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BIOLOGICAL ACTIONS *In vivo* AND *In vitro* OF TWO γ-AMINOBUTYRIC ACID (GABA) ANALOGUES: β-CHLORO GABA AND β-PHENYL GABA

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1 The synthesis of two analogues of γ-aminobutyric acid (GABA), β-chloro GABA and β-phenyl GABA is described.

2 The activity of brain GABA aminotransferase was inhibited by β-chloro GABA (5.7×10^{-5} M) and β-phenyl GABA (4.6×10^{-3} M) in a competitive manner with GABA.

3 β-Chloro GABA exhibited 50% of the inhibitory activity of GABA in blocking the discharge of the crayfish stretch receptor neurone; β-phenyl GABA had no detectable effect.

4 Injection of β-phenyl GABA (200 mg/kg) into normal or epileptic cats (cobalt) caused the appearance of synchronized slow-wave EEG activity.

5 Administration of β-chloro GABA (200 mg/kg) to epileptic cats (cobalt) produced a temporary diminution or abolition of epileptic discharges while causing no alteration in normal EEG activity.

6 β-Chloro GABA and β-phenyl GABA had no effect on the concentrations of catecholamines or of amino acids in mouse brain.

7 The results suggest that both β-chloro GABA and β-phenyl GABA may pass the blood-brain barrier.

Introduction

The exclusion of exogenous γ-aminobutyric acid (GABA) from the central nervous system (CNS) by the blood-brain barrier systems (van Gelder & Elliott, 1958; van Gelder, 1968) as well as the structural specificity of the compound in exerting inhibitory action on neurones (Curtis & Watkins, 1960), poses unsolved questions. Previous work has shown for example that substances closely related to GABA with respect to molecular structure, charge distribution or water solubility, present no problem in penetrating into the CNS (Wallach, 1961; van Gelder, 1968). Yet, despite the similarity to GABA, even small modifications in the GABA structure appear sufficient either to diminish or to abolish the physiological action (van Gelder, 1971). On the other hand, such GABA analogues very often demonstrate a high affinity for GABA aminotransferase (EC 2.6.1.19, 4-aminobutyrate; 2-oxoglutarate aminotransferase) thereby causing strong inhibition of the enzyme *in vitro* and, occasionally, *in vivo*.

The present paper describes the synthesis and some of the physiological properties of two new analogues of GABA: β-chloro GABA and β-phenyl GABA.

Methods

Synthesis of 3-chloro-4-aminobutyric acid

(a) Methyl 3-chloro-4-aminobutyrate hydrochloride. A solution of 3-hydroxy-4-aminobutyric acid (5 g) in methanol (50 ml) was bubbled with a stream of anhydrous HCl for a period of 2 hours. Evaporation of the solvent at reduced pressure left a transparent, viscous oil which was washed twice with acetone and once with anhydrous ether. The oil was then dissolved in 50 ml of chloroform. To this solution was added quickly, with rapid stirring, an excess amount (16 g) of PCl_5. Stirring was continued for an additional hour after completion of the addition. The precipitate was then filtered, washed with ether and recrystallized from a solution of alcohol-ether to yield white crystals with a m.p. of 166-167° C.

Analysis calculated for $C_5H_{11}NO_2Cl_2$: C, 31.9; H, 5.9; Cl, 37.7.
Found: C, 31.7; H, 5.8; Cl, 37.5.

(b) 3-Chloro-4-aminobutyric acid hydrochloride. A solution of methyl 3-chloro-4-aminobutyrate in 50 ml of 2N HCl was refluxed for 3 hours. Cooling and evaporation at reduced pressure of the solvent left a white solid residue. Recrystallization from a solution of acetic acid-ether yielded 2 g of white crystals with a m.p. 206-207° C (dec.).

Analysis calculated for $C_4H_9NO_2Cl_2$: C, 27.6; H, 5.3; N, 8.0.
Found: C, 27.6; H, 5.6; N, 8.0.

Synthesis of 3-phenyl-4-aminobutyric acid

(a) Ethyl 3-cyano-3-phenyl-propionate. A solution of sodium ethoxide was freshly prepared from 2.0 g of sodium and 30 ml of absolute ethanol. Exactly 11.7 g of phenylacetonitrile was added rapidly and the solution was cooled to 0-5° C. Ethyl bromoacetate (16.7 g) was introduced dropwise, with stirring. The reaction mixture was stirred for an additional hour, then poured over ice-water. The organic layer was extracted three times with 30 ml of ether, and the combined ether extracts were dried over $MgSO_4$. The solvent was evaporated at reduced pressure and the liquid residue was distilled *in vacuo*. The fraction which had a $b.p._{1\,mm}$ at 150-160° C (4.2 g) gave infrared and n.m.r. spectra which were in agreement with the structure of ethyl 3-cyano-3-phenylpropionate.

