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EFFECTS OF ANTICONVULSANTS ON THE *IN VIVO* AND *IN VITRO* RELEASE OF GABA

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Abstract—The actions of various anticonvulsant compounds on GABA release *in vivo* and *in vitro* were studied. An *in vivo*, superfusion of sensorimotor cerebral cortex was employed and drugs were administered either by intraperitoneal injection, or in superfusion fluid, and release of endogenous amino acids was measured. The *in vitro* method involved superfusion of synaptosomes, with drugs dissolved in superfusate, with monitoring of the release of pre-loaded [U^{14}C]-GABA. Two alkyl-GABA analogues, γ-acetylenic GABA and γ-vinyl GABA caused enhanced release of GABA to superfusate both *in vivo* and *in vitro*. However, phenobarbitone, diphenyl hydantoin, sodium n-dipropyl acetate and carbamazepine were without effect on GABA release in either test system. Taurine caused no detectable GABA release *in vivo*, or from purified synaptosomes *in vitro*, but did stimulate release *in vitro*, from crude synaptosome preparations containing mitochondria in large quantities, though histidine and leucine were equally effective.

Agents which raise brain GABA content are of special interest as potential anticonvulsants because of the potent inhibitory action of this compound, and also because of the observed aberrations in the storage and metabolism of γ-aminobutyrate (GABA) in focal epileptic tissue obtained from experimental animal models, and in cases of human epilepsy [1]. The now widely used anticonvulsant sodium dipropyl acetate Epilim [2, 3] raises brain GABA content 1–2-fold by inhibiting the enzyme succinic semialdehyde dehydrogenase (EC1.2.1.24:SSADH) and GABA transaminase (4-amino-butyrate: 2-oxoglutarate amino transferase, EC2.6.1.19), two catabolic enzymes of GABA metabolism [3–7]. It also protects against pentylenetetrazole and electroshock convulsions [8]. Other agents which raise brain GABA content also commonly have anticonvulsant actions. Thus GABA transaminase inhibition by amino-oxyacetic acid [9, 10] and by γ-vinyl and γ-acetylenic GABA [11, 12], leads to a rise in GABA levels and correlating anticonvulsant action [13–18]. The question, therefore, arises as to whether commonly employed clinically effective anticonvulsants, and potential candidates for this role, produce their effects partially, or entirely, by raising brain GABA content, or by causing a rise in the concentration of this inhibitory agent in the extracellular fluid of brain. This could, for example, be achieved by preventing its re-uptake following its release from brain cells. This possibility was tested by following the release of endogenous and exogenous GABA both *in vivo* and *in vitro*.

MATERIALS AND METHODS

In vivo *superfusion technique.* This was carried out on adult female hooded Rowett rats (200–250 g body weight), which were implanted with a special superfusion cannula in the skull over the exposed sensorimotor cortex. The design, construction and method of implantation of the cannula have been described in detail elsewhere [19]. This method allows monitoring of the *in vivo* release of amino acids and other neurotransmitters from small areas of cerebral cortex of awake, unrestrained and behaviourally normal animals. The small size (4 mm diameter) and dead space of the cannula (15 μl) allows continuous superfusion of the local cortical surface over long periods. The superfusion fluid employed was sterilized saline (0.85% w/v) containing 1.3 mM $CaCl_2$.

After complete recovery of the animals from surgery and anaesthesia (48 hr) the anticonvulsant drugs were administered by i.p. injection whilst cortical superfusion was continued at 0.1 ml/min. Samples were collected in tubes containing HCl and norleucine to give final concentrations of 0.25 M and 5 pmoles/ml respectively. The samples were analysed employing a sensitive amino acid analyser as described elsewhere [21].

Sources of drugs tested. γ-Vinyl GABA (4-amino-hex-5-enoic acid RMI 71754) and γ-acetylenic GABA (4-amino-hex-5-ynoic acid RMI 71645) were synthesised and supplied by Centre de Recherche Merrell International, Strasbourg, France. Sodium-n-di-propyl acetate was supplied by Rickett and Coleman Ltd., Hull. Carbamazepine (Tegretol) was supplied by Geigy Pharmaceuticals, Macclesfield, Cheshire. Phenobarbitone and phenytoin sodium were supplied by May and Baker, Dagenham, Essex, England. All of these anticonvulsants were gifts for which we are most obliged. Radioactive 4-amino-n-

