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EFFECTS OF γ-ACETYLENIC GABA AND γ-VINYL GABA ON SYNAPTOSOMAL RELEASE AND UPTAKE OF GABA

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Abstract—The effects of the irreversible inhibitors of GABA-transaminase (E.C.2.6.1.19), γ-acetylenic GABA (4-amino-hex-5-ynoic acid, RMI 71645) and γ-vinyl GABA (4-amino-hex-5-enoic acid, RMI 71754) on the release and uptake of endogenous and exogenous GABA by rat cerebral cortical synaptosomes were studied. γ-Acetylenic GABA increased the release of preloaded [$U^{14}C$]-GABA by 12 to 46% over the concentration range 0.5 to 5 mM. With γ-vinyl GABA, the concentration range 0.25–5 mM caused an increase of 22–95% above controls. All concentrations of γ-vinyl GABA were more effective than equivalent levels of γ-acetylenic GABA. The two agents had similar effects on the release of endogenous GABA, no change was observed in any other amino acid. The effect of both drugs on [$U^{14}C$]-GABA release was inhibited significantly by tetrodotoxin and blocked by Verapamil.

When animals were pretreated with γ-acetylenic GABA (100 mg/kg) for 8 hr or γ-vinyl GABA (1000 mg/kg) for 14 hr, the control release of GABA from synaptosomes prepared subsequently was increased, but other amino acids showed no change. The GABA content of the tissue was also greatly increased. The veratrine-stimulated release of GABA, glutamate and aspartate were increased by this pretreatment, but no change occurred in the rates of release of other amino acids. All veratrine-stimulated changes in the release of endogenous GABA, glutamate and aspartate were prevented by tetrodotoxin.

γ-Acetylenic GABA and γ-vinyl GABA are two catalytic irreversible inhibitors of GABA-transaminase [1–3]. They cause a sharp decrease of whole brain GABA-T activity and a dose-dependent increase in brain GABA concentration [1, 4]. *In vivo*, both drugs elicit a complex response with features such as sedation and lowered body temperature [5].

These agents have been shown to have strong anti-convulsant properties [6–8], and this has been attributed to their ability to raise brain GABA content, though the precise mechanism for this remains obscure. This laboratory has reported that these agents administered intraperitoneally caused the appearance of GABA in superfusates washing the cerebral cortex [9, 10] and, other laboratories [11, 12] have shown an increase of GABA levels in the cerebrospinal fluid and blood following intraperitoneal injection of both γ-acetylenic GABA and γ-vinyl GABA. Whether these elevated extracellular concentrations of GABA are due to enhancement of its release or result from a decreased ability to take up GABA once released becomes a question of special interest. The present study reports the effects of the agents on the release and on the uptake of GABA by synaptosomes isolated from the cerebral cortex.

MATERIALS AND METHODS

Materials. γ-Acetylenic GABA (4-amino-hex-5-ynoic acid, RMI 71645) and γ-vinyl GABA (4-amino-hex-5-enoic acid, RMI 71754) were a gift of Dr. J. Wilkins and his colleagues at the Centre de Recherche Merrel International, Strasbourg, whilst Verapamil was a gift of Abbott Laboratories, Queenborough, Kent. Radioactive 4-amino-*n*-[$U^{14}C$]butyric acid (224 Ci/mmole) was purchased from Amersham, England.

Methods. Whole cerebral cortex tissue was rapidly dissected from female Sprague–Dawley rats (200–250 g) following death by exsanguination, and placed in ice cold 0.32 M sucrose. Synaptosomes were subsequently prepared by the method of Gray and Whittaker [13] as modified by Bradford and Thomas [14]. The synaptosomal pellet was resuspended in a suitable volume of Krebs-phosphate medium of composition (mM): NaCl, 124; KCl, 5; KH_2PO_4, 1.2; $CaCl_2$, 0.75; $MgSO_4$, 1.3; Na_2HPO_4, 20; glucose, 10; and at pH 7.4. The medium was thoroughly gassed with oxygen prior to resuspension.