(b) 3-Phenyl-4-aminobutyric acid hydrochloride. A solution of ethyl 3-cyano-3-phenylpropionate (2 g) in acetic anhydride (50 ml) was hydrogenated under pressure (50 psi) for a period of 24 h, with a Paar shaker hydrogenator and PtO (Adam's catalyst) as catalyst. The solution was filtered and evaporated at reduced pressure. The liquid residue was dissolved in 50 ml of concentrated HCl and refluxed for 8 hours. Evaporation of the solvent at reduced pressure left a white solid residue which after recrystallization from ethanol ether yielded 0.9 g of white crystals m.p. 189-190° C (dec.).

Analysis calculated for $C_{10}H_{14}NO_2Cl$: C, 55.7; H, 6.5; N, 6.5.
Found: C, 55.9; H, 6.6; N, 6.7.

Brain GABA aminotransferase (4-aminobutyrate: 2-oxoglutarate aminotransferase EC 2.6.1.19)

Mice were decapitated and the brains were dropped within 45 s into petroleum-ether which was cooled to −70° C by a dry ice-ethanol bath. The tissue was homogenized in 9 vol. of sodium phosphate buffer (pH 8.3) containing mercapto-ethanol (0.05%), triton X-100 (1%) and pyridoxal phosphate (0.3 mM). The homogenate was centrifuged at 15-20,000 *g* for 30 minutes. The supernatant was diluted to four times its volume with the same buffer and the clear enzyme solution was kept on ice.

GABA aminotransferase activity was measured by adding 0.1 ml of the crude enzyme preparation to 0.9 ml of 0.1 M sodium phosphate buffer (pH 8.3). The buffer routinely contained NAD (33.3 mM) pyridoxal phosphate (0.11 mM), α-ketoglutaric acid (44.4 mM) and GABA (2.5-15 mM). Solutions were incubated for 30 min at 38° C, transferred to an ice-bath and NADH formation was determined by reading the optical density of the solution in a Zeiss spectrophotometer at 340 nm exactly 2 min following the transfer. Solutions serving as controls lacked either GABA or α-ketoglutaric acid; no NADH formation occurred under these circumstances. At the concentrations of GABA chosen, a plot of optical density at 30 min versus substrate concentrations (*S*) resulted in a rectangular hyperbola, thus allowing use of the Michaelis-Menten equation to determine the apparent K_m (Lineweaver & Burke, 1934). Finally, to establish that the rate measured after 30 min of incubation represented the initial rate, another set of experiments was done in which portions were taken every 5 min, their optical densities measured and plotted versus time. The resulting curve was linear over the 30 min period.

Inhibition of brain GABA aminotransferase by β-chloro GABA and β-phenyl GABA was investigated over a range of GABA concentrations (2.5 mM to 15 mM), first with no inhibitor and subsequently in the presence of two different concentrations of the inhibitor.

The possibility that β-chloro GABA and β-phenyl GABA might act as substrate of brain GABA aminotransferase was investigated by replacing GABA with either of the analogues at equivalent concentrations under identical conditions. The incubation period was either 30 or 60 minutes. No NADH formation was observed when either of the GABA analogues served as potential substrate.

Crayfish stretch receptor assay

The physiological activity of β-chloro GABA and β-phenyl GABA on the crayfish stretch receptor was investigated according to the procedure described by Elliott & Florey (1956). The discharge of the slow-adapting neurone in this organ is inhibited upon application of low concentrations of GABA (4-9 μg/ml).

Catecholamine determination

Mice, weighing 30 g, were injected subcutaneously with 200 mg/kg of β-chloro GABA and β-phenyl GABA, and killed by stunning 30 min after the injection. The brains were rapidly (45 s) removed and dropped into a dry-ice alcohol mixture. The frozen brains were weighed and homogenized in 0.4 M perchloric acid and catecholamine content was determined according to the procedure described by Shellenberger & Gordon (1971).

Analysis of amino acids

Mice, weighing 30 g, were injected subcutaneously with 500 mg/kg of β-chloro GABA and β-phenyl GABA, killed 45 min after injection and amino acid content of the brain (minus cerebellum) was determined according to the procedure of van Gelder (1969).

