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INHIBITION BY PYRIDOXAL-5'-PHOSPHATE OF γ-AMINOBUTYRIC ACID RECEPTOR BINDING TO SYNAPTIC MEMBRANES OF CAT CEREBELLUM

Godfrey Tunnicliff

Evansville Center for Medical Education
Indiana University School of Medicine
8600 University Boulevard, Evansville, IN 47712

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SUMMARY

The binding of [^{3}H]γ-aminobutyric acid to cat cerebellar membranes is reversibly inhibited in a competitive manner by pyridoxal-5'-phosphate present during the binding assay. Structural analogues of the inhibitor have no such effect. If, on the other hand, the membranes are preincubated with pyridoxal-5'-phosphate followed by the addition of sodium borohydride, a rapid, irreversible inhibition of subsequent γ-aminobutyric acid binding is observed. Since pyridoxal-5'-phosphate is known to inactivate certain enzymes by reacting with essential lysine residues, the present results suggest that such a lysine residue may be present within the γ-aminobutyric acid receptor.

INTRODUCTION

As the major inhibitory neurotransmitter, γ-aminobutyric acid plays a crucial role in brain function [1]. The inhibitory action of this amino acid is achieved by an interaction with a specific receptor on the post-synaptic membrane [2]. In contrast to the acetylcholine receptor, very little is known of the binding site for γ-aminobutyric acid. A greater knowledge of this receptor will increase our understanding of the part played by γ-aminobutyric acid in the functioning of the central nervous system.

Evidence is presented in this paper that pyridoxal-5'-phosphate inhibits the binding of γ-aminobutyric acid to its receptor and that this is possibly achieved by the reaction with a lysine residue within the receptor.

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MATERIALS AND METHODS

Pyridoxal-5'-phosphate was obtained from Sigma Chemical Company, St. Louis, Missouri. γ-Amino[2,3-^{3}H]butyric acid (66 Ci/mmol) was purchased from Amersham, Arlington Heights, Illinois.

Preparation of membranes. Cerebella of adult male cats, anaesthetised wih chloralose, were homogenised in 9 vol of ice-cold 0.32 M sucrose containing 1 mM HEPES buffer (pH 7.4). The homogenate was centrifuged at 4°C for 10 min at 1000 g. The supernatant was diluted 4-fold with 1 mM HEPES buffer (pH 7.4) and then centifuged at 30,000 g for 15 min at 4°C. The pellet was washed by being resuspended in 20 vol of the HEPES buffer. After a further centrifugation at 30,000 g, the pelleted membranes were frozen overnight at -20°C. It was found that this step increased subsequent binding considerably.

Measurement of γ-aminobutyric acid binding. The frozen membranes were resuspended in 20 vol of the HEPES buffer and centrifuged for 20 min at 30,000 g. Finally the pellet was suspended in 50 mM TRIS-citrate buffer (pH 7.4). Five ml of buffer was used for each 2 g of original cerebellum.

Fifty microlitres of membranes (approximately 0.5 mg of protein) were added to 1.5 ml plastic centrifuge tubes containing 1 ml of the TRIS-citrate buffer and 20 nM [^{3}H]-γ-aminobutyric acid (1000,000 dpm). The mixture was incubated for 10 min at 4°C, and then centrifuged for 10 min in an Eppendorf 3200 centrifuge to terminate binding. The supernatant was discarded and the pellet briefly washed with 2 ml of the TRIS-citrate buffer. The membranes were solubilised in 0.5 ml of NCS (Amersham). After the addition of 10 ml of toluene containing 0.1% PPO and 0.03% POPOP, the radioactivity was counted in a Nuclear Chicago Unilux IIA liquid scintillation counter. Binding of γ-aminobutyric acid is defined as that radioactivity able to be displaced by 1 mM non-radioactive γ-aminobutyric acid. It should be noted that this binding assay is Na^+-free to distinquish binding from transport. Preliminary experiments determined that binding was proportional to tissue concentration over the range used in this study. It was established that maximum binding was reached by 5 min of incubation.