[$U^{14}C$]-*GABA and endogenous GABA release studies (*in vitro*).* Synaptosomes were incubated (3 mg protein/ml phosphate medium) for 20 min in the presence of 10 μM [$U^{14}C$]-GABA (final sp. act. 0.25 μCi/mmole) and sedimented in a bench top-centrifuge prior to resuspension in phosphate medium containing either Ca^{2+} or the calcium antagonist 5-[(3,4-dimethoxy phenethyl) methyl amino]-2-(3,4-dimethoxyphenyl)-2-iso propyl valeronitrile (Verapamil) (100 μM). These suspensions were

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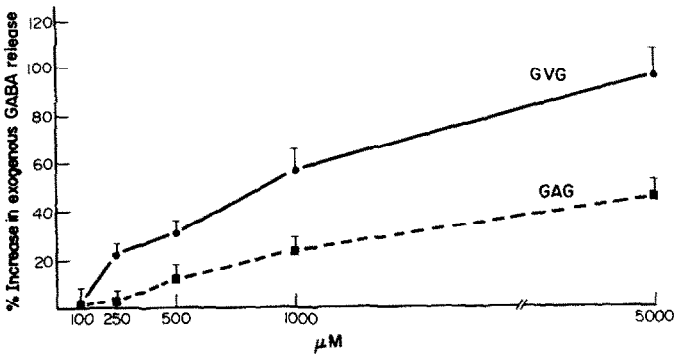

Fig. 1. Effect of GABA-analogues on release of preloaded [U^{14}C]-GABA. Experimental details are as for Fig. 2. The values are mean ± S.E.M. for ten experiments (GVG = γ-vinyl GABA; GAG = γ-acetylenic GABA).

incubated for 30 min before tetrodotoxin (TTX; 1.0 μM), γ-acetylenic GABA (0.5–5 mM) or γ-vinyl GABA (0.25–5 mM) were added as appropriate. Incubation continued for a further 10 min prior to sedimentation of the synaptosomes in a bench ultracentrifuge. The supernatants were added to vials. A toluene-based liquid scintillant was added and the vials were taken for radiolabel counting.

Endogenous GABA release studies (in vivo). Rats were injected intraperitoneally with γ-acetylenic GABA (100 mg/kg) or γ-vinyl GABA (1000 mg/kg) in saline, or with a similar volume of saline in control animals at 6 hr or 14 hr respectively, before preparation of cerebrocortical synaptosomes. These synaptosomes were incubated in Krebs-phosphate medium for 30 min prior to addition of tetrodotoxin (1 μM) or veratrine (75 μM) as appropriate. Incubation continued for a further 10 min. Synaptosomes were sedimented in a bench ultracentrifuge and the supernatants and pellets were extracted with 10% TCA. The TCA extracts were purified on a semi-automatic ion exchange apparatus to collect amino acids. The eluates containing amino acids were taken to dryness on a Buchler Evapomix. Samples were then dissolved in 0.025 M HCl and analysed on an automatic amino acid analyser [15].

Uptake studies. In uptake studies, synaptosome suspension volumes of 1 ml containing approximately 3 mg/ml protein were allowed an equilibration period of 20 min before incubation with or without the drugs, or tetrodotoxin (1 μM), as appropriate at 37° for 5 min. Then [U^{14}C] GABA (final sp. act. 224 μCi/μmole) was added to a final concentration of 1 μM and this incubation proceeded for a further 2 or 5 min. The suspensions were then sedimented in a bench ultracentrifuge and the clear supernatants were discarded. The synaptosomal pellets were extracted with 1 ml 10% (w/v) TCA and resedimented. The supernatants were dissolved in 20 ml of liquid scintillant (toluene, 1 litre; methoxyethanol, 800 ml; 2(4-tert-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole, 6 g) and counted using a Packard-Tricarb scintillation counter. The extracted pellets were assayed for protein according to the method of Lowry *et al.* [16].

Purity and stability of drug samples. No GABA contamination of the γ-vinyl GABA used could be detected when 5 mM samples were analysed (i.e. 0.1% GABA present at the limit of detection). A check was made on the breakdown, during incubation of γ-vinyl GABA and γ-acetylenic GABA to GABA. For this purpose synaptosomes (3 mg protein/ml) were incubated at 37° for 20 min. Tissue was then sedimented and lysed in ice-cold water (1.5 ml water/3 mg protein). Membranes and lysate were collected and stored. The drugs were then incubated for 5 min at 37° with each of these fractions, including the original incubation medium.