Electroencephalogram (EEG) recording

The technique used to render cats epileptic by application of cobalt powder to the cortex, as well as the characterization of the epilepsy which develops has been described in previous publications (Courtois, 1972; van Gelder & Courtois, 1972). Mature cats of 2.5-3.5 kg were anaesthetized by intravenous injection of pentobarbitone (25 mg/kg). The area of the left anterior motor cortex was uncovered by means of a trephine hole (5 mm in diameter) and after opening the dura, 50 mg of cobalt powder was placed on the exposed cortex. The bone was replaced and the skin was sutured. Subsequent development of seizure activity (16 h after operation) was monitored by EEG.

Results

The structures assigned to 3-chloro-4-aminobutyric acid and 3-phenyl-4-aminobutyric acid (β-phenyl GABA) were consonant with their elemental analyses, their infra-red and nuclear magnetic resonance spectral properties, and the fragmentation pattern of their mass spectra. One of the analogues, 3-chloro-4-aminobutyric acid, is a new compound while the synthesis of 3-phenyl-4-aminobutyric acid has not been hitherto reported in *Chemical Abstracts* despite reports that a parent compound (β(*p*-chloro-phenyl)GABA) may cause muscle relaxation (Burke, Andrews & Knowles, 1971).

Both β-phenyl GABA and β-chloro GABA, at concentrations of 4.6×10^{-3} M and 5.7×10^{-5} M respectively, significantly inhibited the activity of GABA aminotransferase. Removal of the co-enzyme, pyridoxal phosphate, by the analogues could be ruled out as a possible cause of enzyme inhibition since no change in the K_i occurred when the concentration of pyridoxal phosphate was increased twofold with respect to inhibitor concentrations. Neither β-chloro GABA nor β-phenyl GABA appeared to serve as substrate of the enzyme, even at a concentration as high as 6×10^{-3} M and 4×10^{-2} M respectively. In the case of β-chloro GABA this lack of reaction precludes the possibility that the analogue was hydrolysed to β-hydroxy GABA during incubation. The latter compound may serve as a substrate of the mammalian enzyme (Albers & Jakoby, 1960).

The Lineweaver-Burke plots shown for β-chloro GABA (Fig. 1) and for β-phenyl GABA (Fig. 2) indicate that inhibition is of a competitive nature. The calculated K_i was 5×10^{-5} M for β-chloro GABA and 7×10^{-3} M for β-phenyl GABA. These values were representative of at least three sets of experiments. Under the experimental conditions employed the apparent K_m for GABA was 3×10^{-3} M.

Physiological activity

At pH 6.8, β-chloro GABA exhibited approximately 50% of the inhibitory activity of GABA in blocking the neuronal discharge in the crayfish stretch receptor organ. As with GABA, the inhibitory action of β-chloro GABA was promptly reversible upon rinsing of the preparation with buffered crayfish saline. When half the inhibitory concentration of GABA was combined with half the inhibitory concentration of β-chloro GABA no potentiation or antagonism was observed. No inhibition of the neuronal discharge in the crayfish stretch receptor organ by β-phenyl GABA was detectable, even at concentrations which exceeded the inhibitory concentration of GABA three hundredfold.

Effect of β-phenyl GABA and β-chloro GABA on the EEG of the cat

Injection of β-phenyl GABA (200 mg/kg s.c.) to normal cats caused the appearance of synchronized, slow-wave EEG activity. The effect occurred approximately 30 min after administration of the drug and persisted for at least 3 hours. In cats rendered epileptic by application of cobalt, β-phenyl GABA did not influence either the frequency or the amplitude of the epileptic discharges although it caused the appearance of identical synchronized slow-wave EEG activity (Figure 3). The injection of β-chloro GABA, on

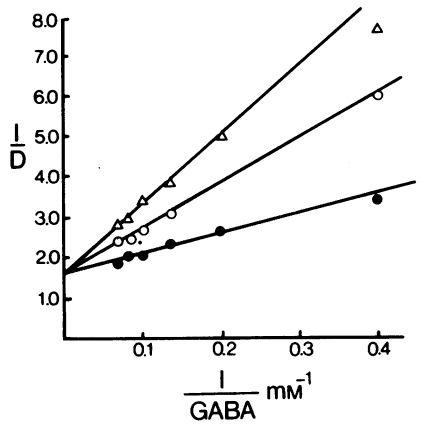

Fig. 1 Lineweaver-Burke plot of inhibition of brain aminobutyrate transferase by β-chloro GABA. Reaction was initiated by addition of the enzyme solution to a solution containing (mM): NAD (33.3 mM); pyridoxal phosphate (0.11 mM); α-keto-glutaric acid (44.4 mM); and GABA (2.5 to 15 mM). The mixture was incubated for 30 min at 37°C and optical density (*D*) measured at 340 nm. The data are representative of at least three experiments. (△) β-chloro GABA (0.114 mM); (○) β-chloro GABA (0.057 mM); (●) control (GABA).

