British Journal of Pharmacology (1997) 122, 539–545

Effect of chronic treatment with the GABA transaminase inhibitors γ-vinyl GABA and ethanolamine O-sulphate on the *in vitro* GABA release from rat hippocampus

[1]M. Qume & L.J. Fowler

Department of Pharmacology, School of Pharmacy, University of London, London

1 The effects of 2, 8 and 21 day oral treatment with the specific γ-aminobutyric acid transaminase (GABA-T) inhibitors γ-vinyl GABA (GVG) and ethanolamine O-sulphate (EOS) on brain GABA levels, GABA-T activity, and basal and stimulated GABA release from rat cross-chopped brain hippocampal slices was investigated.

2 Treatment with GABA-T inhibitors lead to a reduction in brain GABA-T activity by 65–80% compared with control values, with a concomitant increase in brain GABA content of 40–100%.

3 Basal hippocampal GABA release was increased to 250–450% of control levels following inhibition of GABA-T activity. No Ca^{2+} dependence was observed in either control or treated tissues.

4 GVG and EOS administration led to a significant elevation in the potassium stimulated release of GABA from cross-chopped hippocampal slices compared with that of controls. Although stimulated GABA release from control tissues was decreased in the presence of a low Ca^{2+} medium, GVG and EOS treatment abolished this Ca^{2+} dependency.

5 GABA compartmentalization, Na^+ and Cl^- coupled GABA uptake carriers and glial release may provide explanations for the loss of the Ca^{2+} dependency of stimulated GABA release observed following GVG and EOS treatment.

6 Administration of GABA-T inhibitors led to increases in both basal and stimulated hippocampal GABA release. However, it is not clear which is the most important factor in the anticonvulsant activity of these drugs, the increased GABA content 'leaking' out of neurones and glia leading to widespread inhibition, or the increase in stimulated GABA release which may occur following depolarization caused by an epileptic discharge.

Keywords: GABA; γ-vinyl GABA; ethanolamine O-sulphate; epilepsy; GABA transaminase; release

Introduction

Enhancement of γ-aminobutyric acid (GABA)-mediated inhibition has been shown to produce anticonvulsant effects (Meldrum, 1985). Ethanolamine O-sulphate (EOS) (Fowler & John, 1972) and γ-vinyl GABA (GVG; vigabatrin) (Jung *et al*., 1977) are both specific mechanism based inhibitors of GABA-transaminase (GABA-T, EC 2.6.1.19), the enzyme responsible for the catabolism of GABA. Administration of these compounds leads to elevations in brain GABA levels (Fowler, 1973; Jung *et al*., 1977), and it is thought that this is the reason behind their anticonvulsant activity. Indeed, GVG is a well-established antiepileptic drug (Sabril) with proven efficacy in several types of seizure (see Grant & Heel, 1991).

GABA-T occurs in the mitochondria of both neurones and glia, hence inactivation of this enzyme leads to increases in the intracellular concentration of GABA. However, the pharmacological actions of this neurotransmitter are presumed to act via receptors located extracellularly, on the cell membrane. The increased GABA content must therefore be released to the exterior of the cell in order to produce antiseizure activity. Indeed, GVG administration has been shown to increase GABA release from cerebral cortex synaptosomes (Abdul-Ghani *et al*., 1981), slices of rat cortex, spinal cord and retina (Neal & Shah, 1989) and mouse embryonic cultured neurones (Gram *et al*., 1988). We have previously described the effect of treatment with both GVG and ethanolamine O-sulphate (EOS) on the *in vivo* release of GABA from the rat hippocampus (Qume *et al*., 1995), demonstrating that these compounds increase both basal and K^+ stimulated GABA efflux (by a maximum of over 500% of control values) and that the exclusion/decrease of extracellular Ca^{2+} had no effect on release. It has been proposed that the GABA uptake carrier system terminating neurotransmitter action may operate in the reverse direction, functioning as a means of releasing GABA in a non-vesicular and calcium-independent fashion (see Adam-Vizi, 1992; Bernath, 1992; Attwell *et al*., 1993). In this study we have used *in vitro* release to examine the effects of GABA-T inhibition following 2, 8 and 21 day EOS or GVG treatment on basal and K^+ stimulated GABA release. The effect of decreasing the Ca^{2+} content of the medium on both basal and stimulated release was also examined. The drug regime has been previously shown to produce inhibition of GABA-T activity by 60–85% and the elevation of total brain GABA content to 200% of control values (Qume & Fowler, 1996). These data have been published previously in abstract form (Qume *et al*., 1992; Qume & Fowler, 1992).

Methods

Animals

Adult male Wistar rats (weighing 245–285 g at the beginning of the experiment) were used and housed in groups of 6 with a 12 h light-dark cycle at a temperature of 22±2°C. Access to diet (rat and mouse expanded or compressed diet No. 3, Quest Nutrition) and drinking water (tap water containing drugs) was *ad libitum*. All animal use procedures were in accordance with Home Office Guidelines and licensed under the Animals (Scientific Procedures) Act 1986.

[1]Author for correspondence at: Department of Pharmacology, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT

Drug administration

EOS was purified as described by Phillips and Fowler (1982). GVG was a gift from the Marion Merrell Dow Research Centre (Winnersh, Berkshire, U.K.). Fifty four male Wistar rats (weighing 267 ± 7 g) were divided into 3 groups, receiving in their drinking water, vehicle (1 g l^{-1} sucrose), 3 g l^{-1} GVG or 3 g l^{-1} EOS. The use of sucrose has been found necessary in previous studies (John *et al.*, 1987) to overcome the animals' aversion to drinking the GVG solution. Body weights and fluid consumption were monitored and drug solutions changed regularly.