The calcium antagonist Verapamil at 100 μM had no measureable effect on the performance of synaptosomes in the control condition, but did prevent the depolarisation-induced release of amino acid neurotransmitters from synaptosomes caused by veratrine (75 μM).

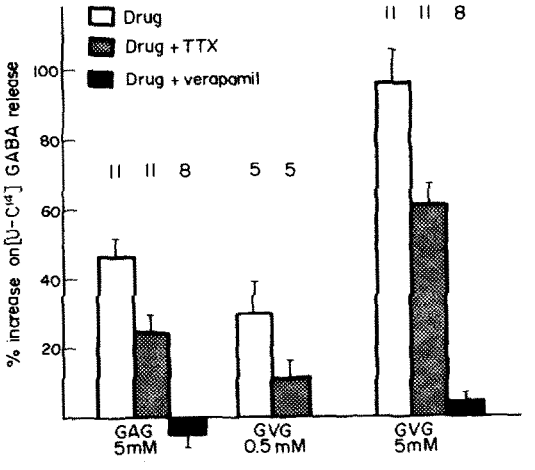

Fig. 2. Effect of tetrodotoxin and Verapamil on the release of preloaded [U^{14}C]-GABA from synaptosomes incubated with GABA-analogues. Synaptosomes were incubated (3 mg protein/ml phosphate medium) for 20 min in the presence of [U^{14}C]-GABA (final sp. act. 0.25 μCi/ml) and sedimented in a bench centrifuge prior to resuspension in phosphate medium containing either Ca^{2+} of 100 μM Verapamil. These suspensions were incubated for 30 min, before addition of TTX (1 μM), γ-vinyl GABA (0.25–5 mM), or γ-acetylenic GABA (5 mM) as appropriate. Incubation continued for a further 10 min prior to sedimentation of the synaptosomes in a bench ultracentrifuge and analysis of radioactivity in the supernates as described under Methods. Data represent mean ± S.E.M. for the number of experiments indicated above.

RESULTS

Release of preloaded [$U^{14}C$]-*GABA*

γ-Acetylenic GABA and γ-vinyl GABA at 1 mM concentrations caused a significant increase of preloaded [$U^{14}C$]-GABA release from incubated synaptosomes corresponding to 23 per cent ($P < 0.025$) and 57 per cent ($P < 0.005$) respectively (Fig. 1). This effect increased with drug concentration to 46 per cent with γ-acetylenic GABA (5 mM) and to 96 per cent with γ-vinyl GABA (5 mM).

The minimum effective dose with γ-acetylenic GABA was 0.5 mM (12%) and with γ-vinyl GABA, 0.25 mM (22%).

Tetrodotoxin (1 μM) added to the incubated synaptosomes one minute before γ-acetylenic GABA (5 mM) and γ-vinyl GABA (5 mM and 0.5 mM) significantly inhibited the release of [$U^{14}C$]-GABA by 46, 35 and 60 per cent respectively (Fig. 2).

The release of [$U^{14}C$[-GABA from synaptosomes incubated in the presence of either drug at 5 mM concentration was substantially inhibited (>90%) by Verapamil when added at 100 μM.

Release of endogenous GABA

When γ-vinyl GABA at a final concentration of 5 mM was added at the start of a 5 min incubation period to suspensions of cerebral cortical synaptosomes, GABA was greatly increased in concentration in the medium, from 22 ± 12 nmoles/100 mg protein to 171 ± 17 nmoles/100 mg protein, though no change was detectable in the release of other amino acids (Table 1). Tetrodotoxin (1 μM) reduced this GABA-releasing action by 58 per cent. Similar results were obtained with γ-acetylenic GABA, although it was not possible to separate γ-acetylenic GABA from GABA itself in the analytical system employed.