Protein determination. The procedure of Lowry et al [3] was employed. Bovine serum albumin was used to prepare standards.

RESULTS

Competitive inhibition of binding.

Cerebellar membranes were incubated in the absence or presence of 20 mM pyridoxal-5'-phosphate over a concentration range of γ-aminobutyric acid of 10^{-8} to 10^{-6}M. The

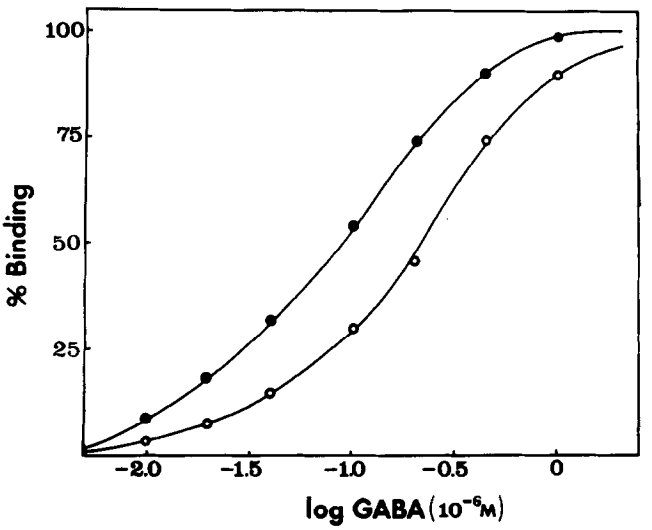

Fig. 1. Saturation curve of γ-aminobutyric acid binding in the absence (closed circles) or presence (open circles) of 20 mM pyridoxal-5'-phosphate. Membranes (about 0.5 mg protein) were incubated at 4°C for 10 min in the presence of [^{3}H]γ-aminobutyric acid and varying concentrations of non-radioactive amino acid (between 10^{-8}M and 10^{-6}M). The presence of inhibitor shifted the binding curve to the right without altering the maximum binding. This is a characteristic of competitive inhibition. Maximum binding (100%) was 3.5 pmoles per mg protein. Each point represents the mean of 3 experiments. In no instant did the standard error exceed 15%.

results are presented in Fig. 1 and show that the presence of pyridoxal-5'-phosphate gives rise to a competitive inhibition of γ-aminobutyric acid binding. This inhibition was shown to be reversible by incubating membranes in 20 mM pyridoxal-5'-phosphate for 10 min at 4°C in the absence of γ-aminobutyric acid. The membrane suspensions were then dialysed for 15 hours at 4°C against 4 litres of 50 mM TRIS buffer (pH 7.4). No inhibition of subsequent γ-aminobutyric acid binding was detectable.

Effects of pyridoxal-5'-phosphate on binding.

The binding of γ-aminobutyric acid was assayed in the absence or presence of several compounds structurally rel-

TABLE 1. Effects of pyridoxal-5'-phosphate analogues on the binding of γ-aminobutyric acid to cat cerebellar membranes.

Compound (20 mM)	% Inhibition
Pyridoxal-5'-phosphate	28.4 ± 2.5
Pyridoxal HCl	0.0 ± 3.1
Pyridoxine-5'-phosphate	-1.9 ± 2.5
Pyridoxine HCl	-2.2 ± 2.0
Pyridoxamine-5'-phosphate	1.2 ± 1.8
Pyridoxic acid	3.0 ± 2.5

Membranes (approximately 0.5 mg protein) were incubated with 10 nM [^{3}H] γ-aminobutyric acid at 4°C for 10 min in the absence or presence of each compound. The values represent the mean (±S.E.M.) of 4 experiments.

ated to pyridoal-5'-phosphate. As can be seen in Table 1, pyridoxal-5'-phosphate was the only compound to produce an inhibition of binding. The structural analogues had little or no effect.

