Neurochemical Research, Vol. 19, No. 2, 1994, pp. 183–187

Convulsions and Inhibition of Glutamate Decarboxylase by Pyridoxal Phosphate-γ-Glutamyl Hydrazone in the Developing Rat

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(Accepted June 14, 1993)

We have previously shown that in the adult rat the inhibition of brain glutamate decarboxylase (GAD) activity by pyridoxal phosphate-γ-glutamyl hydrazone (PLPGH) administration does not result in convulsions, whereas in the adult mouse intense convulsions invariably occur. In the present study we report that, surprisingly, immature rats from 2 to 20 days of age treated with PLPGH (80 mg/kg) showed generalized tonic-clonic convulsions, whereas no convulsions at all were present in 30 days-old or older rats. GAD activity, measured by enzymic determination of GABA formed in forebrain homogenates, was inhibited by about 60% at the time of convulsions in 15 days-old and younger rats, whereas the inhibition was between 40 and 50% in older animals. The addition of the coenzyme pyridoxal 5′-phosphate to the incubation medium completely reversed this inhibition. In all treated animals GABA levels were lower compared to controls. The results indicate that the susceptibility of GAD in vivo to a diminished cofactor concentration decreases with age. It seems possible that changes in the expression of enzyme forms are reflected in developmental variations in the susceptibility to seizures induced by vitamin B_6 depletion, but alterations of other B_6-dependent biochemical pathways cannot be discarded.

KEY WORDS: Glutamate decarboxylase; pyridoxal phosphate; convulsions; brain development; γ-aminobutyric acid.

INTRODUCTION

The inhibition of brain glutamate decarboxylase (GAD) activity in vivo is recognized as a causal factor in experimental epilepsy (1–4). Particularly important in this respect is the involvement of the coenzyme pyridoxal 5′-phosphate (PLP), since GAD activity is very sensitive to a decrease in its concentration in vivo (1,5–7) and in humans vitamin B_6 deficiency seems to be linked to some type of epileptic seizures (8). Kinetic and inhibition studies led to the proposal of the existence of two types of GAD, differing in their dependence on free PLP (9–12). This has been recently confirmed by molecular cloning of two forms of the enzyme protein, termed GAD_{65} and GAD_{67} to indicate their molecular weight (in kdaltons) (13–14). The activity of GAD_{65} seems to be more dependent than that of GAD_{67} on the availability of free PLP and is located predominantly at nerve endings (15–17).

In previous work we have synthesized and tested a hydrazone derivative of PLP, formed by the carbonyl trapping of the latter by the hydrazide group of L-glutamyl-γ-hydrazide, termed PLP-glutamyl-γ-hydrazone

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0364-3190/94/0200-0183$07.00/0

(PLPGH). This drug has proven to be a powerful convulsant in adult mice, an effect that was well correlated with the inhibition of pyridoxal kinase activity and the consequent decrease of PLP levels and inhibition of GAD in brain (1,3,5,18). However, when PLPGH was administered to adult rats, although GAD was inhibited by about 35% convulsions were not observed (1,19). In the present work we describe that, surprisingly, immature rats show intense convulsive behavior when injected with PLPGH, and that GAD is more notably inhibited than in mature rats.

EXPERIMENTAL PROCEDURE

Animal Handling. Wistar rats of 2, 5, 10, 15, 20, 25, and 30 days of age of both sexes, and male 90 days-old rats were used throughout the study. PLPGH was synthesized from L-glutamyl-γ-hydrazide and pyridoxal 5'-phosphate (obtained from Sigma Chemical Co., St. Louis, MO), as previously described (19) and was dissolved in water. Experimental animals were injected i.p with PLPGH at a dose of 80 mg/kg (5,6). Control litter-mates were injected with isotonic saline and were handled in parallel throughout the experiment. PLPGH-treated rats were decapitated at the time of the first generalized tonic-clonic convulsion (see Results) or, at those ages (30–90 days) when no convulsions occurred, at equivalent times. The absence of convulsions in the latter was assessed previously in groups of animals injected with the drug and observed continuously during several hours.

Determination of GAD Activity. Initially, a radiometric procedure based on the release of $^{14}CO_2$ from [1-^{14}C]glutamate (20) was used to measure GAD activity. However, it was observed that, as previously described (21), in 2–10 days-old pups the CO_2 production was 2–5-fold higher than the amount of GABA formed. Therefore, GAD activity was determined by measuring GABA production (21). Briefly, forebrains from PLPGH-treated and control animals were quickly dissected out and homogenized in 10 mM phosphate buffer, pH 6.8, (10% wt/vol) on ice. Aliquots (0.25 ml) of the homogenates were transferred in duplicate to 1.5 ml microfuge tubes containing 10 mM phosphate buffer (pH 6.8), 20 mM L-glutamate and 0.5 mM PLP (when added) in a total volume of 0.7 ml. The reaction mixture was incubated for 30 min at 37°C, and was stopped by the addition of 0.1 ml of 7% perchloric acid. To determine the initial endogenous GABA content, in parallel tubes of each homogenate the reaction was stopped at zero time. This value was subtracted from that of the 30 min-incubated tubes. After protein removal by centrifugation, the extracts were neutralized with KOH and the potassium perchlorate was spun down. GABA levels in the supernatants was quantified by the spectrophotometric GABAse method described by Jakoby (22), using a GABAse preparation purchased from Sigma (St. Louis, MO). The zero time GABA values were used as an indication of the endogenous GABA content of the tissue, although the possibility of some postmortem increase cannot be discarded.