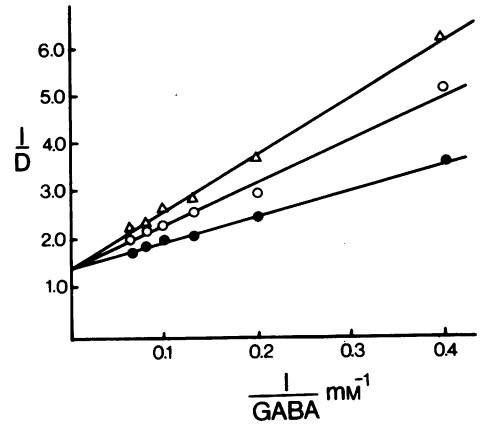

Fig. 2 Lineweaver-Burke plot of inhibition of brain aminobutyrate transferase by β-phenyl GABA. The experimental conditions were identical to those described in Figure 1. The data are typical of three experiments. (△) β-phenyl GABA (9.3 mM); (○) β-phenyl GABA (4.65 mM); (●) control (GABA).

the other hand, did not seem to have any effect on the EEG of normal cats even when the cat was injected with a large dose (500 mg/kg). However, in four out of five epileptic animals, epileptic discharges were either sharply diminished or abolished for approximately 2 h following injection.

Catecholamine content

Both cats and mice were prostrate after injection with β-chloro GABA or β-phenyl GABA. The catecholamine content of whole brain was measured in order to determine whether or not the drugs might cause interference with the metabolism of the catecholamines. The results showed no differences between the cerebral concentrations of dopamine or noradrenaline in the control group and the drug-injected group.

Amino acid content

Previous reports have shown that injection of inhibitors of GABA aminotransferase, such as aminooxyacetic acid and β-hydrazinopropionic acid, affect the metabolism of amino acids in mouse brain (van Gelder, 1966, 1969). Unlike these very effective inhibitors of GABA aminotransferase, the new analogues had no effect on the concentrations of amino acids in mouse brain, even when injected at doses up to 500 mg/kg.

Discussion

The experimental data presented here show that β-phenyl GABA and β-chloro GABA inhibited the activity of GABA aminotransferase of mouse brain in a competitive manner. By comparison with various other inhibitors of GABA-aminotransferase which have been previously studied (Wallach, 1961; van Gelder, 1968; Beart, Uhr & Johnston, 1972), it appears that β-phenyl GABA is a relatively weak inhibitor, and β-chloro GABA a moderately effective inhibitor of GABA-amino-transferase.

There was no indication that either β-phenyl GABA or β-chloro GABA could act as a substrate of the brain aminotransferase. Substitution may increase the rigidity and the degree of hydrogen bonding of the apoenzyme-substrate complex and prevent it from undergoing the necessary conformational change for subsequent trans-amination. Thus a rigid analogue of GABA, 4-aminotetrolic acid, has been reported to behave as a dead-end inhibitor of GABA transaminase activity (Beart *et al.*, 1972).

