IN VITRO STUDIES INTO THE EFFECT OF INHIBITION OF RAT BRAIN SUCCINIC SEMIALDEHYDE DEHYDROGENASE ON GABA SYNTHESIS AND DEGRADATION

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1. Introduction

The inhibitory role of GABA in certain central nervous system mechanisms is well known [1]. As GABA itself does not normally traverse the blood–brain barrier various inhibitors of GABA transaminase have been used in order to increase its cerebral level [2]. However, most of these inhibitors which cross the blood–brain barrier act on the coenzyme, pyridoxal phosphate, and hence are very toxic and not specific for GABA-T. Some branched chain fatty acids inhibit purified GABA-T [3–5] as well as succinic semialdehyde dehydrogenase [6–7].

We describe the results of experiments designed to explore the effect of inhibition of SSADH on GABA synthesis and degradation in a rat brain 900 X *g* supernatant fraction.

2. Methods

2.1. *Preparation of [^{3}H]SSA*

This was performed enzymatically. 20 μg purified GABA-T from rat brain were incubated with [^{3}H] GABA (1 μmol–100 μCi) and 3 X 10^{-4} 2-oxoglutarate in 0.3 M Tris–HCl, pH 8.6, for 4 h at 37°C. The reaction was stopped by acidification to pH 2.0 with 1 M HCl. One μmol of non radioactive SSA was added to the medium. This was obtained by hydrolysis of γ-ethoxybutyrolactone in boiling water [8] and assayed as the 2–4 dinitrophenylhydrazone using a solution of sodium pyruvate as a standard. The incubation medium (about 1 ml) was placed on a 1 cm by 4 cm column of Dowex 50 WX8, H^+ form, eluted with distilled water and collected in 0.3 ml fractions. The SSA was detected in the fractions by the method of Salvador and Albers [9], whereby SSA reacts with 3,5-diaminobenzoate under acid conditions to give a fluorescent quinaldehyde. The fractions containing SSA were pooled and assayed as indicated above. About 2 μCi of labelled SSA were obtained giving a yield of about 2%.

Abbreviations: GABA-T, 4-aminobutyrate-2-oxoglutarate transaminase (EC. 2.6.1.19). SSADH, Succinic semialdehyde deshydrogenase (EC. 1.2.1.16). GABA, γ-aminobutyric acid. SSA, Succinic semialdehyde. DPA, *n*-dipropyl acetate.

2.2. *Preparation of the 900 X g supernatant fraction*

The brains of two adult Wistar rats, killed by decapitation, were rapidly removed and homogenised in 0.32 M sucrose at 4°C. After 5 min centrifugation at 900 X *g*, the supernatant was immediately employed for the incubation experiments in the presence of [^{3}H]SSA.

2.3. *Incubation of the 900 X g supernatant fraction with SSADH inhibitors and [^{3}H]SSA*

For these experiments, 4 fractions of 200 μl (A, B, C and D) of the 900 X *g* supernatant fraction, containing 2.16 mg protein as determined by the Lowry method [10] were preincubated in Potter microhomogeniser tubes for 3 min at 37°C with 500 μl of Krebs-Ringer solution, pH 7.4, using gentle agitation. The following compounds were added to a final concentration of 10 mM, to fractions A, B and C in 10 μl: (A) sodium dipropylacetate; (B) sodium 5,5-dipropylpentanoate; (C) sodium 2-propyl-2-pentenoate. 10 μl of Krebs-Ringer were added to fraction D, which served as a control. After a time lapse of 3 min to allow for temperature equilibration and diffusion

of inhibitors, 100 μl of [^{3}H]SSA containing about 0.3 μCi were added to each fraction. The concentration of SSA was thus 3.3 × 10^{-4} M in each fraction. The incubation was continued for 10 min at 37°C using gentle agitation and then the reaction was stopped by the addition of 200 μl of 1 M HCl. The precipitate of protein formed in the homogeniser tubes was homogenised with a Teflon-pestle and centrifuged for 5 min at 1000 × *g*.

