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Evidence of Stimulation of the γ-Aminobutyric Acid Shunt by Valproate and *E*-Δ²-Valproate in Neonatal Rat Brain

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SUMMARY

The effect of valproate and its more active metabolite *E*-Δ²-valproate on the rate of glucose oxidation through different metabolic pathways in neonatal rat brain slices was studied. The presence of valproate or *E*-Δ²-valproate did not change the rate of [3,4-^{14}C]glucose or [6-^{14}C]glucose incorporation into CO_2, suggesting that glucose oxidation through the pyruvate dehydrogenase-catalyzed reaction and through the tricarboxylic acid cycle was not affected by these drugs. However, both drugs significantly enhanced the rate of [2-^{14}C]glucose oxidation, supporting the notion that the activity of the γ-aminobutyric acid (GABA) shunt is specifically stimulated by valproate and, to a greater extent, by *E*-Δ²-valproate. The presence of methionine sulfoximine or γ-hydroxybutyrate did not change the GABA shunt activity. Brain glutamate decarboxylase activity was significantly increased after incubation of the brain slices in the presence of valproate. Consequently, our results suggest that the mechanism of action of valproate is related to the increase in the levels of the inhibitory neurotransmitter GABA caused by the enhancement of flux through the glutamate decarboxylase-catalyzed reaction.

It is widely accepted that the mode of action of the antiepileptic drug VPA is related to the increase in brain concentrations of the inhibitory neurotransmitter GABA (see Refs. 1–3 for reviews). This hypothesis arises from the measurement of brain GABA concentrations after *in vivo* administration of VPA in rodents (4, 5). Enhancement of the incorporation of [^{14}C]glucose into GABA caused by VPA has also been reported *in vivo* (6–8). However, the mechanism through which VPA increases GABA levels is unknown, although the drug has been reported to inhibit GABA aminotransferase (EC 2.6.1.19), succinate-semialdehyde dehydrogenase (EC 1.2.1.24) (9, 10), and α-ketoglutarate dehydrogenase (EC 1.2.4.2) (11) and to stimulate GAD (EC 4.1.1.15) (12, 13) activity. However, the absence of side effects of the drug at therapeutic doses suggests a specific mode of action in which GABA concentrations increase with no large accumulations of intermediate metabolites. Despite this, direct stimulation of the GABA shunt by VPA has never been demonstrated and, hence, the mode of action of the drug is elusive. In the present work, we report evidence supporting stimulation of the GABA shunt by VPA and by its more active metabolite (14, 15), *E*-Δ²-VPA.

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Materials and Methods

Pregnant Wistar rats fed on a stock laboratory diet and of known gestational age were used, as described previously (16–19). Virgin females were caged with males overnight and conception was considered to occur at 1:00 a.m.; this was verified the next morning by the presence of spermatozoa in vaginal smears. On day 21.5 of gestation (full gestation requires 21.7 days) neonates, weighing 5.2 ± 0.1 g (mean ± standard error), were delivered by rapid hysterectomy after cervical dislocation of the mother. Neonates were carefully wiped and their umbilical cords were cut. Newborns were kept in an incubator at 37° in a continuous stream of water-saturated air, without feeding. After 1 hr, the newborns were decapitated and the right hemispheres of the forebrain were removed and immediately sliced in a water-saturated cabin.

Brain slices (70 mg of wet weight) were incubated as described previously (16, 17), in 2 ml of phosphate-buffered saline containing 0.5 μCi of D-[1-^{14}C]-, D-[2-^{14}C-, D-[6-^{14}C]-, or D-[3,4-^{14}C]glucose (New England Nuclear, Boston, MA) and 5.4 mM unlabeled glucose (0.046 Ci/mol), in the absence (control) or in the presence of 1 mM sodium VPA (Sigma Chemical Co., St. Louis, MO), 1 mM sodium *E*-Δ²-VPA (generously donated by Dr. H. Nau, Institut für Toxikologie und Embryopharmakologie, Freie Universität Berlin, Germany), 1 mM MSO (Sigma), or 1 mM sodium GHB (Sigma). The flasks were gassed with O_2, sealed with rubber caps, and incubated in a shaken water bath at 37°. Incubations were stopped after 2 hr by injection of 4.75 M $HClO_4$ through the rubber cap into the main well, but shaking was continued for an additional 20 min. The $^{14}CO_2$ evoked by the slice was trapped by 3.56 M KOH placed in a center well; the radioactivity was measured by liquid scintillation counting (LS 1800; Beckman, Palo Alto, CA).