In vitro *release*

Hippocampal dissection and tissue preparation Following 2, 8 and 21 days drug administration, 6 animals from each group were stunned, decapitated and the brain removed onto an ice-cold petri dish. The hippocampii were gently dissected free and placed onto a filter soaked in Krebs solution (composition (mM): NaCl 120, KCl 3, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11 and $CaCl_2$ 2). Cross-chopped hippocampal slices were produced by slicing (250 μm) the hippocampii lengthways and then at an angle of 45° with a McIlwain tissue chopper. The remaining brain tissue was homogenized in 3 ml of ice-cold distilled water and stored at −20°C for subsequent determination of GABA-T activity (Salvador & Albers, 1959), amino acid and protein content (Bradford, 1976).

Incubation The tissue slices were suspended in 9 ml warmed (37°C), constantly gassed (95% O_2/5% CO_2) low calcium (0.1 mM $CaCl_2$) Krebs for 10 min. The slices were allowed to settle for 30 s and the Krebs aspirated off and replaced with 6 ml fresh low Ca^{2+} Krebs. The suspension was evenly distributed onto 2.5 cm, 0.2 μm Whatman cellulose filters which were under vacuum in the 12 wells of a Millipore multiwell filter unit. The tissue was washed, under vacuum, with 10×2 ml of either normal or low Ca^{2+} Krebs, depending on the conditions required. A 5 min preincubation with the relevant Krebs solution was also performed, the solution drained under vacuum and discarded.

Basal and stimulated release Basal release of amino acids was determined by 5 min incubation of the tissue with 500 μl of the appropriate Krebs solution (normal or low Ca^{2+}) which was then drained under vacuum into ice cold test-tubes. Stimulated amino acid release was determined in exactly the same way using an elevated potassium Krebs (20, 50 and 100 mM K^+, with reduced Na^+ content to maintain osmolarity) and an elevated K^+ (50 mM)/low Ca^{2+} Krebs. The released samples were transferred to eppendorf vials and stored at −20°C for subsequent determination of amino acid content by high performance liquid chromatography (h.p.l.c.).

Preparation of brain tissue samples for h.p.l.c. Brain tissue homogenates (90 μl) were precipitated with 100% TCA (10 μl) and centrifuged (11,000 *g*, Anderman eppendorf centrifuge 5413, 10 min) followed by subsequent neutralisation of 10 μl of the supernatant with 0.2 M $NaHCO_3$, pH 9 (90 μl).

H.p.l.c. analysis of samples Analysis of samples was carried out by precolumn derivatization with *o*-phthaldialdehyde/2 mercaptoethanol followed by reverse-phase gradient h.p.l.c., based on a modified version of the method of Lindroth and Mopper (1979). A C18 analytical column (Rainin Dynamax; 15 cm × 0.4 cm i.d.; particle size 5 μm) was maintained at a constant temperature of 30°C by a column heater. Both derivatization and the subsequent injection were performed by a Gilson model 231 automatic sampler. A 41 min gradient (including column wash) was used to allow the elution of GABA (approx. 28–30 min). Data were collected and peak-area analysis was carried out by a PC-based system (Drew Systems chromatographic software), and quantification was achieved by constructing calibration graphs with external standards. The detection limit for the system was in the order of 300–400 fmol of amino acid. H.p.l.c. analysis of both release and tissue samples led to incomplete separation of the serine and histidine doublet and of the arginine, glycine and threonine triplet, incomplete separation of the alanine and tyrosine doublet was also observed in release samples. In total, 13 amino acids were analysed, the main emphasis of this study is on GABA, so to maintain brevity, the other amino acid levels were measured after 2 days treatment only.

For statistical analysis, measurements were compared for significant differences from control, at corresponding time points with either ANOVA followed by *post hoc* Dunnet's test, or paired Student's *t* test as appropriate, with a level of significance of 5%.

Results

Body weights and fluid consumption

Animals receiving GVG gained little or no weight, EOS consumption on the other hand caused no significant difference in weight gain compared to control animals (Qume *et al.*, 1995; Qume & Fowler, 1996), these data are summarized in Figure 1. The mean drug consumption ($\pm$s.d.) of treated animals throughout the study for those receiving GVG or EOS was 263 ± 47 mg kg^{-1} day^{-1} and 366 ± 49 mg^{-1} kg^{-1} day^{-1}, respectively.

Behaviour

Animals treated with GVG and to a lesser extent with EOS exhibited classic signs associated with GABA-T inhibition (Baxter *et al.*, 1973), lethargy, disinterest, hypokinesia, diarrhoea, piloerection and a slightly hunched posture. These symptoms grew worse with prolonged treatment.

Brain tissue analysis

GABA-T activity of the brain (minus hippocampus) was reduced by 65–80% of control values (Figure 2).

GABA content was significantly elevated at all time points, with both drugs, with the increases ranging from approximately 40–100% (Figure 3). The tissue content of amino acids other than GABA was similar throughout the time course, 2 day levels are shown in Table 1.

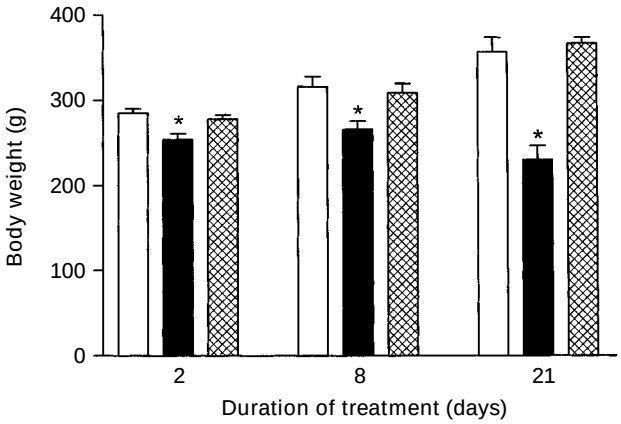

Figure 1 The effect of treatment with 3 g l^{-1} GVG (solid columns) or EOS (cross-hatched columns) compared with vehicle (open columns) on terminal body weights. Means $\pm$ s.d. are shown, $n = 6$ animals per group at each time point. *Indicates a significant difference from control (ANOVA followed by *post hoc* Dunnet's test, $P < 0.05$).