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Table 1. Amino acid release from sensorimotor cortex following treatment with anticonvulsant drugs (release rate pmoles/cm^2/min)

Treatment	Dose (mg/kg)	GABA‡	Glutamate	Aspartate	Glutamine and serine	Glycine	Alanine	Valine	Leucine
Control	—	—	48 ± 9 (27)	37 ± 6 (18)	304 ± 27 (14)	186 ± 20 (12)	119 ± 14 (25)	46 ± 8 (18)	64 ± 12 (22)
γ-Vinyl GABA	1000–1500†	8.31 ± 0.87 (28)	42 ± 5 (23)	39 ± 7 (12)	342 ± 23 (13)	185 ± 17 (23)	128 ± 14 (23)	52 ± 5 (23)	58 ± 8 (23)
γ-Acetylenic GABA	100	42.26 ± 4.16 (14)*	51 ± 4 (14)	47 ± 5 (14)	332 ± 22 (14)	153 ± 13 (14)	142 ± 11 (14)	76 ± 12 (14)	57 ± 4 (12)
Di-n-propyl acetate	200–400	—	51 ± 7 (12)	33 ± 5 (12)	270 ± 34 (12)	206 ± 30 (12)	131 ± 12 (12)	68 ± 11 (12)	46 ± 11 (12)
Phenobarbitone	50	—	50 ± 9 (3)	30 ± 4 (3)	283 ± 41 (3)	160 ± 20 (3)	120 ± 16 (3)	58 ± 7 (3)	73 ± 15 (3)
Phenytoin	100–150	—	49 ± 2 (2)	29 ± 5 (2)	288 ± 16 (2)	204 ± 17 (2)	117 ± 10 (2)	64 ± 5 (2)	61 ± 2 (2)
Carbamazepine	35	—	47	32 (1)	320 (1)	172 (1)	123 (1)	55 (1)	65 (1)
Taurine	200	—	51 ± 5 (4)	34 ± 2 (4)	301 ± 35 (4)	218 ± 27 (4)	159 ± 16 (4)	68 ± 11	69 ± 2 (4)

Release of amino acids from sensori-motor cortex was determined in 8 min fractions (0.8 ml) of superfusate, collected 30–120 min after i.p. administration of the drugs at doses as indicated above. Taurine (1–5 ml), and di-n-propyl acetate (1 mM) were also applied through the cannulae without causing any significant change.

Values are mean ± S.E.M. for the number of experiments indicated in brackets.

* The peak represents GABA and γ-acetylenic GABA, since we were not able to separate between them.

† When γ-vinyl GABA 100 μM was injected through the cannulae, the amount of GABA detected was only 4 pmoles/cm^2/min.

‡ The absence of GABA means <2 pmoles/cm^2/min.

[$U^{14}C$]-butyric acid (224 mCi/mmole) was purchased from Amersham Radio Chemicals, Amersham, Buckinghamshire, England.

Administration of drugs. All the drugs were administered i.p. as neutral isotonic solutions. Taurine, di-*n*-propyl acetate and γ-vinyl GABA were also introduced directly onto the superfused cortex, through the cannula as solutions in superfusion fluid.

The amino acids in the superfusate were measured using the amino acid analyser described previously [20, 21], and the concentrations, were expressed as pmoles/min/cm^2 of cortical surface exposed.

Preparation and superfusion of synaptosomes. Purified synaptosomes (P_2B) or crude synaptosomes (P_2) were prepared from the cerebral cortex of female Sprague Dawley rats (200–250 g body weight) by the method of Gray and Whittaker [22] as modified by Bradford *et al.* [20]. Following resuspension in a Krebs phosphate medium (1–3 mg protein/ml) of composition (mM): NaCl 124; KCl 5.0; KH_2PO_4 1.2, $CaCl_2$ 0.75; $MgSO_4$ 1.3; Na_2HPO_4 20; glucose 10; pH 7.4, the synaptosome preparations were pre-equilibrated for 30 min at 37° and then incubated with [$U^{14}C$]-GABA 2 μM (Amersham Radio Chemical Centre) at a final activity concentration of 0.5 μCi/ml. Portions of the suspension (0.5 ml) were then placed on Millipore filters (0.8 μm pores) situated at the bottom of 5 parallel superfusion chambers thermostated at 37° as described by Raiteri and Levi [23]. After the excess radioactivity had been washed off with oxygenated medium, the synaptosome deposit was superfused for an 8 min control period at 0.5 ml/min. Subsequently, oxygenated medium containing the test substance at various concentrations (100 μM–1 mM) was introduced. Fractions (0.5 ml) were collected, and the radioactivity was measured by liquid scintiallation counting employing a Beckman scintillation counter. The radioactivity remaining in the tissue was also measured.