Over an *in vitro* incubation period of 50 min in the presence of γ-vinyl GABA (5 mM) the intrasynaptosomal pool of GABA increased by 55 per cent over control levels. (Control level: 1060 nmoles GABA/100 mg protein, drug-treated 1640 nmoles/100 mg protein). However, after 10 min the increase was only 11 per cent (not significant) and a pool size increase is not, therefore, likely to be a partial cause of the GABA "releasing" action of the drugs at this time.

Following *in vivo* (i.p.) administration of γ-acetylenic GABA (100 mg/kg) or γ-vinyl GABA (1000 mg/kg) for periods of 6 and 14 hr respectively, animals were sacrificed and cerebral cortical synaptosomes were prepared and incubated in a Krebs-phosphate medium for 30 min. Measurement of the total amino acid content of cerebro-cortical synaptosomes from treated and untreated rats showed that γ-acetylenic GABA and γ-vinyl GABA adminsitered *in vivo*, caused an increase of 3 to 4-fold in the content of GABA in the synaptosome preparation. No detectable changes were found with other amino acids (Table 2).

The spontaneous release of endogenous GABA from synaptosomes of animals pretreated with one of the drugs was much higher than from controls treated with saline. No change in release was observed in any of the other amino acids measured.

In the presence of depolarising concentrations of veratrine (Fig. 3), a large increase in the release of glutamate, aspartate and GABA occurred. Other amino acids remained unaffected. The GABA releasing action of Veratrine was approximately twice as great as that due γ-vinyl GABA over the same period.

However, the extent of this well established veratrine-stimulated release of putative neurotransmitter amino acids was larger (150–200%) when the animals were pretreated with γ-acetylenic GABA or γ-vinyl GABA. The stimulated release of endogenous glutamate, aspartate and GABA in all cases was totally inhibited when the synaptosomes were incubated with tetrodotoxin (1 μM; Fig. 3).

Effects of the agents on [$U^{14}C$]-*GABA uptake*

In addition to their effect on its release, γ-acetylenic GABA and γ-vinyl GABA at 5.0 mM concentrations caused a significant ($P < 0.001$) inhibition of [$U^{14}C$]-GABA uptake corresponding to 30 per cent and 52 per cent respectively (Fig. 4). This inhibition was reduced at 1 mM (Fig. 4) and only just detectable at 0.5 mM of either drug. Tetrodotoxin (1 μM) significantly reduced the GABA uptake-blocking actions of both drugs (Fig. 4).

Table 1. Amino acid accumulation in medium containing suspensions of rat cerebrocortical synaptosomes and γ-vinyl GABA or γ-acetylenic GABA to final concentrations of 5 mM*

	Control	γ-Vinyl GABA (5 mM)	γ-Acetylenic GABA
Aspartate	71 ± 25	77 ± 10	72 ± 12
Threonine	134 ± 13	198 ± 25	154 ± 16
Serine	293 ± 35	343 ± 19	324 ± 18
Glutamate	207 ± 85	233 ± 60	246 ± 50
Glutamine	34 ± 13	31 ± 8	37 ± 13
Glycine	159 ± 12	213 ± 26	174 ± 11
Alanine	222 ± 15	231 ± 24	250 ± 11
GABA	22 ± 12	171 ± 17	n.d.

* Synaptosome suspensions were incubated at 37° in a shaking water bath for 20 min prior to the addition of γ-vinyl GABA or γ-acetylenic GABA to final concentrations of 5 mM. Incubation continued for a further 5 min before separation of tissue and medium as described under Methods. All values are expressed as nmoles amino acid/100 mg protein and represent means ± S.E.M. for six or more experiments.