Protein was determined by the Folin method (23).

RESULTS

Behavioral Observations. All PLPGH-treated animals 2-20 days of age showed generalized tonic-clonic convulsions following a period of hyperactivity, tremor, rigidity of the tail, grooming, lateral movements of the head, loss of posture, and sporadic clonic movements of the four limbs. As shown in Table I, the latency to the first generalized convulsion varied with the age of the animals, from 2 h in the 2 days-old rats to less than one h in the 20 day-old rats. The convulsant effect of PLPGH disappeared with age, since only 25% of the 25 days-old rats showed convulsions and no signs of hyperexcitability were observed in 30 or 90 days-old animals.

GAD activity. As shown in Fig. 1, GAD activity in control animals progressively increases with age. Both in the absence and in the presence of saturating PLP concentration, a six fold increase occurs from 5 to 25 days of age (GAD activity could not be measured at 2 days because it was in the limits of sensitivity of the method used) and from this age to adulthood a plateau is reached, although in the absence of the cofactor a slight decrease was observed between 30 and 90 days. It is noteworthy that the percent activation by the cofactor was considerably higher at 5 and 10 days of age (117% and 140%, respectively) than at older ages (40%-90%).

PLPGH treatment resulted in a significant inhibition of GAD activity, when measured without added PLP, at all ages studied, and this inhibition was, with the exception of 30 days-old rats, completely reversed by the addition of the cofactor to the incubation medium. The percent inhibition at the time of convulsion was 57-62% in the youngest animals (5 to 15 days), and it decreased to 36-48% at 20-90 days, although the experimental variation was relatively large (Figs. 1 and 2).

GABA Levels. As described in Methods, an estimate of endogenous GABA concentrations was calculated from the zero time GABA levels present in the homogenates used to measure GAD activity. The control values, expressed as related to the protein content, were relatively constant from 5 to 30 days of age, and in-

Table I. Convulsant Action of PLPGH in the Developing Rat

Age (days)	No. convulsant rats/ No. total rats	Latency to first convulsion (min ± SEM)
2	14/14	120.5 ± 14.8
5	19/19	89.5 ± 12.4
10	26/26	60.5 ± 9.2
15	17/17	53.9 ± 9.0
20	20/20	53.9 ± 9.5
25	5/20	54.6 ± 11.3
30	0/18	—
90	0/10	—

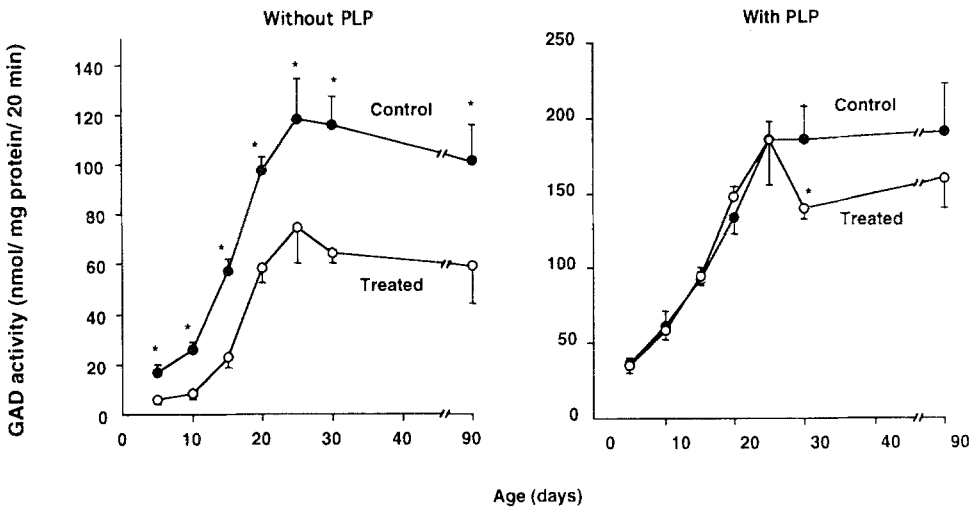

Fig. 1. GAD activity in control and PLPGH-treated rats of different ages, in the absence (left panel) and in the presence (right panel) of PLP. Values are means ± SEM for 5-7 rats in each group. In the absence of PLP, the differences between control and treated animals were significant at all ages (asterisks, $p < 0.01$ or < 0.05, t-test).