The findings that β-chloro substituted GABA

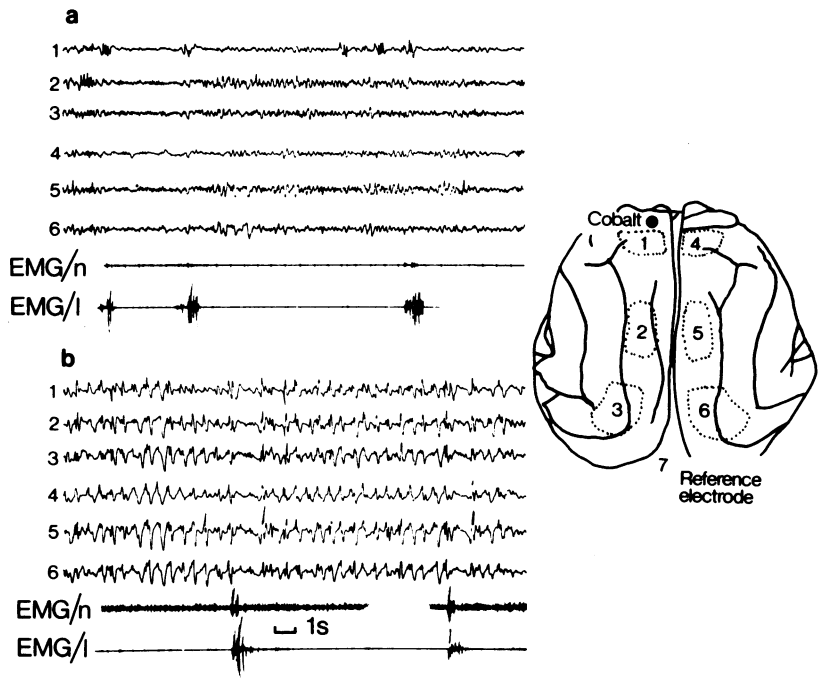

Fig. 3 The effect of injection of β-phenyl GABA on EEG activity of epileptic cat (cobalt), before (a) and after (b) injection. Onset of symptoms occurred 30 min after administration of the drug and continued 2-3 hours. EMG activity was recorded from neck (EMG/n) and left leg (EMG/l). The data shown were obtained 1 h after an injection of β-phenyl GABA (200 mg/kg, s.c.). Leads 1 to 6 refer to positions of electrodes on the cortex (see insert). Note that epileptic discharges were not affected by the drug. Synchronized EEG activity involves whole cortex, suggesting a generalized change in CNS conditions (metabolic and/or physiological).

exhibited only 50% of the inhibitory activity of GABA and that β-phenyl substituted GABA was devoid of physiological activity on the crayfish neurone, were consistent with previous studies of Bazemore, Elliott & Florey (1957) which showed that substitution or modification of the GABA molecule invariably leads to a decrease of its physiological activity. In the present study such a decrease or loss of activity may be ascribed in part to the increasing interference of the β substituent with the two zwitterionic centres of GABA.

No apparent correlation seems to exist between the affinity of compounds for GABA aminotransferase and their inhibitory activity on neurones (Table 1). Thus aminooxyacetic acid (Wallach, 1961) and hydrazinopropionic acid (van Gelder, 1968), two inhibitors with a very strong affinity for the enzyme receptor, exhibit little or no activity on the crayfish neurone. Alternatively, GABA itself and several analogues with a weak affinity for the enzyme (i.e. β-hydroxy GABA, β-chloro GABA) exert moderately strong inhibition on neurones.

No direct evidence is available as to whether or not these compounds pass the blood-brain barrier systems. However, β-phenyl GABA, when injected in normal animals, caused identical symptoms to those seen in cats with minimal brain damage from implantation of cortical surface electrodes. The persistent and striking synchronized EEG activity

Table 1 Drug-enzyme dissociation constants (K_m or K_i) for GABA aminobutyrate transferase and inhibitory activity on crayfish stretch receptor of GABA and some GABA analogues

	K	*Inhibitory (crayfish)*
GABA	3×10^{-3} M	++++
Aminooxyacetic acid	3×10^{-4} M	0
Hydrazinopropionic acid	3.4×10^{-7} M	+
β-Hydroxy GABA	?	++
β-Chloro GABA	5×10^{-5} M	++
β-Phenyl GABA	7×10^{-3} M	0

in such normal animals strongly suggests that this analogue readily penetrates into the CNS. The reports that the parent compound, β-(*p*-chlorophenyl) GABA, specifically causes muscle relaxation (Burke *et al.*, 1971) may require reappraisal in the light of the pronounced alterations in brain wave patterns observed with unsubstituted β-phenyl GABA.

Epileptic cats injected with GABA (200 mg/kg) continued to exhibit unchanged epileptic discharges. Subsequent administration in the same animals of β-chloro GABA effectively diminished or abolished the epileptic signs temporarily. Since GABA, when applied directly to the cortex, does antagonize the action of cobalt, the present results suggest that β-chloro GABA passes the blood-brain barrier systems.

In conclusion, these studies indicate that β-phenyl GABA and β-chloro GABA are sufficiently similar to GABA itself to have an affinity for the apoenzyme of GABA aminotransferase equal to or surpassing the natural substrate. However, the alterations in GABA structure appear at the same time to limit sharply the affinity of the compounds for the physiological receptors of GABA in the neuronal membranes. Despite rather exhaustive investigations, no biochemical alterations in the brain could be detected following administration of the analogues. The cause of the 'epileptic' action of β-phenyl GABA and the opposite, 'anti-epileptic' action of β-chloro GABA, remains unexplained at present.

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