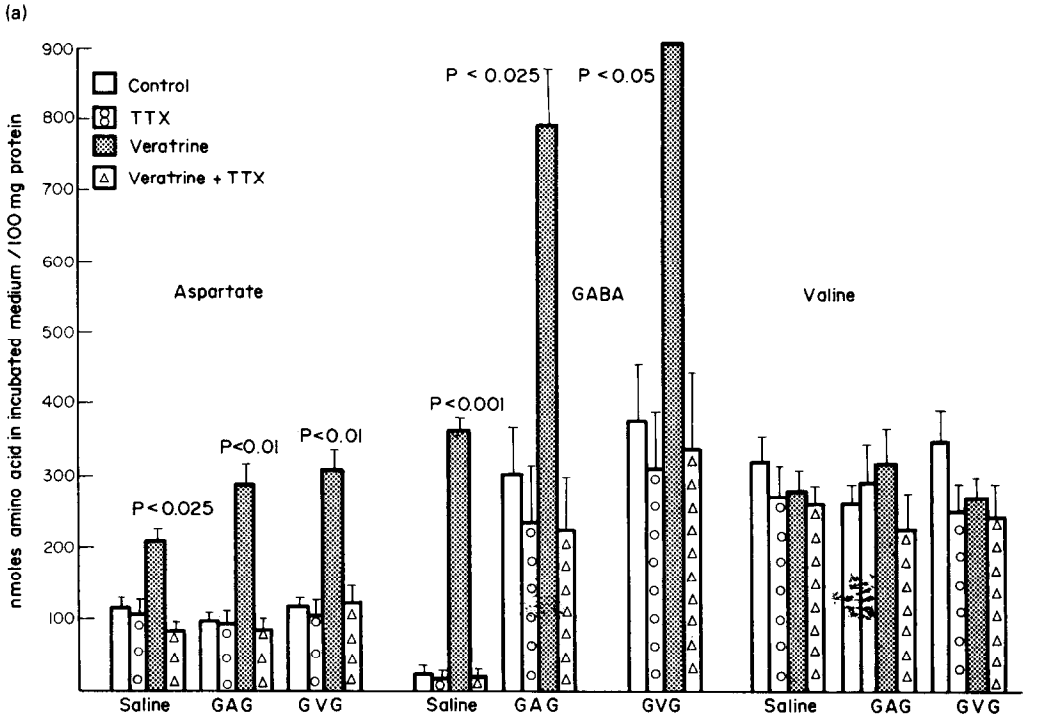
(a)
nmoles amino acid in incubated medium / 100 mg protein
Control
TTX
Veratrine
Veratrine + TTX
Aspartate
GABA
Valine
P < 0.025
P < 0.05
P<0.001
P<0.01
P<0.01
P<0.025
Saline
GAG
GVG
Saline
GAG
GVG
Saline
GAG
GVG

Fig. 3(a).

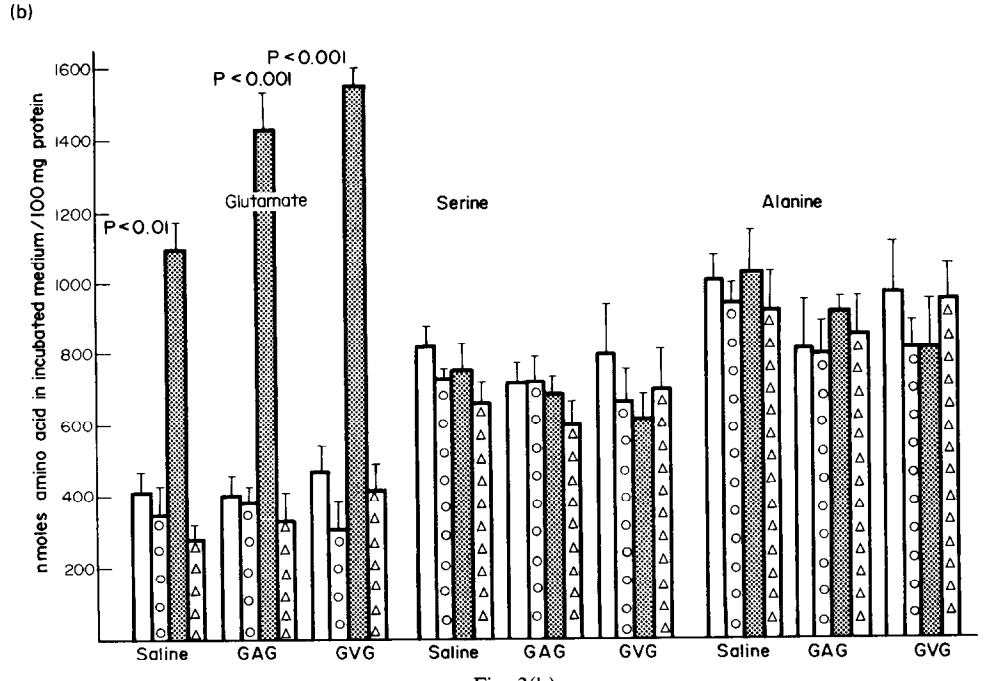
(b)
nmoles amino acid in incubated medium / 100 mg protein
P<0.001
P<0.001
P<0.01
Glutamate
Serine
Alanine
Saline
GAG
GVG
Saline
GAG
GVG
Saline
GAG
GVG

Fig. 3(b).

