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Effect of the 'antidementia drug' pantoyl-GABA on high affinity transport of choline and on the contents of choline and acetylcholine in rat brain

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1 Effect of pantoyl-γ-aminobutyric acid (pantoyl-GABA) on high affinity transport of choline into synaptosomes and on the choline (Ch) and acetylcholine (ACh) concentrations of rat brain were studied.

2 Pantoyl-GABA was injected intraperitoneally four times at a dose of 500 mg kg^{-1} at intervals of 30 min. One hour after the last injection, rats were killed by decapitation for measurement of high affinity transport of Ch into synaptosomes or by microwave irradiation for the measurement of Ch and ACh concentrations.

3 Transport of Ch was increased into synaptosomes prepared from the cerebral cortex and hippocampus, but not into those from the striatum.

4 In the cerebral cortex and hippocampus, Ch concentration was increased and ACh concentration decreased.

5 Since treatments that enhance the activity of cholinergic neurones *in vivo* are reported to increase high affinity transport of Ch measured *in vitro*, the present results suggest that pantoyl-GABA may increase cholinergic activity *in vivo*. This action of the drug may be related to changes in the Ch and ACh concentrations.

Introduction

There is much interest in drugs that facilitate learning and memory or overcome the deficits in cognition and memory observed in aged humans and in patients with Alzheimer's disease. Several drugs have been shown to facilitate memory and cognition in animal models, and some of them have been examined in clinical studies (Heise, 1987). Pantoyl-γ-aminobutyric acid (pantoyl-GABA) is commercially available and is widely used in Japan for treating cognitive and memory impairments in pathological states (Kaneda *et al.*, 1980).

We have found that pantoyl-GABA increases acetylcholine (ACh) release and high affinity transport of choline (Ch) in slices of rat brain (Nakahiro *et al.*, 1985; Nakahiro & Yoshida, 1986). Since disorder of cholinergic neurones in the central nervous system (CNS) is thought to be a major factor in Alzheimer's disease (Terry & Davies, 1980; Bartus *et al.*, 1982; Coyle *et al.*, 1983), these observations on the effect of pantoyl-GABA *in vitro* might contribute to its action as an 'antidementia drug'. *In vivo*, pantoyl-GABA has been reported to reverse the action of cholinergic antagonists on locomotor activity of mice (Nakahiro *et al.*, 1985). However, the activity of

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cholinergic neurones after treatment with pantoyl-GABA *in vivo* has not yet been examined.

Drugs which increase ACh turnover and release *in vivo* are reported to increase the high affinity transport of Ch *in vitro* and measurement of this transport has been proposed as an index of the relative activity of cholinergic neurones *in vivo* (Atweh *et al.*, 1976; Simon *et al.*, 1976). In this context, the effect of *in vivo* treatment of the rat with pantoyl-GABA on high affinity transport of Ch into brain synaptosomes was examined.

Recently, a new procedure has been introduced for the measurement of ACh and Ch (Fujimori & Yamamoto, 1987). This method involves use of liquid chromatography-electrochemistry with an immobilized-enzyme reactor and provides a simple, rapid and sensitive means for measuring ACh and Ch. Using this method, we have also measured the concentrations of ACh and Ch as indices of other cholinergic activity.

Methods

Assay of high affinity transport of choline

Male Sprague-Dawley rats (Charles River Japan, Inc) were injected intraperitoneally with 500 mg kg^{-1} pantoyl-GABA four times at intervals of 30 min. One hour after the last injection, the rats were decapitated. Cerebral cortex, hippocampus and striatum were removed and homogenized in 10 volumes of ice-cold 0.32 M sucrose. The homogenates were centrifuged at 1,000 *g* for 10 min and the supernatants were recentrifuged at 10,500 *g* for 30 min. The resultant pellets were resuspended in the original volume of 0.32 M sucrose.

Samples of 100 μl of suspension were mixed with 1 ml of Krebs-Ringer buffer (composition in mM: NaCl 170, KCl 5, $MgCl_2$ 1.2, KH_2PO_4 1.24, $CaCl_2$ 0.8, Tris-HCl (pH 7.4) 25 and glucose 10) containing 1 μCi [^{3}H]-choline and non-radioactive Ch to give a final concentration of 0.5 μM. Samples were incubated at 30°C for 5 min, and the reaction was terminated by filtration through a glass filter (Whatman GF/F). The filter was washed 3 times with 2 ml of ice-cold buffer and radioactivity was measured in a liquid scintillation counter. For determination of Na^+-independent transport, buffer containing 340 mM sucrose instead of 170 mM Na^+ was used. High affinity transport of Ch was defined as Na^+-dependent transport; that is, total transport in a solution containing Na^+ minus Na^+-independent transport in Na^+-free solution.

For assay of protein, an aliquot of synpatosome suspension was solubilized in 0.05% Triton X-100. Protein was determined by measuring the absorbance shift of Coomassie Brilliant Blue G-250 bound to protein (Bradford, 1976) with a Bio-Rad Protein Assay Kit.