Basal amino acid release

Table 2 details the effects of GVG and EOS treatment on GABA release in the presence of normal and low Ca^{2+} medium. After 2 days of GVG treatment, the GABA efflux was significantly elevated to over 300% of control values in both Ca^{2+} mediums. EOS on the other hand produced no significant changes compared with control. Following 8 day treatment with GVG, and 21 days of treatment with both GABA-T inhibitors, significant elevations in the basal release of GABA in both Ca^{2+} environments, increases ranging from 250 to over 500% were observed. No Ca^{2+} dependent basal GABA release was obtained in either control or treated tissues. Following two day (Table 3), 8 and 21 day (data not shown) GVG and EOS treatment, no consistent changes in the basal release of amino acids other than GABA were observed.

Stimulated amino acid release

Table 4 details the effect of 2, 8 and 21 day treatment with GABA-T inhibitors on K^+ stimulated GABA release (expressed as stimulated minus basal release). Stimulated GABA

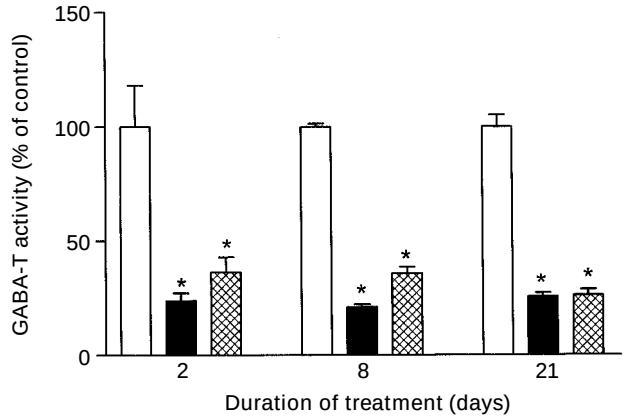

Figure 2 The effect of treatment with 3 g l^{-1} GVG (solid columns) or EOS (cross-hatched columns) compared with vehicle (open columns) on brain (minus hippocampus) GABA-T activity. Data are expressed as a percentage of control. Means ± s.e.mean are shown, $n=6$ animals per group at each time point. *Indicates a significant difference from control (ANOVA followed by *post hoc* Dunnet's test, $P<0.05$).

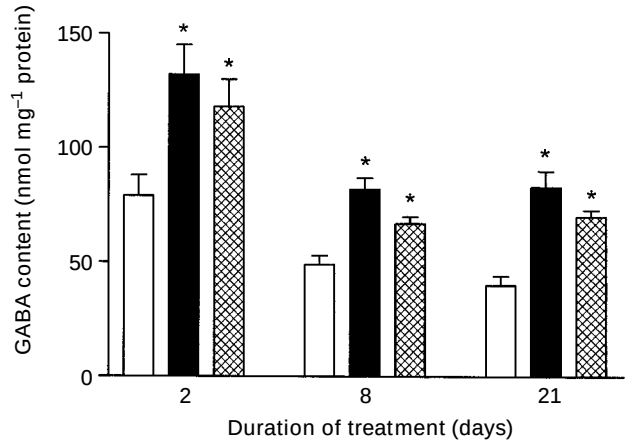

Figure 3 The effect of treatment with 3 g l^{-1} GVG (solid columns) or EOS (cross-hatched columns) compared with vehicle (open columns) on brain (minus hippocampus) GABA content. Data are expressed as nmol mg^{-1} protein. Means ± s.e.mean are shown, $n=6$ animals per group at each time point. *Indicates a significant difference from control (ANOVA followed by *post hoc* Dunnet's test, $P<0.05$).

Table 1 Whole brain (minus hippocampus) amino acid content following 2 day treatment with 3 g l^{-1} GVG or EOS, compared with vehicle (control)

Amino acid	*Tissue content* (nmol mg^{-1} protein) *Control*	*GVG*	*EOS*
Asp	63 ± 6	47 ± 5	63 ± 6
Glu	110 ± 12	116 ± 5	103 ± 4
Asn	4 ± 0	4 ± 1	4 ± 1
Ser/His	13 ± 2	13 ± 2	18 ± 2
Gln	84 ± 6	82 ± 3	85 ± 6
A/G/T†	14 ± 2	15 ± 2	13 ± 2
Tau	93 ± 7	83 ± 2	93 ± 5
Ala	18 ± 2	15 ± 1	19 ± 2

Values are mean ± s.e.mean, $n=6$ animals per group for each time point. Statistical analysis (ANOVA followed by *post hoc* Dunnet's test $P<0.05$) indicated no difference between control and treated groups. †Arginine-glycine-threonine triplet.

Table 2 The basal GABA release from hippocampal slices after 2, 8 and 21 days of treatment with GVG or EOS (3 g l^{-1}), compared with control

Duration (days)	Ca^{2+} *content*	*Basal release* (pmol 500 μl^{-1} mg^{-1} tissue) *Control*	*GVG*	*EOS*
2	Normal	97 ± 18	299 ± 60*	99 ± 8
	Low	106 ± 12	279 ± 57*	135 ± 21
8	Normal	88 ± 19	372 ± 49*	236 ± 31*
	Low	86 ± 20	275 ± 46*	190 ± 33
21	Normal	59 ± 4	205 ± 39*	156 ± 15*
	Low	50 ± 7	193 ± 40*	186 ± 33*

Values are mean ± s.e.mean, $n=6$ animals per group for each time point. *Indicates a significant difference from control, (ANOVA followed by *post hoc* Dunnet's test, $P<0.05$). No differences were found after the Ca^{2+} content of the incubation medium had been decreased (Student's paired *t* test, $P<0.05$).