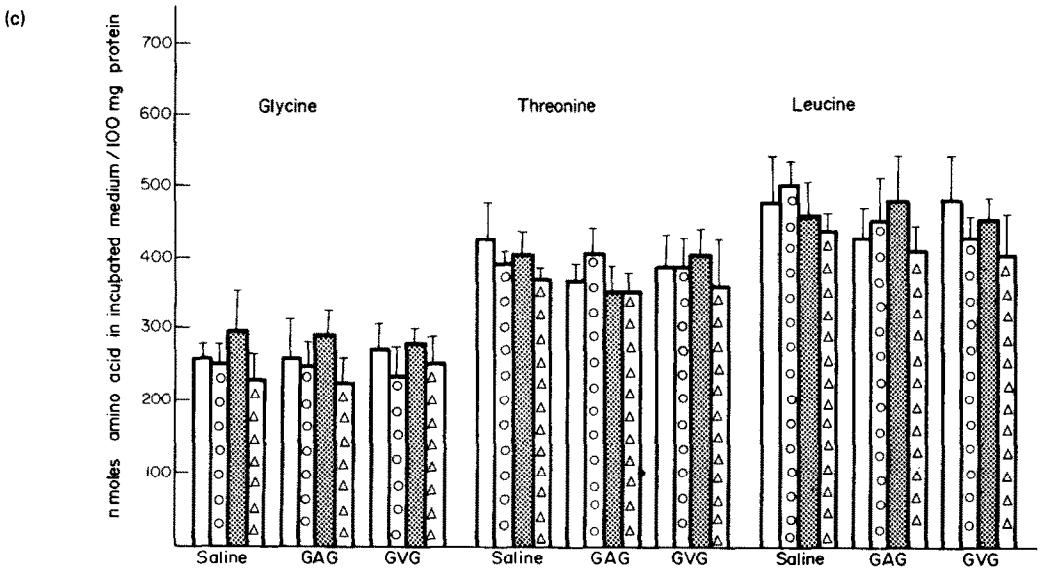

Fig. 3. Influence of the GABA-analogues on endogenous amino acid release. Fourteen hours prior to synaptosome preparation, rats were injected with γ-vinyl GABA (1000 mg/kg) or γ-acetylenic GABA (100 mg/kg, 6 hr) or with equivalent volumes of saline. Cerebral cortical synaptosomes were subsequently prepared and incubated (3 mg protein/ml medium) in Krebs-phosphate medium for 30 min prior to addition of TTX (1 μM) or Veratrine (75 μM) as appropriate. Incubation continued for a further 10 min. Synaptosomes were sedimented on a bench ultracentrifuge, and the supernatants and pellets were separated, and the amino acid content of supernatants was determined as described under Methods. Values are mean ± S.E.M. for four experiments.

In order to test whether this uptake inhibition property was due to the GABA analogue nature of the drugs, the action of non-isotopic GABA itself (1 or 5 mM) on [$U^{14}C$]-GABA uptake was examined. Although, as expected, it caused an 80–95 per cent reduction in [$U^{14}C$]-GABA uptake, added tetrodotoxin was without influence.