Samples of superfusate were analysed by autoanalysis [21] for their total GABA content, and the pattern of release followed as counts and as total GABA were found to be very similar. Also the inclusion of alpha amino oxyacetic acid to inhibit GABA metabolism did not alter the pattern of responses observed. Taken together these observations suggest that the released radioactivity was principally present as GABA.

RESULTS

In vivo GABA release. GABA is not normally detectable (i.e. present at <10 nm) in superfusates of rat cerebral cortex [19, 24] but 1 to 3 hr after i.p. injection of γ-vinyl GABA (1500 mg/kg) the concentration of GABA in the superfusate rose to 6 to 9 pmoles/min/cm^2 (Table 1). Equally, GABA efflux levels reached 4 pmoles/min/cm^2 when γ-vinyl GABA was introduced into the superfusion fluid at 100 μM. The congener, γ-acetylenic GABA appeared to show similar patterns of GABA release, but it was not separated from GABA itself by our analytical method.

Neither alkyl GABA analogue caused any change in the rates of efflux to the superfusion fluid of any of seven other amino acids measured (Table 1).

Both γ-vinyl GABA and γ-acetylenic GABA raise brain GABA content about 5-fold [16, 17]. The anticonvulsant drug, sodium di-*n*-propylacetate causes a 1.5 to 2-fold change in brain GABA content [3, 7], but when given i.p. (200–400 mg/kg) in the present experiments, caused no detectable efflux of GABA over 1 to 3 hr. When delivered in the superfusion fluid (1 mM) it was also without action. Several other common anticonvulsants, namely, carbamazepine (36 mg/kg), phenobarbitone (50 mg/kg) and phenytoin (100–150 mg/kg) were also without detectable effect on GABA efflux rates over 1–6 hr periods.

Taurine, which has been reported to have substantial anti-convulsant actions [25–32], was also without effect on GABA levels in superfusion fluid when administered i.p. (200 mg/kg) or in the superfusion fluid (1–5 mM) *in vitro*. In addition, neither taurine nor any of the other anticonvulsants tested influenced the patterns of release of the other amino acids measured (Table 1).

In vitro GABA release. The actions of these compounds was tested on the release of preloaded radiolabelled GABA from rat brain synaptosome preparations. Continuous superfusion of these fractions was employed for this purpose. As shown by others [23, 33] high concentrations of K^+ ions (56 mM) caused a 3–3.5-fold increase in release of pre-loaded [$U^{14}C$]-GABA (Fig. 1). The two alkyl GABA derivatives at 100 μM also evoked substantial [$U^{14}C$]-GABA release (1.5–2.2-fold). At 200 μM γ-vinyl GABA, this rate could be increased by 6-fold.

None of the other anticonvulsants (sodium di-*n*-propyl acetate, carbamazepine, phenobarbitone, ethosuccimide, phenytoin) tested had any detectable influence on the rate of release of pre-loaded GABA to the superfusion fluid, even at concentrations up to 1 mM.

Actions of amino acids. Taurine at 100 μM doubled the rate of [$U^{14}C$]-GABA efflux from crude synaptosome fractions over a 5 min period (Fig. 2) but this was not specific to taurine, since histidine and leucine at 100 μM were equally effective. Unlabelled GABA itself at this concentration was 2 to 3 times as effective as the other amino acids in releasing [$U^{14}C$]-GABA.

However, pure synaptosomal preparations did not show this pattern of taurine-induced GABA release, or any changes with histidine or leucine.

