Table 1. The injection of GME increases the amount of central GABA by 68%, while the injection of GABA induces an increase of only 2.7%. The actual amount of GME transformed into GABA appears to range from 15 to 20%.

The results of incubation experiments of GME with serum, brain homogenates and isolated synaptosomes documenting the possibility of biotransformation of GME to GABA *in vitro* are shown in Table 2.

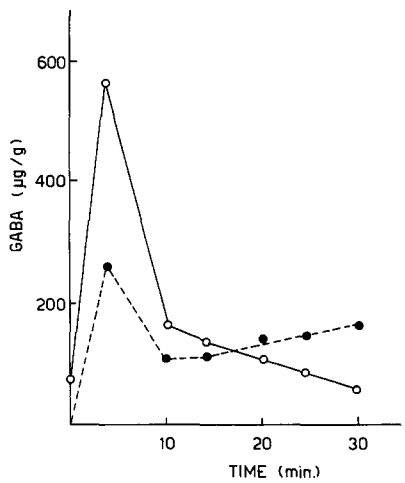

Fig. 2. Amounts of GABA found per g of fresh tissue and blood after intracardiac administration of GME. GABA was measured at different times in brain (○——○) and blood (□---□). Experimental points are mean values of assays in triplicate.

Table 1. Amount of GABA determined by TLC and found in brain of controls, GABA- and GME-injected rats

	GABA (µg/g wet tissue)	Significance
Controls	245.4 ± 28.8 (10)	—
+ GABA (1.5 mg)	252.2 ± 12.6 (7)	n.s.
+ GME (1.5 mg)	413.6 ± 37.0 (10)	$P < 0.01$

Means ± S.D. Statistical significance by paired Student's *t*-test. Number of animals in parentheses.

Table 3 shows that GME causes an increase in the release and a decrease in the uptake of [^{14}C]-GABA in rat synaptosomes and modifies in a parallel way the Na/K ratio in the supernatant.

The experiment on the effect of GME on GABAse *in vitro* (data not reported) shows that GME is a slight competitive inhibitor of the GABAse system with a K_i value of 1.81 mM, while, under our conditions, the apparent K_m of the GABAse is 0.504 ± 0.08 mM (mean ± S.D. of five experiments).

The correlation of TLC vs GABAse ($r = 0.90$; $b = 1.08$; $P = 0.01$), TLC vs MFG ($r = 0.54$; $b = 1.20$; $P = 0.05$) and MFG vs GABAse ($r = 0.80$; $b = 0.80$; $P = 0.05$) were evaluated to check the analytical method used.

DISCUSSION

The slight modification introduced in the GABA molecule renders it capable of crossing the blood–brain barrier. Once arrived at a central level, the GABA derivative is converted back to GABA by a hydrolytic activity which from Table 2 can be roughly estimated as of the order of 19.5 µg GME/min/g of homogenate. The hydrolytic activity of serum as well as that of synaptosomes is, on the contrary, undetectable. The injected GME is therefore probably transformed into GABA by a brain esterase.

The prolonged effect of GME on the aggressive behaviour cannot be explained on the basis of the observed increase of brain GABA and evidently involves other transmitter systems although the inhibitory effect of GME on GABAse is consistent with an antiaggressive action [14].

Table 2. Biotransformation of GME to GABA induced *in vitro* by serum, isolated synaptosomes (1.5 mg) and total brain homogenates after 15 min incubation at 37°

	Controls	+ GME (2.5 mg)
Serum (µg/ml)	50 ± 5	43 ± 4
Synaptosomes (µg/mg)	150 ± 23	153 ± 24
Brain (µg/g)	268 ± 38	561 ± 43

Means ± S.D. of four experiments. The total amount of GABA found in serum, synaptosomes and brain is given. The samples were treated with perchloric acid, acidification and ion exchange after the incubation and before measurement of GABA by TLC.

Table 3. [^{14}C]-GABA released in 5 min by synaptosomes after pre-loading and [^{14}C]-GABA taken up in 1 min

	Release (dpm/min/mg)	Na/K	Uptake (dpm/min/mg)	Na/K
Controls	4570 ± 250	0.19 ± 0.01	1215 ± 130	1.1 ± 0.02
+ GME	5986 ± 360	0.23 ± 0.02	1032 ± 210	0.31 ± 0.01

Means ± S.D. of 10 experiments. The Na/K ratio was measured in the supernatant by atomic absorption. Final GME concn was 5 mM.