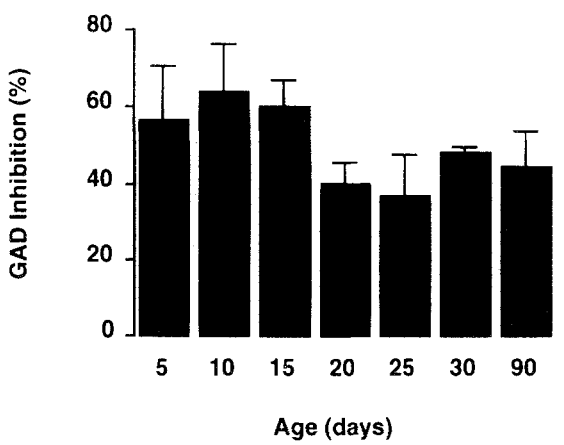

Fig. 2. Percent inhibition of GAD activity by PLPGH treatment, in the absence of PLP, at different ages. Values are means ± SEM for 5-7 animals in each group. Differences were not significant ($p > 0.05$).

Table II. Brain GABA Levels in Control and PLPGH-Treated Rats

	GABA (nmol/mg protein)	
Age (days)	Control	PLPGH
5	35.5 ± 4.9	23.7 ± 2.9*
10	39.9 ± 3.0	27.8 ± 5.6*
15	33.0 ± 9.0	28.7 ± 8.8
20	50.5 ± 3.4	29.6 ± 3.4*
25	38.9 ± 5.7	19.0 ± 2.7*
30	43.0 ± 2.4	29.2 ± 2.4*
90	67.5 ± 10.7	57.7 ± 6.6

The figures are mean ± SEM for 5-7 rats in each group
* $P < 0.05$ as compared with the corresponding control (t -test)

creased at 90 days (Table II). As expected, GABA levels were decreased in PLPGH-treated animals at all ages studied, but no clearcut correlation was found between such decrease and the extent of GAD inhibition, due to the variability of the results and possibly also because of postmortem GABA changes.

DISCUSSION

The occurrence of seizures after the administration of PLPGH in immature rats is surprising, since no signs of hyperexcitability were observed in 30 days-old or older animals. Also interesting is the considerably longer latency to seizures observed in 2-5 days-old as compared to 10-20 days-old rats, for which it is difficult to offer an explanation. The susceptibility to convulsions decreases markedly at approximately 25 days, since at this age only 25% of the rats convulsed, whereas at earlier ages 100% of the rats showed intense convulsive behavior. This finding is in contrast with the effects of PLPGH in the mouse, since in this species the same dose used here induces convulsions in the adult animal even more potently than in immature mice (24). This kind of differences between mice and rats, regarding the susceptibility to drug-induced convulsions, has been described previously (1).

In the mouse and in other mammals a clear corre-

lation between convulsions and the extent of GAD inhibition induced by various mechanisms, including treatments capable of decreasing PLP concentration or blocking its coenzymatic role, has been established. Furthermore, this correlation seems to be independent of the total levels of GABA, suggesting that GAD activity is an important factor regulating the synaptic inhibitory role of GABA at the motor system. Thus, in the adult mouse, an inhibition of about 45% has been calculated as the threshold value at which convulsions almost invariably occur (1,3,25). In the adult rat such threshold is higher since, in agreement with previous work (1,19), a 40-50% decrease was observed in 30 and 90 days-old animals treated with PLPGH and no seizures occurred.

It is interesting that at all ages studied the inhibition of GAD was reversed by the addition of PLP to the incubation mixtures, and that this reversal was complete at early ages. This indicates that, as previously demonstrated in the mouse, the decrease of GAD activity produced by PLPGH is secondary to a diminution of PLP concentration (5,18) and, therefore, that the smaller and more free PLP-dependent form of the enzyme (GAD_{65}, see the Introduction) is predominantly inhibited. Consequently, the fact that the extent of inhibition of GAD activity in the absence of PLP was higher (57-62%) in the youngest rats might indicate that during the early postnatal period GAD_{65} is more abundant than GAD_{67} and thus seizures occur because the threshold GAD activity value is reached. In fact, embryonic rat brain expresses a GAD mRNA transcript distinct from the adult form (26). However, the differences in percent inhibition in rats of 5-15 days of age, as compared to rats of 25-90 days, are not significant. In addition, this explanation cannot account for the finding that in 20 days-old rats GAD activity was decreased by only 39% and convulsions were present in 100% of the animals.

In view of this, the higher sensitivity of immature rats to PLPGH-induced seizures seems to be due not to a greater reduction in the rate of GABA synthesis but rather to a higher dependence on GABAergic transmission for an adequate regulation of motor excitability. Alternatively, other neurotransmitter systems or metabolic pathways might be affected by the decrease of PLP induced by PLPGH. For example, a notable increase in the brain concentration of 3-hydroxykynurenine has been observed in 14 and 18 days-old rats, but not in mature animals, with decreased brain PLP levels due to vitamin B_6-deficient diet (27). This compound, as well as other kynurenine derivatives, can produce seizures when administered intracerebroventricularly in mature rats or intraperitoneally in immature animals (28,29).

ACKNOWLEDGMENTS

This work was supported in part by DGAPA-UNAM (Project IN201089).

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