Table 3 The basal amino acid release from hippocampal slices after 2 days of GVG or EOS (3 g l^{-1}) treatment compared with control

Amino acid	Ca^{2+} *content*	*Basal release* (pmol 500 μl^{-1} mg^{-1} tissue) *Control*	*GVG*	*EOS*
Asp	Normal	81 ± 14	89 ± 15	74 ± 9
	Low	96 ± 19	74 ± 12	87 ± 15
Glu	Normal	172 ± 17	217 ± 31	160 ± 12
	Low	223 ± 32	205 ± 7	211 ± 26
Asn	Normal	46 ± 6	49 ± 5	39 ± 2
	Low	44 ± 5	35 ± 4	33 ± 4
Ser/His	Normal	299 ± 75	261 ± 42	165 ± 26
	Low	295 ± 64	203 ± 47	175 ± 30
Gln	Normal	353 ± 41	365 ± 43	284 ± 18
	Low	367 ± 66	231 ± 15‡	283 ± 24
A/G/T†	Normal	153 ± 26	149 ± 16	103 ± 11
	Low	159 ± 26	111 ± 15	95 ± 15
Tau	Normal	311 ± 35	468 ± 68	285 ± 38
	Low	445 ± 81	385 ± 43	331 ± 39
Ala/Tyr	Normal	330 ± 49	308 ± 34	235 ± 15
	Low	356 ± 59	233 ± 37	246 ± 29

Values are mean ± s.e.mean, $n=6$ animals per group for each time point. Statistical analysis (ANOVA followed by *post hoc* Dunnet's test $P<0.05$) indicated no significant difference between control and treated groups. ‡Indicates a significant difference after the Ca^{2+} content of the incubation medium had been decreased (Student's paired *t* test, $P<0.05$). †Arginine-glycine-threonine triplet.

release was increased after 2 day treatment with GVG for all stimulation parameters except 20 mM K^+, which although doubled was not statistically significant ($P=0.1$) compared with control. The release from EOS treated tissues was not elevated to a significant level at this time point. The Ca^{2+}-dependence of stimulated release observed in the control tissue was not maintained following GABA-T inhibitor treatment.

Eight day treatment with GVG led to a significant increase in release with all stimulated parameters, except 50 mM K^+ (elevated non-significantly by 75%). EOS caused increased stimulated release of GABA, except with that elicited in the low Ca^{2+} medium. Calcium dependence was observed in both control and EOS-treated tissues.

After 21 days of administration of GVG, all stimulation parameters caused a release greater than that in control tissues. Increases following EOS treatment reached significance following 100 mM K^+ and 50 mM K^+/low Ca^{2+}, the other 2 conditions caused the release to double, but this was not statistically significant. Calcium-dependence was observed in control tissues, but not following treatment with GABA-T inhibitors.

Two day (Table 5), 8 and 21 day (data not shown) GVG and EOS treatment caused no consistent changes in the stimulated release of amino acids other than GABA.

Table 4 The stimulated GABA release from hippocampal slices after 2, 8 and 21 days of treatment with GVG or EOS (3 g l^{-1}) compared with control

Duration (days)	K^+ (mM)	*Stimulated release* (pmol 500 µl^{-1} mg^{-1} tissue) *Control*	*GVG*	*EOS*
2	20	65±12	105±19	84±22
	50	214±38	531±87*	399±90
	100	528±73	1027±169*	621±55
	50/lowCa^{2+}	152±18‡	405±19*	278±40
8	20	71±16	383±88*	162±28*
	50	351±57	613±166	686±149*
	100	436±81	950±131*	830±21
	50/lowCa^{2+}	190±33‡	628±100*	346±121‡
21	20	52±8	125±22*	113±34
	50	159±25	263±27*	273±84
	100	210±19	556±139*	638±127*
	50/lowCa^{2+}	86±16‡	279±61*	228±38*

Values are mean±s.e.mean $n=6$ animals per group for each time point. *Indicates a difference from control relevant to that time point (ANOVA followed by *post hoc* Dunnet's test, $P<0.05$). ‡Indicates a difference in 50 mM K^+-evoked stimulated release after the Ca^{2+} content of the incubation medium had been decreased (Student's paired *t* test $P<0.05$).

Discussion

Tissue GABA-T activity and amino acid content

As previously shown (Qume & Fowler, 1996), this treatment regime (3 gl^{-1} GVG/EOS in the drinking water) lead to an inhibition of brain GABA-T activity of 65–80%, with subsequent elevations in GABA content in the region of 40–100%.

Basal GABA release

The unstimulated release of GABA was found to be increased following GVG and EOS treatment to 240–500% of control. Lowering the Ca^{2+} content of the medium did not alter this release either in control or treated tissues, correlating with that observed by Neal and Shah (1989). This increase in basal GABA efflux may be interpreted as a 'pre-loading' of the nerve terminal by the prevention of GABA metabolism and the subsequent 'leakage' (passive diffusion) of GABA out of the cell. However it is uncertain where the extra GABA is stored, either in an increased number of synaptic vesicles, increased content in synaptic vesicles or perhaps just in the nerve terminal.

The spontaneous release of transmitter is thought to reflect the resting levels in intracellular Ca^{2+}, with mitochondria playing an important role (Alnaes & Rahaminoff, 1975). Indeed, treatments known to elicit Ca^{2+} loss from mitochondria have been shown to stimulate transmitter release from isolated nerve endings, (Sandoval, 1980) and the frog neuromuscular junction (Alnaes *et al.*, 1974). The results of this study indicate that external Ca^{2+} is not required for the basal efflux of GABA from cross-chopped hippocampal slices, either in control tissues or those with elevated GABA content due to markedly reduced GABA-T activity.