Irreversible inhibition of binding.

Membranes were incubated with 5 mM to 40 mM pyridoxal-5'-phosphate for up to 30 min at 23°C. Sodium borohydride was then added to a final concentration of 20 mM. The mixture was allowed to stand for 20 min before being centrifuged at 17,000 g for 15 min at 4°C. The resulting pellet was washed once with 10 ml of the TRIS buffer and then suspended in and dialysed against 4 litres of the same buffer for 15 hours. Aliquots of these membranes were taken for binding measurements. These results are shown in Fig. 2. It is apparent that an irreversible inhibition of γ-aminobutyric acid binding has been brought about by the treatment with sodium borohydride. This inhibition is first-order dependent (Fig. 2). If 2 mM γ-aminobutyric acid (non-radioactive)

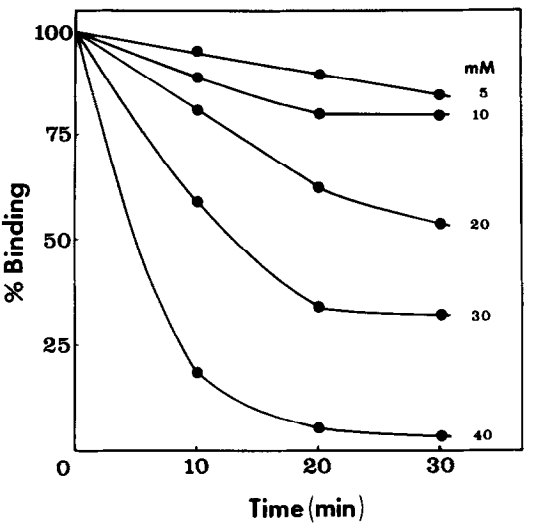

Fig. 2. Relationship between increasing inhibitor concentration and time upon the irreversible inhibition of γ-aminobutyric acid binding. Membranes (about 0.5 mg protein) were exposed to pyridoxal-5'-phosphate followed by sodium borohydride as described in the Methods section. The concentration of inhibitor is given on the figure adjacent to each curve. Maximum binding represents 3.2 pmoles per mg protein. Each point is the mean of 3 experiments. In no instant did the standard error exceed 12%.

was present during the preincubation of the membranes with 20 mM pyridoxal-5'-phosphate for 20 min, the irreversible inhibition of binding was reduced from 37.5% to 10.5% (data not shown).

DISCUSSION

It has previously been reported that a number of enzymes can be inactivated if incubated with pyridoxal-5'-phosphate [4-7]. Furthermore, this inactivation has been shown to be the result of the formation of a Schiff base between the ε-amino group of a lysine residue and the carbonyl group of the pyridoxal-5'-phosphate. A feasible interpretation of the present results is that a similar Schiff

base formation occurs at the γ-aminobutyric acid binding site. This could be manifested as the reversible competitive inhibition seen in Fig. 1. This Schiff base formation would also likely occur during the preincubation of the membranes with pyridoxal-5'-phosphate. However, the addition of sodium borohydride reduces the Schiff base to yield a covalent bond. This could give rise to the observed irreversible inhibition of binding. These data are consistent with the idea that a lysine residue exists at or near the γ-aminobutyric acid binding site. The fact that γ-aminobutyric acid can protect against both reversible and irreversible inhibition lends credence to this idea.

It is unlikely that these observations represent any physiological mechanism for the regulation of the binding of γ-aminobutyric acid to its receptor. There is no evidence that pyridoxal-5'-phosphate is present in the synapse, especially in millimolar concentrations. Instead, pyridoxal-5'-phosphate has been used here as a tool to tentatively demonstrate the existence of lysine residues within the γ-aminobutyric acid receptor. That such residues are required for the binding of γ-aminobutyric acid seems a likelihood. More definitive evidence will have to await the purification of the receptor.

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