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INHIBITION OF MOUSE BRAIN SYNAPTOSOMAL γ-AMINOBUTYRIC ACID TRANSPORT BY PYRIDOXAL 5'-PHOSPHATE

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SUMMARY

Pyridoxal 5'-phosphate (PLP) inhibited the uptake of [^{3}H]γ-aminobutyric acid into mouse brain synaptosomes in a competitive manner. If, however, the synaptosomes were preincubated with PLP, treated with sodium borohydride and washed, an irreversible inhibition of subsequent γ-aminobutyric acid (GABA) uptake was noted. Moreover, this time- and concentration-dependent inhibition was markedly reduced if GABA was present during the preincubation with the inhibitor. PLP had a similar effect on the binding of [^{3}H]GABA to its carrier. These results suggest that the reversible and irreversible inhibition of GABA uptake is the result of an interference with the initial binding of the amino acid to its carrier. Since it is known that PLP can form Schiff bases with lysine residues of proteins, the data presented here point to the idea that essential lysine residues are present at the GABA binding site of its transporter.

INTRODUCTION

There is little question that GABA is the major chemical transmitter in the mammalian brain [1]. Evidence is available that the action of this amino acid is terminated by its rapid removal from the synaptic cleft by an active uptake mechanism [2,3]. At present remarkably little is understood of the molecular aspects of GABA transport, especially the nature of the interaction of GABA with its transporter. On the other hand, our knowledge of physiological and pharmacological factors affecting uptake is far more advanced.

Recent work from this laboratory has shown that PLP can inhibit the binding of GABA to its receptor, and it was suggested that this inhibition involves a lysine residue at the GABA binding site [4]. In addition to binding to its receptor, the GABA molecule is recognized by the binding site of its transporter. Indeed, preliminary work using cat brain has demonstrated that PLP is able to affect GABA

GABA = γ-aminobutyric acid; pyridoxal-P = pyridoxal 5'-phosphate.

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TABLE 1. Effect of pyridoxal-P and analogues on synaptosomal GABA uptake.

Compound	% Inhibition
Pyridoxal-P	26.1 ± 3.5
Pyridoxal	1.1 ± 0.9
Pyridoxine phosphate	0.6 ± 1.1
Pyridoxine	-1.3 ± 2.1
Pyridoxamine phosphate	0.9 ± 0.9
Pyridoxamine	4.8 ± 2.9
Pyridoxic acid	2.4 ± 0.7
Acetaldehyde	-0.7 ± 1.0

The values represent the mean (± S.E.M.) of 4 determinations. Each compound was present during the assay at 20 mM.

transporter binding [5]. The present experiments were carried out to investigate in greater detail this observation and to study the affects of PLP on synaptosomal GABA uptake in mouse brain.

MATERIALS AND METHODS

γ-Amino-2,3-[^{3}H]butyric acid (66 curies/mmol) and NCS were purchased from Arlington Heights, IL. Pyridoxine phosphate was obtained from ICN Pharmaceuticals, Cleveland, OH.

Preparation of Synaptosomes. Sprague Dawley male mice of 12 weeks of age were killed by cervical dislocation and synaptosomes prepared by the procedure of Gray and Whittaker [6].

[^{3}H]*GABA Uptake.* The method described by Martin [7] was followed. The reaction was allowed to proceed for 2 min at 37°C. Uptake was linear for at least 10 min and was proportional to tissue concentration up to 1 mg protein. About 0.1 mg of protein was present in each assay tube. Blanks were determined by the amount of radioactivity associated with the tissue after incubation in the presence of 200 mM non-radioactive GABA.

Preparation of Synaptic Membranes, and [^{3}H]*GABA Binding.* The procedure of Roberts et al. [8] was used. Membranes were incubated for 10 min at 37°C. It was established that maximum sodium-dependent binding was reached by 5 min and that the assay was linear over the protein concentration range used.

Protein Determination. The procedure of Lowry [9] was employed.