Stimulated GABA release

An elevation (of up to 3 times control levels) of stimulated GABA release was observed following GVG and EOS treatment. These results are consistent with data obtained by Neal and Shah (1989) following GVG administration. Decreasing the Ca^{2+} content of the incubation medium (to 0.1 mM) caused a significant decrease in the hippocampal efflux of GABA following stimulation with 50 mM K^+ in control tissues. However, following GVG or EOS treatment the potassium stimulated release of GABA showed little or no

Table 5 The stimulated amino acid release from hippocampal slices after 2 days of GVG or EOS (3 g l^{-1}) treatment compared with control

Amino Acid	K^+ (mM)	*Stimulated release* (pmol 500 µl^{-1} mg^{-1} tissue) *Control*	*GVG*	*EOS*
Asp	20	31±15	35±8	42±13
	50	162±33	138±36	203±44
	100	447±77	447±84	438±56
	50/lowCa^{2+}	102±24	129±28	121±32‡
Glu	20	97±17	160±31	149±40
	50	504±51	573±120	596±57
	100	938±51	949±102	936±64
	50/lowCa^{2+}	403±61	541±100	411±62‡
Asn	20	12±9	48±20	24±7
	50	15±10	26±9	21±4
	100	32±20	38±10	75±34
	50/lowCa^{2+}	16±5	12±8	22±6
Ser/His	20	28±42	39±12	103±29
	50	108±46	113±49	133±69
	100	123±40	110±63	113±19
	50/lowCa^{2+}	−4±35	27±31	100±36
Gln	20	75±31	82±30	168±52
	50	139±48	235±63	262±72
	100	369±91	343±77	425±66
	50/lowCa^{2+}	57±36	105±71‡	156±52‡
A/G/T†	20	21±19	27±8	46±19
	50	81±27	67±30	77±23
	100	68±23	69±28	75±13
	50/lowCa^{2+}	16±14	22±14	61±17
Tau	20	96±32	12±33	81±29
	50	187±68	143±70	120±30
	100	137±51	78±37	106±18
	50/lowCa^{2+}	34±58‡	21±54	107±36
Ala/Tyr	20	80±30	84±11	143±36
	50	186±56	145±52	232±61
	100	355±64	297±74	333±31
	50/lowCa^{2+}	76±41	104±43	186±50

Values are mean±s.e.mean, $n=6$ animals per group for each time point. Statistical analysis (ANOVA followed by *post hoc* Dunnet's test, $P<0.05$) indicated no significant difference between control and treated groups. ‡Indicates a difference in 50 mM K^+ evolved stimulated release after the Ca^{2+} content of the incubation medium had been decreased (Student's paired *t* test $P<0.05$). †Arginine-glycine-threonine triplet.

Ca^{2+}-dependence. This is in contrast to the findings of Neal and Shah (1989); they observed a decrease in the K^+ stimulated cortical efflux of GABA in GVG-treated rats after the Ca^{2+} content of the medium (0.2 mM) had been decreased and the magnesium (20 mM) content elevated.

The observed increase in stimulated release of GABA would be expected, given both the elevated GABA content of the brain and increased basal GABA release. A point of interest is the lack of Ca^{2+} dependence of stimulated GABA release observed following the GVG or EOS-evoked increase in GABA content.

The use of high extraneuronal concentrations of K^+ is a widely used method for evoking both endogenous and exogenous radiolabelled release of neurotransmitters from CNS preparations, with the increase in external K^+ leading to depolarization of the nerve terminal resulting in transmitter release. An increase in free cytoplasmic Ca^{2+} is generally regarded as the critical event required for the initiation of neurotransmitter release (Sandoval, 1980). However, depolarizing K^+ concentrations have been shown to stimulate GABA and glutamate release from brain slices and synaptosomes, in Ca^{2+} free media (Srinivasan *et al*., 1969; Raiteri *et al*., 1975; Nadler *et al*., 1976; Redburn *et al*., 1976).

Work by Sandoval (1980) on [^{3}H]GABA efflux from whole brain synaptosomes suggested that, in the absence of external Ca^{2+}, the accumulation of Na^+ at the nerve endings elicited an increased efflux of Ca^{2+} from the mitochondria providing the internal Ca^{2+} required for transmitter release increased. The release of intracellular Ca^{2+} is thought to occur through mitochondrial Na^+/Ca^{2+} exchange, demonstrated in the mitochondria of excitable tissues (Carafoli *et al*., 1974; Silbergeld, 1977).

It has been concluded that newly taken up, labelled transmitters preferentially enter pools from which they can be released (Ryan & Roskoski, 1975; Levi *et al*., 1981; Szerb *et al*., 1981; Szerb, 1983; Waldmeir *et al*., 1989). Guinea-pig cortical synaptosomes have provided evidence for the existence of two functionally different compartments for glutamate and GABA; one, cytosomal, rapidly labelled by exogenous radioligands which may be depleted by Ca^{2+}-independent mechanisms (as with Sandoval, 1980), the other pool slowly equilibrates with exogenous transmitters, releases transmitters in a Ca^{2+}-dependent manner and is resistant to changes in total amino acid levels (Nicholls & Sihra, 1986; Nicholls *et al*., 1987; Sihra & Nicholls, 1987; McMahon & Nicholls, 1990). The following data provide evidence that the different pools may be released in the absence or presence of Ca^{2+}. Studies on primary cerebellar cultures suggest that transmitters may be released from different compartments following depolarizing stimuli (Rogers *et al*., 1987). The Ca^{2+}-independent release of GABA from synaptosomes, stimulated by potassium was shown to occur preferentially from the accumulated, cytoplasmic pool, compared to Ca^{2+}-dependent release where GABA from both the accumulated and endogenous pools was released (Haycock *et al*., 1978). Szerb (1979) observed veratridine-stimulated release of radiolabelled GABA from cortical slices in the absence of Ca^{2+}, after the Ca^{2+}-dependent pool had been depleted by K^+.

