EVIDENCE THAT A SPECIFIC SUCCINIC SEMIALDEHYDE REDUCTASE IS RESPONSIBLE FOR γ-HYDROXYBUTYRATE SYNTHESIS IN BRAIN TISSUE SLICES

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1. Introduction

γ-Hydroxybutyric acid (GHB) is a minor brain catabolite of γ-aminobutyric acid (GABA) which, when administered systematically to animals and man, induces in both multiple neuropharmacological effects (review [1]). From rat and human brain, two enzymes have been isolated which can reduce succinic semialdehyde (SSA) to GHB [2–4]. In each case, one of these enzymes was non-specific for this reaction and could reduce a wide range of aldehydes, whereas the other enzyme showed a high degree of specificity for SSA reduction. The non-specific SSA reductase from both species was strongly inhibited by barbiturates and various branched-chain fatty acids, whereas the specific enzyme was little affected by most of these compounds.

It is of great interest to determine to what extent these two enzymes participate in GHB formation in vivo. To investigate this, we studied the biosynthesis of GHB from labelled GABA in rat brain cerebellar slices, an area rich in SSA reductase enzymes [5]. These experiments were performed in the absence and presence of 3 compounds differentially inhibiting the purified enzymes capable of catalyzing the oxidation of SSA by succinic semialdehyde dehydrogenase and its reduction to GHB.

2. Materials and methods

SSA was obtained from Sigma and its concentration assayed using purified rat brain succinic semialdehyde dehydrogenase [6]. 2,3-Di-*n*-propylhexan 3-ol-oic acid (GT 16) and 4-*n*-propylheptanoic acid (GT 43) were gifts from Dr G. Taillandier (Laboratoire de Chimie et de Toxicologie, Université Scientifique et Médicale de Grenoble). [2,3-^{3}H]GABA (spec. act. 45 Ci/mmol) and [2,3-^{3}H]GHB (spec. act. 40 Ci/mmol) were purchased from the CEA (Saclay).

2.1. *Enzyme assays used to test the effects of the three potential inhibitors*

(1) GABA-transaminase (GABA-T, EC 2.6.1.19): This activity was determined by the radiometric method in [7] using a 30 000 × *g* supernatant solution of rat brain, but with GABA at 2 mM, which is similar to the level of GABA found in brain tissue, and α-ketoglutarate at 0.7 mM Krebs-Ringer bicarbonate buffer (pH 7.4) was used.

(2) Succinic semialdehyde dehydrogenase (SSADH, EC 1.2.1.16): Pure SSADH prepared as in [6] was used. The assay conditions were identical to those in [6] except that SSA was 10^{-5} M and that the same buffer as in (1) was used.

(3) Succinic semialdehyde reductases (SSR, EC 1.1.1.2): Pure non-specific and specific SSA reductases from rat brain prepared as in [3] were employed and the assay methods were identical to those in [3].

For the 4 enzymes, inhibitors were tested at 10^{-3} M. Enzyme assays were performed in triplicate.

2.2. *Preparation of tissue slices*

Male Wistar rats (~150 g body wt) were used. The animals were decapitated after stunning and the cerebella were rapidly removed and sliced with a mechanical chopper perpendicularly to the pial surface to give ~0.5 mm sections. Each slice was then chopped into 1 mm sections in both planes.

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2.3. *Incubation method*

For each incubation, ~100 mg of this tissue were weighed and placed in tubes containing 1 ml cold Krebs-Ringer bicarbonate buffer (NaCl 122 mM, KCl 3.1 mM, $CaCl_2$ 1.3 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 0.4 mM, $NaHCO_3$ 24.4 mM, glucose 10 mM) pregassed with 95% O_2: 5% CO_2. The tubes were then sealed after replacing the air with 95% O_2: 5% CO_2. Preincubations were carried out at 37°C for 10 min in the absence and presence of the enzyme inhibitors. Then 25 μCi [2,3-^{3}H]GABA were injected into all the tubes which were incubated at 37°C for a further 30 min with gentle agitation. Incorporation of GABA into GHB is linear during this period. The incubation was terminated by injection of 120 μl 10 M HCl. GHB (2.5 μmol) in 50 μl were added prior to homogenisation for 30 s using a Polytron homogeniser at setting 6. The homogenates were then centrifuged for 20 min at 20 000 × *g*. The resulting pellets were re-extracted with 1 ml of 1 M HCl. The pooled supernatants were heated at 85°C for 30 min to lactonise GHB. After cooling, the γ-butyrolactone was extracted twice with 2 ml chloroform and the pooled extracts were concentrated at room temperature under a gentle stream of N_2 to ~100 μl.

2.4. *Purification and measurement of radioactive GHB*

All of each concentrated extract was subjected to thin-layer chromatography on 20 × 20 cm silica gel plates (Merck) and the chromatograms were developed in benzene/ether (70:30) until the solvent front had reached 12 cm from the origin. After drying, each plate was scanned with a Berthold linear β-counter to determined the position of γ-[^{3}H]butyrolactone. A control run was made using pure γ-[^{3}H]butyrolactone. The spots corresponding to γ-[^{3}H]butyrolactone were scraped off the plates and extracted twice with 1 ml ethyl acetate, the pooled extracts were evaporated to ~1 ml under a stream of N_2, and 100 μg δ-valerolactone were added. The yield of γ-butyrolactone from the extraction procedure was determined by gas chromatography using a flame ionisation detector (Perkin Elmer Sigma 3) as in [8]. A linear calibration plot for the GBL standard in ethyl acetate was obtained by plotting the peak area ratio *vs* the amount of GBL. By linear regression, a straight line was obtained ($r = 0.999$) between 5–100 μg GBL with 100 μg δ-valerolactone. The remainder of the ethyl acetate extracts (99.9% of the total) was subjected to liquid scintillation counting. Disintegrations per minute were obtained using a quenching curve. Statistical analysis of data were carried out using Student's *t*-test.