RESULTS

Reversible Inhibition of [^{3}H]*GABA Uptake.*

Synaptosomes were incubated in the presence of 1 μM [^{3}H]GABA with or without 15 mM PLP. A 26% inhibition of uptake was recorded (Table 1). Various analogues

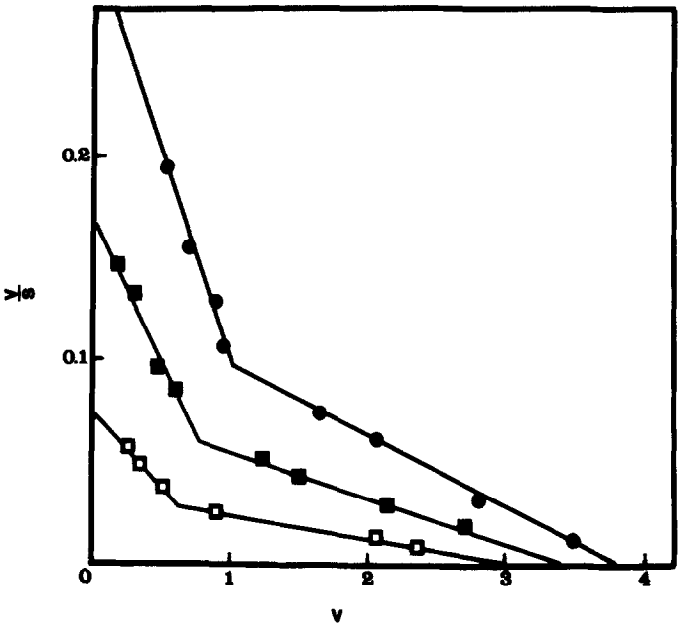

Fig. 1. Eadie-Hofstee plot of the effects of pyridoxal-P on GABA uptake. GABA was varied over the range 10^{-6}M to 10^{-3}M. Circles = control. Closed squares = 5 mM pyridoxal-P. Open squares = 10 mM pyridoxal-P. s = μM GABA; v = nmol. mg. protein^{-1}. min^{-1}.

of PLP were also tested but none was able to inhibit GABA transport to a significant extent.

GABA uptake was also measured over a substrate concentration range of 10^{-6} to 10^{-3}M in the presence or absence of PLP. The results are presented as an Eadie-Hofstee Hofstee plot (Fig. 1). It is apparent that a high-affinity (K_m = 4.9 μM) and a low-affinity (K_m = 30.7 μM) uptake process exists. Each of these uptake mechanisms was inhibited by the PLP in a manner resembling competitive inhibition. This effect of PLP was reversible since no inhibition was detectable if the synaptosomes were washed after exposure to the PLP immediately before the assay.

Irreversible Inhibition of [^{3}H]*GABA Uptake.*

Synaptosomes were suspended in the standard assay medium without [^{3}H]GABA and incubated at 23°C at various concentrations of PLP for periods of time up to 30 min. Sodium borohydride was then added to a final concentration of 20 mM. The incubation was continued for a further 20 min and the synaptosomes were isolated by centrifugation at 4°C for 15 min at 17,000 g. The synaptosomes were resuspended