Measurements of acetylcholine and choline contents

Male Sprague-Dawley rats were treated i.p. with 500 mg kg^{-1} of pantoyl-GABA four times at intervals of 30 min. One hour after the last treatment, they were killed by head-focused microwave radiation (5 kW for 1.5 s). Cerebral cortex and hippocampus were removed, weighed and homogenized in 1 ml 0.1 N perchloric acid containing 0.01% disodium ethylenediamine-tetracetic acid (EDTA) and 10 nmol of ethylhomocholine (EHC) as an internal standard. The homogenates were stored at −80°C until assayed. The frozen homogenate was thawed, sonicated and centrifuged at 10,000 *g* for 20 min. The supernatant was filtered through a 0.2 μm pore membrane, and 20 μl of the sample was applied to the liquid chromatography (lc) system described below for measuring the amounts of ACh and Ch.

The contents of ACh and Ch were measured by lc with electrochemical detection by the method of Fujimori & Yamamoto (1987). The principle of this method was described by Potter *et al.* (1983).

We used an ACh Assay Kit (BioAnalytical Systems Inc.) consisting of the following components: an lc pump, an injector with a 20 μl sample loop, a guard column, an analytical column, a reaction column with a column heater and an amperometric detector with a platinum electrode. Acetylcholinesterase and Ch oxidase are immobilized on the reaction column, and the column is kept at 35°C by the column heater during the assay. After separation of ACh and Ch in the analytical column, ACh is converted to Ch which in turn produces hydrogen peroxide in the reaction column. Production of hydrogen peroxide is monitored continuously with the electrochemical detector and is recorded by a pen-recorder connected to the detector. The electrode potential was set at +0.5 V against a Ag/AgCl reference electrode for the detection of hydrogen peroxide.

The mobile phase consists of 50 mM sodium phosphate buffer (pH 8.3) containing 40 μM sodium octyl sulphate, 1 mM tetramethylammonium and 1 mM EDTA. The solution was degassed by stirring and evacuation before use. The rate of flow of the mobile phase was 1 ml min^{-1}.

We measured areas under peaks instead of peak heights because of broadening of the peaks of ACh. To compensate for variation in the extraction efficiency in preparing samples and change in the detector sensitivity during operation, we used an internal standard of EHC.

Drugs

The calcium salt of pantoyl-GABA and EHC were gifts from Tanabe-Seiyaku Co. Ltd. and Bio-Analytical Systems Inc., respectively. [^{3}H]-choline chloride (80.0 Ci mmol^{-1}) was purchased from New England Nuclear. Other chemicals were obtained commercially.

Results

[^{3}H]-choline uptake into synaptosomes was linear for at least 10 min of incubation. The Na^+-dependent portion of the uptake was approximately half the total uptake, although it varied somewhat in different preparations. Addition of pantoyl-GABA (1 mM) to the reaction mixture had no effect on the transport of [^{3}H]-choline into synaptosomes prepared from untreated rats.

The i.p. injection of pantoyl-GABA significantly increased high affinity transport of Ch into synaptosomes from the cerebral cortex (144%) and hippocampus (109%), but not into those from the striatum (Table 1). Therefore, we examined its effect on the contents of ACh and Ch in the cerebral cortex and hippocampus.

Figure 1 shows representative chromatograms of a standard and samples from the cerebral cortex and hippocampus. In the pantoyl-GABA treated rats, the Ch concentration of the cerebral cortex was three times that of control rats and the Ch concentration of the hippocampus was approximately twice that of controls. In contrast, the ACh concentrations of the cerebral cortex and hippocampus was significantly decreased to 75% and 77% of those of controls, respectively (Table 2).

Discussion

The concentration of pantoyl-GABA in rat brain increases gradually and reaches a maximum within 60 min after i.p. injection (Kodama *et al.*, 1966). Therefore, in this study, rats treated with pantoyl-GABA were killed 60 min after the last treatment for measurements of Ch uptake and contents of ACh and Ch. When rats were treated with a single injection of pantoyl-GABA (500 mg kg^{-1}), no statistically significant effect was observed. Therefore, the protocol of four injections was adopted.

The K_m of Na^+-dependent transport of Ch into synaptosomes is reported to be 0.5–8 μM (Haga & Noda, 1973; Yamamura & Snyder, 1973; Barker, 1976; Murrin & Kuhar, 1976; Simon *et al.*, 1976).

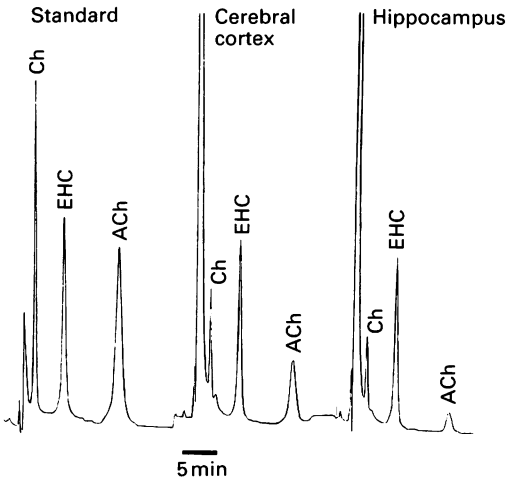

Figure 1 Chromatogram of standard and tissue samples from the cerebral cortex and hippocampus of a control rat. The standard solution contained 100 pmol of choline (Ch) and 200 pmol of acetylcholine (ACh) and ethylhomocholine (EHC). Tissue samples contained 200 pmol of EHC as an internal standard.