Table 1
Percent inhibition

Inhibitor[a]	GABA-T	SSADH	Specific SSR	Non-specific SSR
GT 16	12	86	n.d.	84
GT 43	12	80	40	74
Barbital	n.d.	13	n.d.	80

[a] Final concentration, 10^{-3} M;
n.d., No inhibition detected under our assay condition

Values are the mean of triplicate determinations which varied <10%

3. Results

The percentage inhibition values for the 3 compounds tested by the assay method described for the 4 enzymes, are given in table 1. All 3 compounds strongly inhibit non-specific SSA reductase but in addition GT 16 and GT 43 are also strong inhibitors of SSADH. Only GT 43 is a significant inhibitor of the specific SSA reductase. GABA-T activity is very slightly inhibited by the 2 branched-chain fatty acids and barbital has no effect on this enzyme. Table 2 shows the levels of incorporation of [^{3}H]GABA into GHB after 30 min incubation in the absence and presence of the 3 enzyme inhibitors. Catabolism of [^{3}H]GHB under our incubation conditions was negli-

Table 2
Conversion of [^{3}H]GABA to [^{3}H]GHB in rat brain cerebellar slices

Experimental procedure	GHB formed (10^{-12} mol . min^{-1} . g $tissue^{-1}$, w/w)	Change (%)
Control	612 ± 46 (12)	–
GT 16 (10^{-3} M)	872 ± 78 (4)[a]	+42.5
GT 16 (2×10^{-3} M)	1062 ± 104 (3)[a]	+73.6
GT 43 (10^{-3} M)	550 ± 4 (3)[a]	−10.0
Barbital (10^{-3} M)	654 ± 27 (3)[b]	+6.8

[a] Significantly different from control ($p < 0.001$)
[b] Significantly different from control ($p < 0.01$)

Results are expressed as the mean ± SD (no expt)

gible over 30 min. The levels of GABA incorporation into GHB were calculated assuming a cerebellar level of 1.3 μmol/g wet tissue wt as determined in [9]. Barbital induces a slight increase in [^{3}H]GABA incorporation into GHB, GT 16 induces a large increase in incorporation, that is almost concentration-dependent, whereas GT 43 causes a significant drop in incorporation.

4. Discussion

Under our experimental conditions, [^{3}H]GABA is incorporated into GHB, but for reasons not yet understood, the further catabolism of GHB does not take place to a significant extent. However, in vivo, GHB must be catabolised at about the same rate as it is synthesized, and there are several arguments which indicate that this catabolism does not occur by a direct reversal of GHB synthesis [5]. It might be suggested that some metabolic intermediate or cofactor required for the catabolism of GHB is not produced by cerebellar brain slices which are respiring in Krebs-Ringer bicarbonate glucose medium, although the GABA shunt pathway appears to be functioning normally. The pool of cerebellar GABA has been calculated to be 1.3 μmol/g fresh wt [9] and under our conditions, ~0.05% of this pool is converted to GHB/min. This value is somewhat lower than that found in vivo in [10] where 0.16% of whole brain GABA was incorporated into GHB/min after intraventricular injection of a pulse of labelled GABA. Nevertheless, the effect of certain drugs observed on this system is compatible with their selective action on the enzymes involved in GHB synthesis.

The 3 compounds inhibit only slightly or not at all GABA-T whereas they all strongly inhibit non specific SSA reductase. If this latter enzyme which is present in large excess over the specific enzyme in rat brain tissue [5] was involved in SSA reduction, a large decrease in GHB formation would be expected in GHB formation in the presence of each compound. Evidently this is not the case. The 2 branched-chain fatty acids are inhibitors of SSADH and they would thus be expected to increase the pool of SSA available for reduction to GHB. Indeed, GT 16 does increase the incorporation of GABA into GHB in a dose-dependent manner whereas GT 43 actually elicits a slight decrease. This phenomenon may be explained by the fact that only the latter compound is an inhibitor of specific SSA reductase. Barbital, which is a strong inhibitor only of the non-specific SSA reductase has little effect on the incorporation into GHB. These results strongly suggest that the specific SSA reductase is uniquely involved in SSA reduction to GHB in brain slices, and hence most probably in vivo.

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References

[1] Snead, O. C. (1977) Life Sci. 20, 1935–1943.
[2] Cash, C. D., Maitre, M. and Mandel, P. (1979) J. Neurochem. 33, 1169–1175.
[3] Rumigny, J. F., Maitre, M., Cash, C. and Mandel, P. (1980) FEBS Lett. 117, 111–116.
[4] Hoffman, P., Bendicht, W. and Von Wartburg, J. P. (1980) J. Neurochem. 35, 354–366.
[5] Rumigny, J. F., Maitre, M., Cash, C. D. and Mandel, P. (1981) J. Neurochem. 36, 1433–1438.
[6] Cash, C., Ciesielski, L., Maitre, M. and Mandel, P. (1977) Biochimie 59, 257–268.
[7] Ossola, L., Maitre, M., Blindermann, J. M. and Mandel, P. (1980) J. Neurochem. 34, 293–296.
[8] Lettieri, J. T. and Fung, H. L. (1978) Biochem. Med. 20, 70–80.
[9] Balcom, G. J., Lenox, R. H. and Meyerhoff, J. L. (1975) J. Neurochem. 24, 609–613.
[10] Gold, B. I. and Roth, R. H. (1977) J. Neurochem. 28, 1069–1073.