2.4. *Measurement of the specific activities of the [^{3}H] GABA formed in each fraction*

After centrifugation, the supernatants were each placed on a 1 × 4 cm Dowex 50 WX8, H^+ form, column, and rinsed with 15 ml distilled water. The amino compounds were eluted with 15 ml 2 M ammonia and the eluates lyophilised. The lyophilisates were redissolved in 0.5 ml of 0.2 M phosphate buffer, pH 8.8, and dansylated according to the method of Zanetta et al. [11]. The dansyl-amino compounds were separated by two-dimensional chromatography on thin-layer silica plates in the presence of dansyl-amino markers in both dimensions. This method permits isolation of the majority of dansyl-amino acids in a pure state. The spots corresponding to dansyl-GABA and dansyl-glutamate were scraped off the thin-layer plate and eluted as described by Zanetta. An aliquot of the eluate was taken to measure the fluorescence. Standard curves using pure solutions of dansyl-GABA and dansyl-glutamate were constructed in order to quantify the respective amino acids in the eluates. Other aliquots were used to measure radioactivity by scintillation counting. In all cases, controls were performed using eluates from silica gel samples where there were no spots.

2.5. *Measurement of the rate of GABA degradation*

An aliquot of 200 μl (No. 1–7) of the 900 × *g* supernatant fraction containing 2.16 mg protein was preincubated as for the experiment with [^{3}H]SSA. The following compounds were added respectively to fractions 1, 2 and 3 in 10 μl to a final concentration of 10 mM: (1) sodium dipropylacetate (2) sodium 5,5-dipropylpentanoate (3) sodium 2-propyl-2-pentenoate. 10 μl of Krebs-Ringer was added to fraction 4, which served as a control. To fraction 5, *p*-hydroxybenzaldehyde was added to a final concentration of 0.1 mM. This compound is a very potent inhibitor of SSADH (competitive vis-à-vis SSA, K_i = 2.5 × 10^{-6} M). After a time lapse of 3 min for temperature equilibration, 100 μl of Krebs-Ringer solution was added to each fraction containing the following compounds (final concentration): GABA 2 mM, 2-ketoglutarate 0.5 mM, [U-^{14}C]GABA 5 μCi/ml, aminoethylisothiouronium bromide hydrobromide 1 mM, EDTA 0.1 mM, sodium succinate 0.02 mM, NAD 0.06 mM, pyridoxal phosphate 0.5 mM. After 10 min incubation at 37°C, the reaction was stopped by addition of 200 μl of 10% trichloroacetic acid and the rate of degradation of GABA was measured as described by Dye and Taberner [12] in which the succinic acid product is eluted with 10 ml water from a Dowex 50 WX8, H^+ form column.

3. Results

3.1. *SSA as a precursor in GABA synthesis with and without branched chain fatty acid*

The GABA specific activities are expressed relatively to specific activity of the SSA considered as the precursor (table 1). The radioactivity incorporated into glutamate is not significantly different from the background counts. In table 2, we have reprinted the various K_i values vis-à-vis pure rat brain GABA-T and vis-à-vis purified rat brain SSADH [7]. The ratios of the K_i values: (K_i GABA-T/K_i SSADH) give an indication of the relative importance of the inhibition of the two enzymes by the branched chain fatty acids. The absence of a significant level of radioactivity in the glutamate pool suggests that the radioactivity incorporated in GABA does not arise by metabolism of SSA via the Krebs cycle and decarboxylation of glutamic acid. This radioactivity must thus be due to GABA-T activity. The presence of neither dipropylacetate nor 5,5-dipropyl-pentanoate modifies the quantity of labelled GABA formed. In the case of 2-propyl-2-pentenoate, a diminution of GABA synthesis from SSA is seen. This compound is the stronger inhibitor of GABA-T activity as shown by the measurement of the K_i with purified rat brain GABA-T [7].

3.2. *Rate of degradation of GABA with or without branched chain fatty acids*

The activities measured in the presence or absence of the inhibitor are shown in table 3. The 3 branched

Table 1
Level of incorporation of tritium in GABA with [^{3}H]SSA as precursor, in the presence of various branched chain fatty acids

	Specific activity (GABA/SSA)
Control	0.20 ± 0.04 (4)
Dipropylacetate 10 mM	0.17 ± 0.06 (4) (n.s.)
5,5-Dipropylpentanoate 10 mM	0.20 ± 0.08 (4) (n.s.)
2-Propyl-2-pentenoate 10 mM	0.081 ± 0.023 (4) $P < 0.01$

n.s. non significant

Table 2
Inhibition constants of some branched chain fatty acids for GABA-T and SSADH

	K_i GABA-T (10^{-3} M)	K_i SSADH (10^{-3} M)	K_i (GABA-T/SSADH)
Dipropylacetate	9.5	4	2.7
5,5-Dipropylpentanoate	6	0.3	20
2-Propyl-2-pentenoate	0.5	4	0.12