Table 1 Effect of pantoyl-γ-aminobutyric acid (pantoyl-GABA) on high affinity transport of choline

	Choline (pmol mg^{-1} protein 5 min^{-1})	
	Control	*Pantoyl-GABA*
Cerebral cortex	16.5 ± 1.79 (10)	23.7 ± 1.69** (10)
Hippocampus	30.8 ± 2.58 (11)	33.6 ± 3.15* (11)
Striatum	104.7 ± 6.79 (5)	106.1 ± 6.50 (5)

Values are mean ± s.d. for the number of determinations shown in parentheses. * $P < 0.05$ and ** $P < 0.001$, significance of difference from control value by Student's t test.

Table 2 Effect of pantoyl-γ-aminobutyric acid (pantoyl-GABA) on acetylcholine (ACh) and choline concentration

	ACh (nmol g^{-1} wet weight)	
	Control	*Pantoyl-GABA*
Cerebral cortex	16.7 ± 5.37 (19)	12.6 ± 2.33* (10)
Hippocampus	22.1 ± 5.23 (18)	17.1 ± 2.96* (10)
	Choline (nmol g^{-1} wet weight)	
	Control	*Pantoyl-GABA*
Cerebral cortex	13.5 ± 3.86 (19)	42.5 ± 11.36** (10)
Hippocampus	19.4 ± 4.92 (16)	38.7 ± 4.66** (9)

Values are means ± s.d. for the number of determinations shown in parentheses. * $P < 0.05$ and ** $P < 0.001$, significance of difference from control value by Student's t test.

This high affinity transport of Ch is thought to be a transmembrane passage of Ch into cholinergic neurones, where it is converted to ACh (Haga & Noda, 1973; Yamamura & Snyder, 1973). Since 0.5 μM Ch was used in this study, the radioactivity transported into synaptosomes was probably transferred mainly by this high affinity system.

After the i.p. injection of pantoyl-GABA, the ACh concentration of the cerebral cortex and hippocampus decreased (Table 2). Studies on slices of cerebral cortex and hippocampus have shown that pantoyl-GABA increases ACh release (Nakahiro *et al.*, 1985). As ACh is rapidly converted to Ch after its release, the decrease in the ACh concentration might be a reflection of increased ACh release *in vivo*.

The i.p. administration of pantoyl-GABA increased high affinity transport of Ch into synaptosomes of the cerebral cortex and hippocampus. It can be assumed that ACh concentration inside cholinergic terminals regulates high affinity transport of Ch; in other words, progressive decrease in ACh inside the terminals results in progressive increase in transport of Ch (Jenden *et al.*, 1976; Roskoski, 1978; Marchbanks *et al.*, 1981). Thus, our results can be explained by supposing that pantoyl-GABA enhanced the release of ACh and thereby reduced the ACh content inside cholinergic terminals, as discussed above. This explanation is supported by the finding that high affinity transport of Ch did not increase in the striatum (Table 1), a brain region in which pantoyl-GABA did not enhance ACh release in *in vitro* experiments (Nakahiro *et al.*, 1985).

Kuhar and his colleagues have reported that drugs that increase ACh turnover and release *in vivo*, cause an increase in high affinity transport of Ch *in vitro* and proposed that this transport can be used as a rapid measure of the activity of cholinergic neurones *in vivo* (Atweh *et al.*, 1976; Simon *et al.*, 1976). Thus, the results suggest that pantoyl-GABA increases the cholinergic activity in the cerebral cortex and hippocampus.

The Ch content should increase as the ACh content decreases, and in fact, this was observed. However, as one molecule of ACh is degraded to yield one molecule of Ch, the observed increase in Ch concentration was more than the decrease of ACh (see Table 2). Part of this increase of Ch that cannot be attributed to decrease of ACh may be from some other source(s), for example, phosphatidylcholine which is known to liberate free choline (Blusztajn & Wurtman, 1981), although we have no evidence on this point. Irrespective of its source, the increased Ch taken up may be used to synthesize ACh in cholinergic terminals.

In summary, the present study showed that the i.p. administration of pantoyl-GABA increased high affinity transport of Ch into synaptosomes from the cerebral cortex and hippocampus and that it increased the Ch content and decreased the ACh content in these two regions. As discussed above, these two effects are closely related and strongly suggest that the drug causes overall increase in cholinergic activity. Since the functions of cholinergic neurones in the CNS are assumed to deteriorate in Alzheimer's disease (Terry & Davies, 1980; Bartus *et al.*, 1982; Coyle *et al.*, 1983), these effects of pantoyl-GABA should be beneficial in treatment of dementia.

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