DISCUSSION

Actions of the drugs on amino acid release

The present report demonstrates that the two inhibitors of GABA-T, in addition to raising the GABA content of cortical synaptosomes, also exert a releasing action on both preloaded [$U^{14}C$]-GABA and on endogenous GABA, when presented at concentrations above 0.25 mM (γ-vinyl GABA) or 0.5 mM (γ-acetylenic GABA). Parallel work using the superfusion method for synaptosomes [17] (where re-uptake of neuroactive amino acids is reduced) has shown that smaller concentrations (100 μM) of both drugs are effective in releasing preloaded GABA [18]. Similar releasing actions of the drugs *in vivo* to superfusion fluid, occur when they are administered intraperitoneally in standard doses (γ-vinyl GABA, 1500 mg/kg; γ-acetylenic GABA, 200 mg/kg) which also have anti-convulsant actions and raise brain GABA content [9, 10]. Thus, comparison of these *in vitro* and *in vivo* actions indicates that levels in the range 0.1 to 0.5 mM or 0.25 mM of γ-acetylenic GABA and γ-vinyl GABA respectively are reached in the cerebral cortex after intraperitoneal injection of these doses.

It is not possible to calculate the concentration of the drugs in the cerebral tissue *in vivo* from levels detected in the superfusate. Also higher concentrations may occur in localised tissue regions due to accumulation of the GABA analogues by the GABA transport system.

Although the drugs cause a considerable rise in GABA content of synaptosomes and cortical tissue *in vivo*, this is a relatively long-term process, whilst the GABA-releasing action of the drugs on synaptosomes is seen within a few minutes. Therefore, there is no obligatory coupling between raised GABA content and the GABA-releasing action of the drugs.

Properties of the GABA-releasing process

The specific synaptosomal release of GABA was shown to be partly dependant (50%) on activation of tetrodotoxin-sensitive Na^+ channels, and also Verapamil-sensitive Ca^{2+} receptors or channels [19]. One explanation of this phenomenon is that the alkyl-GABA analogues are depolarizing only the GABA-releasing synaptosomes, presumably by activating a GABA receptor present only on these terminals. Alternatively, the relatively weak releasing action of the drugs added *in vitro* (only 25% of the response due to Veratrine over the same period), could be exerted on glutamate and aspartate as well as GABA, but only the latter was detectable because of the block on its re-uptake by the drugs. The capacity for uptake of glutamate and aspartate (i.e. glutamate = 433; aspartate = 130 nmoles/100 mg protein/5 min) could have reduced their concentration to control levels over the 5 min period of incubation.

Table 2. Effect of γ-acetylenic GABA and γ-vinyl GABA on intrasynaptosomal amino acid content (nmoles/100 mg protein)*

Pre-treatment	Treatment	Aspartate	Glutamate	Glycine	Serine	Valine	Alanine	Leucine	GABA
Saline	Control	3754 ± 248	4835 ± 393	672 ± 38	594 ± 27	165 ± 8	662 ± 23	266 ± 20	1164 ± 45
	Tetrodotoxin	3497 ± 300	4750 ± 661	615 ± 96	575 ± 22	174 ± 8	595 ± 54	238 ± 46	1158 ± 82
	Veratrine	1871 ± 228	4408 ± 381	487 ± 106	441 ± 26	143 ± 9	485 ± 42	197 ± 11	743 ± 15
	Verat. + TTX	3543 ± 606	4393 ± 418	602 ± 95	588 ± 53	180 ± 14	642 ± 53	223 ± 53	223 ± 17
γ-Acetylenic GABA	Control	2877 ± 164	4417 ± 415	749 ± 69	620 ± 86	177 ± 14	518 ± 16	239 ± 3	3698 ± 748
	Tetrodotoxin	2939 ± 225	4010 ± 168	772 ± 99	611 ± 52	204 ± 33	567 ± 30	282 ± 46	3561 ± 456
	Veratrine	1625 ± 135	4307 ± 564	535 ± 123	571 ± 74	166 ± 10	439 ± 72	224 ± 26	2678 ± 167
	Verat. + TTX	2841 ± 222	4683 ± 618	815 ± 134	593 ± 51	179 ± 21	526 ± 77	235 ± 35	4168 ± 252
γ-Vinyl GABA	Control	3396 ± 399	4715 ± 259	879 ± 188	654 ± 46	216 ± 45	690 ± 86	304 ± 15	4628 ± 396
	Tetrodotoxin	2985 ± 169	4227 ± 424	981 ± 224	468 ± 72	203 ± 22	714 ± 36	291 ± 10	4539 ± 837
	Veratrine	1679 ± 177	4176 ± 143	549 ± 100	562 ± 94	183 ± 5	445 ± 25	230 ± 12	3243 ± 60
	Verat. + TTX	3259 ± 322	4704 ± 534	817 ± 100	481 ± 86	191 ± 11	592 ± 22	244 ± 14	4751 ± 691

* The experimental details are exactly as described for Fig. 3.