The experiments on binding of GME to crude synaptic membrane receptors show that its afinity is intermediate between those of the two *N*-protected GABA derivatives studied in our preceding papers [3, 4]. From such binding studies it appears that neither the free amino group nor the free carboxyl group are strictly necessary for the binding. The high rate of hydrolysis of GME in brain is consistent with its short half-life and this can be compared to the slower biotransformation of *N*-protected GABA derivatives which are most resistant to hydrolysis [3].

GME is able to activate the release of preloaded [^{14}C]-GABA from synaptosomes and to inhibit the [^{14}C]-GABA uptake and the orders of magnitude of such activation and inhibition are similar to those observed by others with -vinyl- and -acetylenic-GABA [12]. The parallel effect induced by GME on the Na/K ratio of the supernatant may indicate that the Na-dependent type of transport of GABA is mainly affected.

The ED_{50} for the isoniazid-induced seizures (100 mg/kg) is identical to that found for the electroshock convulsions [6] and a therapeutical index of 9.5 can be calculated for GME. This value can be compared to that of 17.5 found by others [15] for the cetylic ester of GABA and our results indicate that GME can act as an anticonvulsant with an effectiveness comparable to that of other GABA-like compounds such as the -vinyl- and -acetylenic-GABA [12] acting through completely different mechanisms.

Preliminary experiments have shown that, although GME possesses an electrophilic carbon which can be attacked by the nucleophilic NH_2 to yield a cyclized molecule of 2-pyrrolidone, no such reaction occurs "*in vitro*" in the presence of rat serum. We found that the conditions for a cyclization of GME and production of GABA-lactam "*in vitro*" require a 1 M final concn of GME, pH 8 and a partially aqueous environment. This of course does not exclude the possibility that the formation of 2-pyrrolidone may occur at a central level as an active form of GABA [16].

Acknowledgements—The help and assistance of Drs A. Bertazzon and S. Da Oglio in some experiments is gratefully acknowledged.

REFERENCES

1. E. Roberts, T. N. Chase and D. B. Tower (Eds.), *GABA in Nervous System Function*. Raven Press, New York (1976).
2. P. Krogsgaard-Larsen and T. Roldskov-Christiansen, *Eur. J. Med. Chem.* **14**, 157 (1979).
3. L. Galzigna, L. Garbin, M. Bianchi and A. Marzotto, *Archs Int. Pharmacodyn. Thér.* **235**, 73 (1978).
4. L. Galzigna, G. Manani, L. Garbin, C. Aldino and G. Quadro, *Agressologie* **22**, 219 (1981).
5. N. Bodor, *Trends Pharmac. Sci.* **3**, 53 (1982).
6. R. P. Kohli, K. Kishor, P. R. Dua and R. C. Saxena, *Indian J. Med. Res.* **55**, 1221 (1967).
7. E. Spath, F. Kuffner and N. Plotrer, *Ber. chem. Ges. Frankfurt* **68**, 500 (1935).
8. S. J. Enna and S. H. Snyder, *Molec. Pharmac.* **13**, 447 (1977).
9. J. L. Aldermann and M. K. Shellenberger, *J. Neurochem.* **22**, 937 (1974).
10. D. G. Nicholls, *Biochem. J.* **170**, 511 (1978).
11. M. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
12. H. F. Bradford, in *Regulatory Mechanism of Synaptic Transmission* (Eds. R. Tapin and C. W. Cotman), p. 103. Plenum Press, New York (1981).
13. F. Cattabeni, C. L. Galli and T. Eros, *Analyt. Biochem.* **72**, 1 (1974).
14. J. P. Davanzo and M. Sydow, *Psychopharmacology* **62**, 23 (1979).
15. R. U. Ostrovsakaya, V. V. Parin and N. M. Tsybina, *Byull. eksp. Biol. Med.* **73**, 51 (1972).
16. A. Mori, Y. Katayama, M. Matsumoto and H. Takeuchi, *IRCS med. Sci.* **3**, 590 (1975).