Table 3
Rate of GABA degradation with and without branched chain fatty acid and a SSADH inhibitor

	GABA-T activity (10^{-9} mol/min)	GABA-T activity (% decrease)
Control	0.319 ± 0.012	
DPA 10 mM	0.211 ± 0.014 $P < 0.01$	34%
2-Propyl-2-pentenoate 10 mM	0.193 ± 0.015 $P < 0.01$	40%
5,5-Dipropylpentanoate 10 mM	0.097 ± 0.006 $P < 0.001$	70%
4 Hydroxy benzaldehyde 0.1 mM	0.297 ± 0.004 (n.s.)	7% (n.s.)

chain fatty acids studied bring about a fall in GABA-T activity of at least 34% in the 900 X *g* supernatant from the rat brain homogenate. The concentration of GABA used for these studies (2 mM) is very close to the average in vivo level found in the rat brain. The concentration of fatty acids used (10 mM) is close to the cerebral level of DPA found in the rat brain after the i.p. injection of 200 mg/kg [13]. This concentration was determined using [^{14}C]DPA after it was shown that this compound is 70–80% unchanged in brain 60 min after its administration IP [14].

As can be seen in table 3, the inhibition of SSADH, which is practically complete with 10^{-4} M *p*-hydroxylbenzaldehyde, does not significantly modify the rate of GABA degradation.

4. Discussion

Taking into account the cerebral concentration of SSA which is always very low, it cannot inhibit the degradation of GABA by GABA-T to a significant extent (competitive inhibition, $K_i = 4 \times 10^{-3}$ M, Maitre, unpublished results). As a result, inhibition of SSADH can only bring about an elevation in the GABA level by increasing the synthesis of GABA by GABA-T with SSA serving as a precursor.

From these results, it can be seen that parallel inhibition of both GABA-T and SSADH does not produce an increase in the synthesis of GABA from SSA. In the case where the inhibitor was particularly strong vis-à-vis GABA-T, the GABA produced was actually less than in the control. However, in the presence of the concentrations of GABA and DPA existing in the rat brain 45 min after i.p. injection of 200 mg/kg DPA [13], the rate of GABA degradation is reduced by 34% in comparison with the control. This effect is even greater with the inhibitors which are more potent such as 2-propyl-2-pentenoate and 5,5-dipropyl-pentanoate. The almost complete inhibition of SSADH by *p*-hydroxybenzaldehyde does not significantly affect the rate of degradation of GABA.

In the light of our results, it seems hardly likely that the increase of 30–45% in the cerebral GABA level in Swiss albino mice or Wistar rats after intraperitoneal administration of 400 mg/kg of dipropylacetate [3,4] could be due to SSADH inhibition. Moreover, the relative specific activities of GABA-T and SSADH in a brain homogenate is 1:5 [7]. It is well known that the turnover rate of a NAD linked dehydrogenase is considerably greater than that of a transaminase, particularly GABA-T [15,16]. Consequently, the cerebral SSA level is always very low. Under normal conditions, the limiting factor in GABA degradation to succinic acid is thus GABA-T activity. With dipropylacetate and 2-propyl-2-pentenoate, taking into account the respective K_i values for SSADH and GABA-T, the limiting activity for accumulation of GABA is GABA-T. In the case of 5,5-dipropylpentanoate, the residual SSADH activity is inferior to that of GABA-T. Despite this, the level of SSA incorporation into GABA is not significantly modified in relation to the control. These experiments confirm those of Baxter and Roberts [17] which show the impossibility of raising cerebral GABA levels after inhibition of SSADH in vivo by various inhibitors including analogues of NAD.

Anlezark et al. [18] report the absence of inhibition of GABA-T in a rat brain homogenate containing 2 or 5 mM GABA, by 5–15 mM DPA. This result can be explained by the fact that the method used (measurement of [^{14}C] glutamate formed from [^{14}C] 2-oxoglutarate in the presence of 20 mg of cerebral tissue) does not allow the unique measurement of GABA-T activity, but the sum of the enzymatic activities which transform 2-oxoglutarate into glutamate, particularly aspartate aminotransferase activity which is very high in comparison to GABA-T activity, and uses the aspartate endogenous to the tissue fraction. Moreover, GABA-T activity was measured in the presence of an inhibitory concentration of 2-oxoglutarate (25 mM). As this inhibition is competitive with respect to GABA [15] which was present at low concentrations (2 and 5 mM), the conditions are not ideal to measure the effect of a second inhibitor (DPA) in the incubation medium.

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