The increased GABA content in the brain is due to the inhibition of breakdown of released GABA, it may be postulated that these transmitter molecules are preferentially taken up in a similar manner to exogenously applied GABA, i.e. the pool able to release by an external Ca^{2+}-independent manner, this release occurring via the release of intracellular stores of Ca^{2+} through a Na^+-dependent mechanism. Further studies of interest would be the effect of a Na^+ free medium on evoked release, the result of treatment with ouabain or veratridine, thought to increase the internal sodium concentration (Archibald & White, 1974; Cunningham & Neal, 1981), and the effects of compounds such as quinidine, shown to stimulate Ca^{2+} loss from isolated brain mitochondria (Sandoval, 1980) causing elevated [^{3}H]GABA efflux from synaptosomes.

However, there are other opinions on the Na^+-dependence of GABA release in Ca^{2+} free medium. Haycock *et al*. (1978) have also found that Ca^{2+}-dependent GABA (and noradrenaline) release is abolished following removal of Na^+ from the incubating medium. This may be due to the GABA reuptake carrier, which is Na^+-coupled (Martin, 1973) and electrogenic (Nelson & Blaustein, 1982; Pastuszko *et al*., 1982), normally carrying GABA inside at the expense of Na^+. This carrier could work in reverse order if the nerve was depolarized or the Na^+ gradient favoured outward transport of GABA. The cytoplasmic release and necessity of Na^+ for this suggest the possible involvement of the GABA carrier (Haycock *et al*., 1978). However, the GABA transport process is also dependent on Cl^-, although whether chloride translocates across the membrane is debatable (Pastuszko *et al*., 1982; Radian & Kanner, 1983). Bernath (1992) has reviewed the GABA transporter in greater depth.

Glial release of GABA has been described and excess K^+ concentrations have been shown to release neurotransmitters from glial cells, especially at higher K^+ concentrations (see Bernath, 1992). Sellstrom and Hamberger (1977) found that K^+ stimulated the release of [^{3}H]-GABA from glia, in a Ca^{2+} independent manner, in line with the release we have observed. Elevation in glial GABA would be likely following GABA-T inhibition and it is feasible that this may be a reason for the lack of Ca^{2+}-dependence observed. It has been found that veratridine often fails to promote transmitter release from glial cells (Neal & Bowery, 1979; Minchin, 1975), hence an examination of the effect of veratridine on GABA release and Ca^{2+}-dependence may be of interest.

As previously discussed, these results are in contrast to those of Neal and Shah (1989), who showed a significant Ca^{2+}-dependence of GABA release following GABA-T inhibition. However, their work was carried out on a different brain area (cortex) and magnesium (20 mM) was also included in the low Ca^{2+} (0.2 mM) incubation medium. Interestingly they observed a reduction in GABA release with a Ca^{2+} content double that employed in this study (0.2 mM compared with 0.1 mM), with the increased magnesium. However, Ca^{2+}-dependence of GABA release was observed in control tissues in this study. In addition, the release of glutamate showed Ca^{2+}-dependence irrespective of GABA-T activity, indicating that the conditions used in this study were suitable for demonstrating Ca^{2+}-dependence. Another difference in these two studies was the use of high magnesium (20 mM) in the low Ca^{2+} medium, if external magnesium is elevated several fold relative to its physiological level, it inhibits neurotransmitter release (Nadler *et al*., 1977). The release of neurotransmitters following elevated magnesium conditions is discussed by Bernath (1992).

GABA uptake and ATPase activity are affected in the early phases of hepatic encephalopathy, leading to amino acid transmitter release (Moroni *et al*., 1983; Albrecht *et al*., 1985), with these changes involving glial cells. A Ca^{2+}-independent release of GABA was observed in response to 75 mM KCl depolarization from the astrocytes of thioacetamide-induced early hepatic encephalopathy in rats (Albrecht & Rafalowska, 1987), compared to normal rats evoking a slow GABA efflux. Hepatic encephalopathy seems to produce the same changes in neurotransmitter content as does GABA-T inhibition, perhaps the same reasons for Ca^{2+} independence of release are involved in these two states.

In conclusion, the anti-convulsant activity of γ-vinyl GABA and EOS is presumably due to their ability to increase GABA release, although it is unclear which is the most important factor, the increased intracellular GABA content 'leaking' out of neurones and glia leading to widespread inhibition or the increase in stimulated release following depolarization caused by an epileptic discharge.

M.Q. was supported by a grant from the Medical Research Council.

References

ABDUL-GHANI, A.S., NORRIS, P.J., SMITH, C.C.T. & BRADFORD, H.F. (1981). Effects of γ-acetylenic GABA and γ-vinyl GABA on synaptosomal release and uptake of GABA. *Biochem. Pharmacol.*, **30,** 1203 – 1209.

ADAM-VIZI, V. (1992). External Ca^{2+}-independent release of neurotransmitters. *J. Neurochem.*, **58,** 395 – 405.

ALBRECHT, J. & RAFALOWSKA, U. (1987). Enhanced potassium-stimulated γ-aminobutyric acid release by astrocytes derived from rats with early hepatic encephalopathy. *J. Neurochem.*, **49,** 9 – 11.

ALBRECHT, J., WYSMYK-CYBULA, U. & RAFALOWSKA, U. (1985). The Na^+/K^+ ATPase activity and GABA uptake in astroglial cell-enriched fractions and synaptosomes derived from rats in the early stage of experimental hepatic encephalopathy. *Acta Neurol. Scand.*, **72,** 317 – 320.

ALNAES, E., MEIRI, U., RAHAMIMOFF, H. & RAHAMIMOFF, R. (1974). Possible role of mitochondria in transmitter release. *J. Physiol.*, **241,** 30P.

ALNAES, E. & RAHAMIMOFF, R. (1975). On the role of mitochondria in transmitter release from motor nerve terminals. *J. Physiol.*, **248,** 285 – 306.