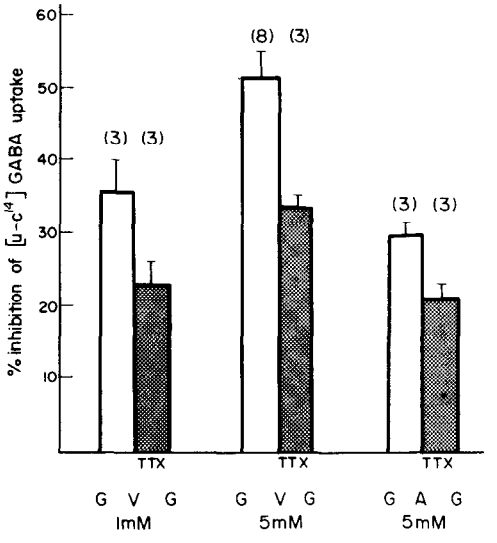

Fig. 4. Effect of Tetrodo toxin on GABA uptake into synaptosomes incubated with γ-acetylenic GABA and γ-vinyl GABA (GAG and GVG). Synaptosomes were incubated in Krebs-phosphate medium, pH 7.4 (containing 10 mM glucose and previously gassed with O_2) for 20 min prior to addition of γ-vinyl GABA (1 mM, 5 mM) or γ-acetylenic GABA (5 mM). Where appropriate, TTX (1 μM) was added to the suspension immediately before addition of the alkyl GABA analogues. The suspensions were incubated at 37° in a shaking water bath for 5 min prior to addition of [U^{14}C]-GABA (final activity 0.25 μCi/ml) to a final concentration of 1 μM. Incubation proceeded for further 5 min. After incubation, tissue and medium were separated and the tissue was analysed for radioactivity as described under Methods. The uptake of radio label in the presence of GVG or GAG is reported as a percentage of the uptake of [U-^{14}C]-GABA by control samples. Values are expressed as means ± S.E.M. with the number of determinations made indicated above the data points.

Action of veratrine

Whilst veratrine released extra quantities of total GABA from synaptosomes containing elevated amounts of GABA (i.e. from drug-treated animals) the *percentage* of total GABA released was similar to that seen for saline controls. However both aspartate and glutamate also showed larger release signals, yet they were *not* increased in their tissue level. These findings suggest that the alkyl-GABA analogues are enhancing the action of Veratrine, a conclusion which would be supported by the tetrodotoxin-sensitive nature of their releasing action discussed above.

The *in vitro*, GABA-releasing, actions reported here were not due to chemical or enzymatic breakdown of the drugs to GABA during incubation since synaptosomal lysates or membranes were unable to generate GABA from the drugs under similar conditions.

Actions of the drugs on amino acid uptake

Using short (1 to 5 min) incubation periods with [U^{14}C]-GABA, both drugs appeared to cause a significant inhibition of GABA uptake by synaptosomes

at those concentrations which increased apparent *release* of preloaded [U^{14}C]-GABA. However, part of this *uptake* inhibition was clearly due to the tetrodotoxin and Verapamil-sensitive release which occurred under the same conditions (Fig. 4).

The same consideration would apply to the finding of other workers (e.g. [20]) that these GABA-analogues inhibit GABA uptake.

Other GABA-T inhibitors such as amino-oxyacetic acid [21], ethanolamine-*O*-sulphate [22], and gabaculine [23] are also potent inhibitors of GABA uptake, though this property has been considered of secondary importance in their actions as anticonvulsants.

In conclusion, the results reported have shown that the alkyl-GABA analogues have *both* GABA-releasing and GABA-uptake inhibition properties which are likely to act together in producing the observed GABA efflux to superfusion fluids *in vivo* which are likely to underlie their anticonvulsant properties.

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