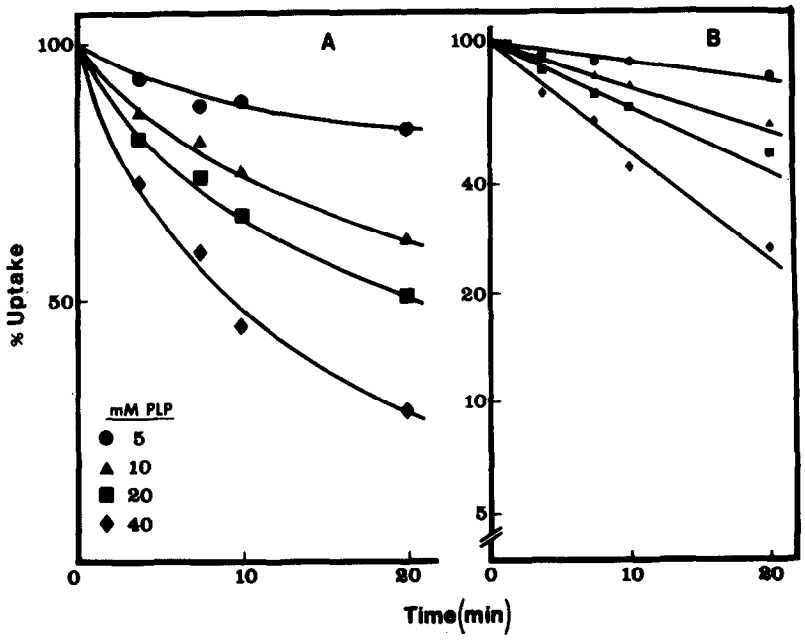

Fig. 2. Time course of the irreversible inhibition of GABA uptake by pyridoxal-P. B is the same data plotted semi-logarithmically. 100% uptake = 15 nmol. mg. protein^{-1}. min^{-1}.

in the same medium and recentrifuged. The pellet was resuspended and the synaptosomes assayed for [^{3}H]GABA uptake. Transport was inhibited in a concentration-dependent manner (Fig. 2A). When the results were plotted on semi-logarithmic paper, a series of straight lines resulted (Fig. 2B). This suggests that the irreversible inhibition of GABA uptake proceeds by a first order dependent reaction. The presence of 1 mM unlabelled GABA during the preincubation with 20 mM PLP gave almost complete protection against inhibition (data not shown).

Reversible Inhibition of [^{3}H]*GABA Binding.*

The Na^+-dependent binding of GABA to synaptic membranes was measured over a substrate range of 10^{-6} to 10^{-3}M. The results were plotted (Fig. 3) by the method of Scatchard [10]. Two binding components were evident. The high-affinity process had a K_d of 6.4 µM and a capacity of 0.16 nmol/mg protein. The low-affinity component had a K_d of 43.7 µM and a maximum binding of 0.52 nmol/mg protein. PLP altered both binding components in a manner resembling competitive inhibition. This effect was shown to be reversible.

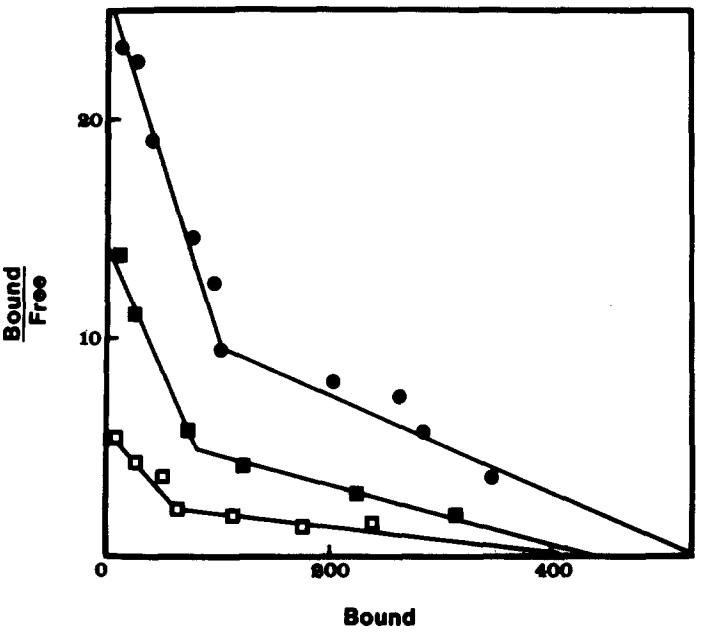

Fig. 3. Effects of pyridoxal-P on GABA transporter binding as a Scatchard plot. GABA was varied over the range 10^{-6}M to 10^{-3}M. Circles = controls. Closed squares = 5 mM pyridoxal-P. Open squares = 10 mM pyridoxal-P. Bound = pmol. mg. protein^{-1}. Free = μM GABA.

Irreversible Inhibition of [^{3}H]*GABA Binding.*

Membranes were suspended in 50 mM Tris-HCl (pH 7.3) and preincubated at 23°C with different concentrations of PLP for up to 30 min. The reaction mixtures were treated with 20 mM sodium borohydride for 20 min and the membranes were pelleted by centrifugation. Each pellet was washed and the membranes resuspended and assayed for GABA binding. As shown in Fig. 4 there was inhibition of binding that was both time- and concentration-dependent. The inhibition of GABA transporter binding shows apparent first order dependence (Fig. 4B). The presence of 1 mM GABA during the preincubation of the membranes with PLP significantly reduced the inhibition (data not shown).