ARCHIBALD, J.T. & WHITE, T.D. (1974). Rapid reversal of internal Na^+ and K^+ of synaptosomes by ouabain. *Nature*, **252,** 595 – 596.

ATTWELL, D., BARBOUR, B. & SZATKOWSKI, M. (1993). Non-vesicular release of neurotransmitter. *Neuron*, **11,** 401 – 407.

BAXTER, M.G., FOWLER, L.J., MILLER, A.A. & WALKER, J.M.G. (1973). Some behavioural and anticonvulsant actions in mice of ethanolamine O-sulphate, an inhibitor of 4-aminobutyrate transferase. *Br. J. Pharmacol.*, **47,** 681P.

BERNATH, S. (1992). Calcium independent release of amino acid neurotransmitters: fact or artifact? *Prog. Neurobiol.*, **38,** 57 – 91.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.*, **72,** 248 – 254.

CARAFOLI, E., TIOZZO, R., LUGLY, G., CROVETTI, F. & KRATZING, C. (1974). The release of calcium from heart mitochondria. *J. Mol. Cell. Cardiol.*, **6,** 361 – 371.

CUNNINGHAM, J. & NEAL, M.J. (1981). On the mechanism by which veratridine causes a calcium independent release of γ-aminobutyric acid from brain slices. *Br. J. Pharmacol.*, **73,** 655 – 667.

FOWLER, L.J. (1973). Analysis of the major amino acids of rat brain after *in vivo* inhibition of GABA transaminase by ethanolamine O-sulphate. *J. Neurochem.*, **21,** 437 – 440.

FOWLER, L.J. & JOHN, R.A. (1972). Active site directed irreversible inhibition of ethanolamine O-sulphate *in vitro* and *in vivo*. *Biochem. J.*, **130,** 569 – 573.

GRAM, L., LARSSON, O.M., JOHNSON, A.H. & SCHOUSBOE, A. (1988). The effect of valproate, vigabatrin and amino-oxyacetic acid on release of endogenous and exogenous GABA from cultured neurones. *Epilepsy Res.*, **2,** 87 – 95.

GRANT, S.M. & HEEL, R.C. (1991). Vigabatrin. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in epilepsy and disorders of motor control. *Drugs*, **41,** 889 – 926.

HAYCOCK, J.W., LEVY, W.B., DENNER, L.A. & COTMAN, C.W. (1978). Effects of elevated $[K^+]_0$ on the release of neurotransmitters from cortical synaptosomes. Efflux or secretion? *J. Neurochem.*, **30,** 1113 – 1125.

JOHN, R.A., RIMMER, E.M., WILLIAMS, J., COLE, G., FOWLER, L.J. & RICHENS, A. (1987). Microvacuolation in rat brains after long term administration of GABA-transaminase inhibitors. *Biochem. Pharmacol.*, **36,** 1467 – 1473.

JUNG, M.J., LIPPERT, B., METCALF, B.W., BOHLEN, P. & SCHECHTER, P.J. (1977). γ-Vinyl GABA (4-amino-hex-5-enoic acid), a new selective inhibitor of GABA-T: Effects on brain GABA metabolism in mice. *J. Neurochem.*, **29,** 797 – 802.

LEVI, G., BANAY-SCHWARTZ, M. & RAITERI, M. (1981). Studies on the release of exogenous and endogenous GABA and glutamate from rat brain synaptosomes. *Neurochem. Res.*, **6,** 275 – 285.

LINDROTH, P. & MOPPER, K. (1979). High performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluoresence derivatisation with *o*-phthaldialdehyde. *Anal. Chem.*, **51,** 1667 – 1674.

MARTIN, D.L. (1973). Kinetics of the sodium-dependent transport of gamma-aminobutyric acid by synaptosomes. *J. Neurochem.*, **21,** 345 – 356.

MCMAHON, H.T. & NICHOLLS, D.G. (1990). Transmitter glutamate release from isolated nerve terminals: evidence for biphasic release and triggering by localized Ca^{2+}. *J. Neurochem.*, **56,** 86 – 94.

MELDRUM, B.S. (1985). GABA and other amino acids. In *Handbook of Experimental Pharmacology: Antiepileptic Drugs*, ed: Frey, M.M. & Hanz, D. pp. 153 – 188. Berlin: Springer-Verlag.

MINCHIN, M.C.W. (1975). Factors influencing the efflux of [^{3}H]gamma-aminobutyric acid from satellite glial cells in rat sensory ganglia. *J. Neurochem.*, **24,** 571 – 577.

MORONI, F., LOMBARDI, G., MONETI, G. & CORTESINI, C. (1983). The release and neosynthesis of glutamic acid are increased in experimental models of hepatic encephalopathy. *J. Neurochem.*, **40,** 850 – 854.

NADLER, J.V., VACA, K.W., WHITE, W.F., LYNCH, G.S. & COTMAN, C.W. (1976). Aspartate and glutamate as possible transmitters of excitatory hippocampal afferents. *Nature*, **260,** 538 – 540.

NADLER, J.V., WHITE, W.F., VACA, K.W., REDBURN, D.A. & COTMAN, C.W. (1977). Characterization of putative amino acid transmitter release from slices of rat dentate gyrus. *J. Neurochem.*, **29,** 279 – 290.

NEAL, M.J. & BOWERY, N.G. (1979). Differential effects of veratridine and potassium depolarization on neuronal and glial GABA release. *Brain Res.*, **167,** 337 – 343.

NEAL, M.J. & SHAH, M.A. (1989). Baclofen and phaclofen modulate GABA release from slices of rat cerebral cortex and spinal cord but not from retina. *Br. J. Pharmacol.*, **98,** 105 – 112.

NELSON, M.T. & BLAUSTEIN, M.P. (1982). GABA efflux from synaptosomes: Effects of membrane potential, and external GABA and cations. *J. Membr. Biol.*, **69,** 213 – 223.