DISCUSSION

None of the common, clinically employed, anticonvulsants tested when administered acutely in doses at or above those known to be clinically effective, caused detectable release of endogenous GABA *in vivo* or of exogenous GABA *in vitro*. This suggests that neither the benzodiazepines (carbamazepine), hydantoins (phenytoin) nor barbiturates (phenobarbitone) exert their anticonvulsant actions by raising extracellular GABA during the periods employed in the experiments reported here (i.e., *in vivo*: 1–3 hr, and *in vitro*: 10–15 min). However, this does not exclude the possibility that longer term *in vivo* treatment (usually required for clinical effectiveness) or superfusion of synaptosomes from such animals could demonstrate a propensity of these

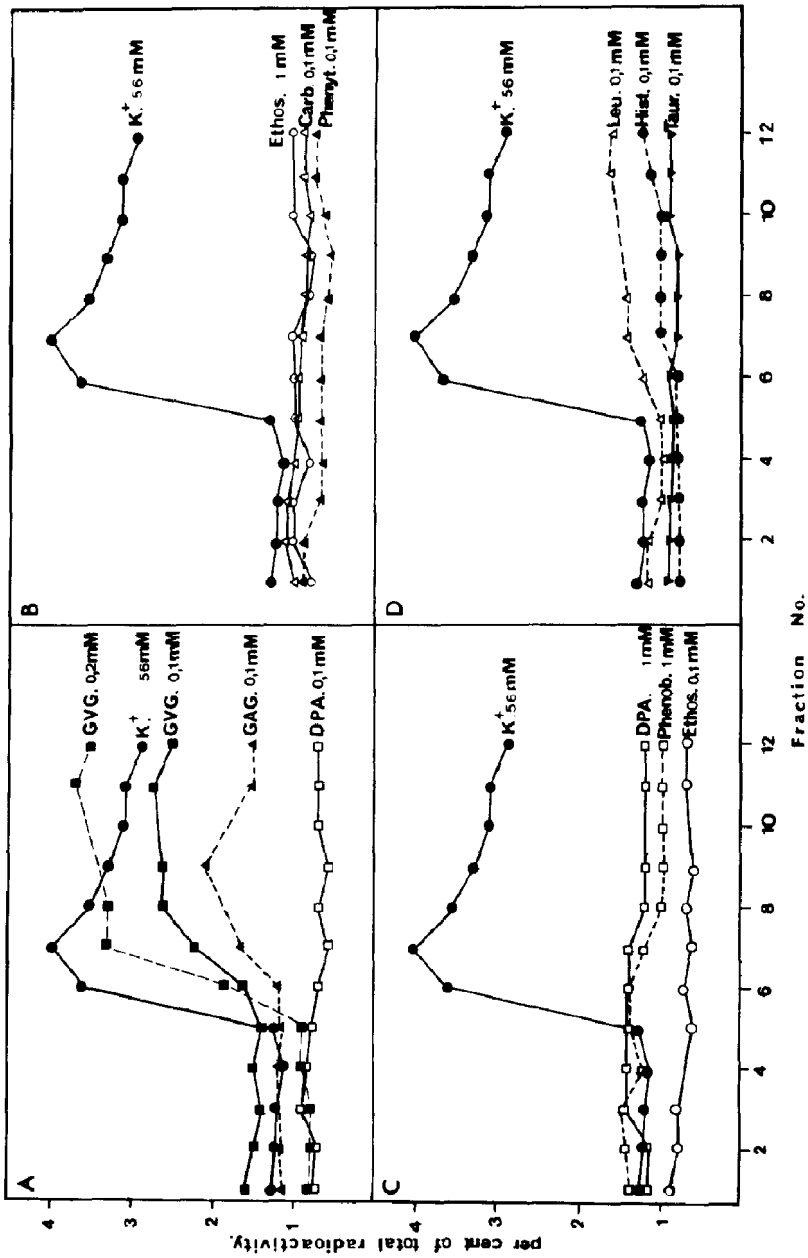

Fig. 1. Effect of anticonvulsants on the release of [U-^{14}C]-GABA from superfused synaptosomes. Rat cortical synaptosomes were resuspended in oxygenated Krebs-phosphate medium (1–3 mg/ml) pH 7.4, containing 10 mM glucose. Following a 30 min preincubation at 37°, the synaptosomes were then pre-loaded with [U-^{14}C]-GABA (final concentration 2 μM, activity concentration 0.5 μCi/mole). Aliquots of the incubates were then superfused as described in Materials and Methods. After 8 min of superfusion with standard medium the test medium was added to give the final concentrations as indicated in the figure. Except for phenytoin and carbamazepine all compounds were prepared with oxygenated Krebs-phosphate medium pH 7.4. Phenytoin was dissolved in a small volume of medium pH > 11.7, followed by medium dilution to pH 7.4. Carbamazepine was dissolved in a few drops of absolute alcohol followed by medium dilution to pH 7.4. GAG: γ-acetylenic GABA; GVG: γ-vinyl GABA; DPA: di-*n*-propyl acetate; Phenob: phenobarbitone; Phenyt: sodium phenytoin; Ethos: ethosuccimide; Carb: carbamazepine; Hist: histadine; Leu: leucine; Taur: taurine.