DISCUSSION

It is clear from these experiments that PLP *reversibly* inhibits synaptosomal GABA uptake when the inhibitor is present during the assay. In addition, transport

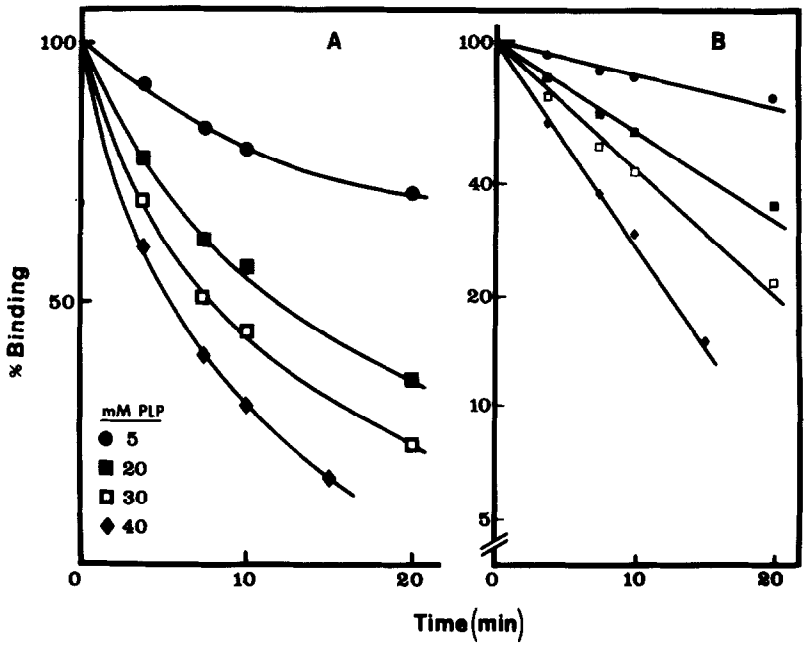

Fig. 4. Time course of the irreversible inhibition of GABA transporter binding by pyridoxal-P. B is the data plotted semi-logarithmically. 100% binding = 112 pmol. mg. protein^{-1}.

is *irreversibly* inhibited if synaptosomes are preincubated with PLP and treated with sodium borohydride prior to the measurement of [^{3}H]GABA uptake in the absence of inhibitor. The effect of PLP appears to be on the binding of the substrate to its transporter rather than on the transport system itself since the presence of GABA can overcome the inhibition. This idea is supported by the observation that PLP can also inhibit GABA sodium-dependent binding to membranes (Fig. 4). There is considerable evidence that Na^+-dependent binding reflects binding to the GABA transporter [8,11].

A physiological role for PLP in the removal of GABA from the synaptic gap is very much in doubt. How then can the inhibition of GABA transport and transporter binding be explained? A feasible explanation is to suppose that a lysine residue resides at the GABA recognition site of its carrier and is vital for the attachment of GABA. PLP could compete with GABA for the lysine under the experimental conditions described here. It is already established that with the vast majority of enzymes requiring PLP as cofactor it is attached to the enzyme by forming a Schiff

base with a lysine residue [12]. Furthermore, several enzymes that contain essential lysine residues for substrate binding can be inactivated with PLP and sodium borohydride [13-17]. Thus if PLP forms a Schiff base with a lysine at the GABA binding site, the irreversible inhibition of uptake and binding could represent the formation of a covalent bond between the PLP and a lysine as in the enzyme inactivation studies. No evidence exists

No evidence exists that PLP is present in the synaptic cleft. Consequently pyridoxal phosphate has not been used here to demonstrate a function for it in the inactivation of GABA at the synapse. Rather, PLP has been employed to tentatively show the existence of a lysine at the GABA binding site of the transporter that is necessary for the attachment of GABA before it can be removed from the synapse.

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