NICHOLLS, D.G. & SIHRA, T.S. (1986). Synaptosomes possess an exocytotic pool of glutamate. *Nature*, **321,** 772 – 773.

NICHOLLS, D.G., SIHRA, T.S. & SANCHEZ-PRIETO, J. (1987). Calcium-dependent and independent release of glutamate from synaptosomes monitored by continuous fluorometry. *J. Neurochem.*, **49,** 50 – 57.

PASTUSZKO, A., WILSON, D.F. & ERECINSKA, M. (1982). Energetics of γ-aminobutyrate transport in rat brain synaptosomes. *J. Biol. Chem.*, **257,** 7514 – 7519.

PHILIPS, N.I. & FOWLER, L.J. (1982). The effects of sodium valproate on 4-aminobutyrate metabolism and behaviour in naive and ethanolamine O-sulphate treated rats and mice. *Biochem. Pharmacol.*, **31,** 2257 – 2261.

QUME, M., DAVIES, J.D. & FOWLER, L.J. (1992). Chronic treatment with the GABA transaminase inhibitors ethanolamine O-sulphate and vigabatrin enhance basal GABA release from cross chopped hippocampal slices. *Br. J. Pharmacol.*, **105,** 170P.

QUME, M. & FOWLER, L.J. (1992). 21 Day treatment with the GABA-T inhibitors ethanolamine O-sulphate and γ-vinyl GABA elevate basal and stimulated GABA release. *Br. J. Pharmacol.*, **106,** 29P.

QUME, M. & FOWLER, L.J. (1996). The effect of chronic treatment with the GABA transaminase inhibitors γ-vinyl GABA and ethanolamine O-sulphate on the peripheral GABAergic system. *Biochem. Pharmacol.*, **52,** 1355 – 1363.

QUME, M., WHITTON, P.S. & FOWLER, L.J. (1995). The effect of chronic treatment with the GABA transaminase inhibitors γ-vinyl GABA and ethanolamine O-sulphate on the *in vivo* release of GABA from rat hippocampus. *J. Neurochem.*, **64,** 2256 – 2261.

RADIAN, R. & KANNER, B.I. (1983). Stoichemetry of sodium- and chloride-coupled γ-aminobutyric acid transport by synaptic plasma membrane vesicles isolated from rat brain. *Biochemistry*, **22,** 1236 – 1241.

RAITERI, M., FEDERICO, R., COLETTI, A. & LEVI, G. (1975). Release and exchange studies relating to the synaptosomal uptake of GABA. *J. Neurochem.*, **24,** 1243 – 1250.

REDBURN, D.A., SHELTON, D. & COTMAN, C.W. (1976). Calcium-dependent release of exogenously loaded γ-amino-[U-^{14}C]butyrate from synaptosomes: time course of stimulation by potassium, veratridine, and the calcium ionophore, A23187. *J. Neurochem.*, **26,** 297 – 303.

ROGERS, K.L., PHILIBERT, R.A., ALLEN, A.J., MOLITOR, J., WILSON, E.J. & DUTTON, G.R. (1987). HPLC analysis of putative amino acid neurotransmitters released from primary cerebellar cultures. *J. Neurosci. Methods*, **22,** 173 – 179.

RYAN, L.D. & ROSKOSKI, R. (1975). Selective release of newly synthesised and newly captured GABA from synaptosomes by potassium depolarisation. *Nature*, **258,** 254–256.

SALVADOR, R.A. & ALBERS, R.W. (1959). The distribution of glutamic-acid-γ-aminobutyric transaminase in the nervous system of the Rhesus monkey. *J. Biol. Chem.*, **234,** 922–925.

SANDOVAL, M.E. (1980). Studies on the relationship between Ca^{2+} efflux from mitochondria and the release of amino acid neurotransmitters. *Brain Res.*, **181,** 357–367.

SELLSTROM, A. & HAMBERGER, A. (1977). Potassium-stimulated γ-aminobutyric acid release from neurons and glia. *Brain Res.*, **119,** 189–198.

SIHRA, T.A. & NICHOLLS, D.G. (1987). 4-Aminobutyrate can be released exocytotically from guinea-pig cerebral cortical synaptosomes. *J. Neurochem.*, **49,** 261–267.

SILBERGELD, E.K. (1977). Na regulates release of Ca sequestered in synaptosomal mitochondria. *Biochem. Biophys. Res. Commun.*, **77,** 464–469.

SRINIVASAN, V., NEAL, M.J. & MITCHELL, J.F. (1969). The effect of electrical stimulation and high potassium concentrations on the efflux of [^{3}H] γ-aminobutyric acid from brain slices. *J. Neurochem.*, **16,** 1235–1244.

SZERB, J.C. (1979). Relationship between Ca^{2+}-dependent and independent release of [^{3}H]GABA evoked by high K^+, veratridine or electrical stimulation from rat cortical slices. *J. Neurochem.*, **32,** 1565–1573.

SZERB, J.C. (1983). The release of [^{3}H]GABA formed from [^{3}H]glutamate in rat hippocampal slices: Comparison with endogenous and exogenous labelled GABA. *Neurochem. Res.*, **8,** 341–351.

SZERB, J.C., ROSS, T.E. & GUREVICH, L. (1981). Compartments of labelled and endogenous γ-aminobutyric acid giving rise to release evoked by potassium or veratridine in rat cortical slices. *J. Neurochem.*, **37,** 1186–1192.

WALDMEIER, P.C., WICKI, P., FELDTRAUER, J.J. & BAUMANN, P.A. (1989). Ca^{2+}-dependent release of endogenous GABA from rat cortical slices from different pools by different stimulation conditions. *Naunyn-Schmiedberg's Arch. Pharmacol.*, **339,** 200–207.

(Received November 22, 1996
Revised June 12, 1997
Accepted June 18, 1997)