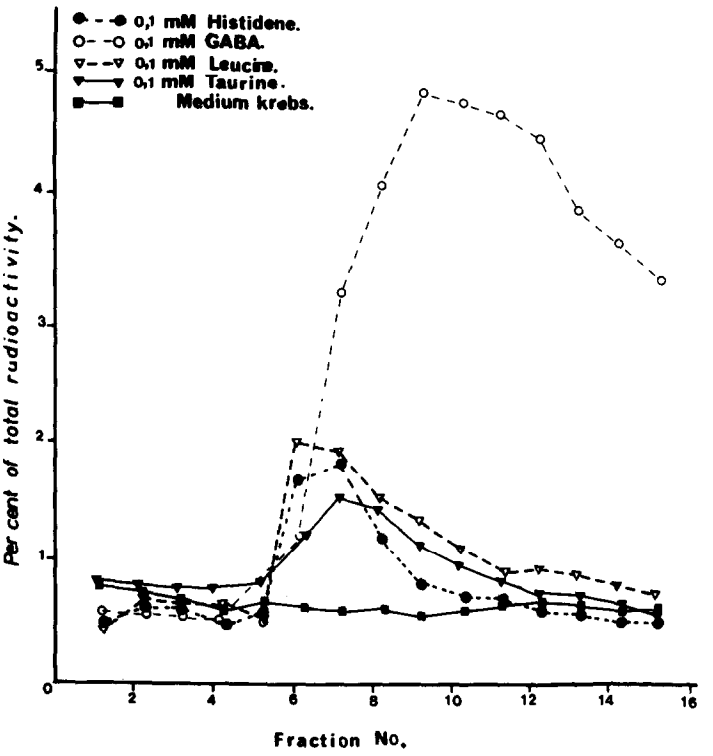

Fig. 2. The release of [U^{14}C]-GABA from crude mitochondrial fractions on superfusion with other amino acids. Experimental conditions as described in legend to Fig. 1. After 8 min of superfusion, with standard medium, the test medium was added to give the indicated final concentrations. All amino acids were prepared in oxygenated Krebs-phosphate medium pH 7.4.

drugs to release GABA. Also an action as GABA agonists at GABA receptors as indicated for benzodiazepines is a clear possibility [34].

Even dipropyl acetate which raises brain GABA by 1–2-fold when given i.p. over the period studied [3] did not cause detectable *in vivo* release at the cortical surface. Re-uptake is unlikely to have prevented an equilibrium level of raised extracellular GABA at deeper levels (layers 4 to 5 where GABA terminals are concentrated) from being detected.

This contrasts with the actions of γ-vinyl and γ-acetylenic GABA which released GABA both *in vivo* and *in vitro*.

The *in vitro* effects on preloaded [U^{14}C]-GABA release are likely to reflect an uptake blocking action of the compounds tested [35] in addition to any boosting of GABA content. It has previously been shown that the alkyl GABA analogues have releasing and uptake blocking components to their role in elevating GABA levels extraterminally [36].

In vivo release of endogenous GABA by the two alkyl-GABA analogues has been reported previously [32], as have its ability to raise GABA levels in cerebrospinal fluid and blood [38, 39].

Taurine. This substance has been found to have anticonvulsant properties in a wide range of test systems [25–28], and also to be clinically effective in controlling fits in human epileptics [30–32, 40, 41].

The absence of an effect of taurine on GABA release in our *in vivo* superfusion system suggests that it is not having any appreciable action in releasing endogenous GABA. The non-specific effect of taurine in releasing preloaded [^{14}C]-GABA from superfused crude synaptosomal preparations suggests some interference with GABA transport processes here, but this was not a potent action when compared with GABA itself, and was not a property of purified synaptosome preparations. A similar action of taurine on GABA release *in vitro* was reported by Pasantes-Morales [42]. Thus, on the evidence presented here, taurine does not appear to exert its anticonvulsant action by raising extracellular GABA levels *in vivo*.

In summary, the *in vivo* and *in vitro* results taken together indicate that there is no correlation between anticonvulsive activity of the drugs and changes in GABA accumulation extracellularly.

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