ABBREVIATIONS: VPA, valproate; GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase; GHB, γ-hydroxybutyrate; MSO, methionine sulfoximine; PPP, pentose-phosphate pathway.

Blanks without slices were carried out in parallel to measure volatile radioactivity, which was subtracted from the sample values. The specific radioactivity of glucose found in the blanks was used for the calculations. In one set of experiments, incubations carried out in the absence and in the presence of 1 mM sodium VPA were not stopped and the slices were removed from the flasks and immediately homogenized in 7 volumes of ice-cold 0.25 M sucrose, in a motor-driven glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 900 × *g* for 15 min at 4°. Potassium phosphate buffer was added to the supernatant to give 50 mM potassium phosphate, pH 7.0. This supernatant was immediately used for the determination of GAD activity.

Experiments *in vivo* were performed by injecting the neonates with a single dose of 200 mg/kg of body weight, intraperitoneally, of sodium VPA (50 μl/neonate of a 20 mg/ml solution in 0.9% NaCl), and controls received the same volume of 0.9% NaCl. After 30 min, neonates were decapitated and the forebrains were removed and homogenized as described above and then immediately used for the determination of GAD activity.

GAD was assayed by measuring the decarboxylation of L-[1-^{14}C] glutamate (New England Nuclear), essentially as described by Wu *et al.* (20). D-Glucose concentration was determined by the method of Bergmeyer *et al.* (21). Protein concentration was determined by the method of Lowry *et al.* (22). The rates of glucose utilization by the brain slices were expressed as μmol of glucose incorporated into CO_2/hr/g of wet weight. Results are means ± standard errors. Statistical analysis was carried out by Student's *t* test.

Results and Discussion

There is a considerable body of evidence to support the notion that VPA enhances the rate of GABA turnover *in vivo* (1, 5, 7), suggesting that the mechanism of action of VPA may be related to the activation of the GABA shunt (3). As far as we know, however, there is no direct evidence supporting the notion that the drug enhances the activity of the GABA shunt. Because the use of pulse labeling with [^{14}C]glucose can provide useful information on the relative rate of GABA synthesis (7), using the method described by Hothersall *et al.* (23) we have estimated the activity of the GABA shunt *in vitro* in the presence of therapeutic concentrations of VPA (1) and of its major metabolite, *E*-Δ^2-VPA. The activity of the GABA shunt has been estimated (23) by calculating the difference between the oxidation of [2-^{14}C]glucose and that of [6-^{14}C]glucose (23, 24) (Fig. 1). However, two thirds of [2-^{14}C]glucose-6-phosphate entering the PPP (25) is recycled and decarboxylated through the 6-phosphogluconate dehydrogenase (EC 1.1.1.44)-catalyzed reaction. Accordingly, two thirds of the activity of the PPP must be subtracted from the oxidation of [2-^{14}C]glucose to properly estimate the GABA shunt activity. This correction was not made by Hothersall and co-workers (23, 24), because under their experimental conditions the activity of PPP was so low that the recycling of [2-^{14}C]glucose through this pathway was assumed to be negligible. However, under our experimental conditions the PPP activity estimated by the difference between the oxidation of [1-^{14}C]glucose and that of [6-^{14}C]glucose (23) was considerably higher (Table 1). Accordingly, we have corrected the values by subtracting two thirds of the observed PPP activity to estimate the GABA shunt activity under our experimental conditions.

The presence of VPA or *E*-Δ^2-VPA significantly enhanced (44 and 141%, respectively) the activity of the GABA shunt (Table 1), suggesting that the pharmacological action of these drugs may be accounted for in terms of an increase in GABA availability. The enhancement of the GABA shunt by these

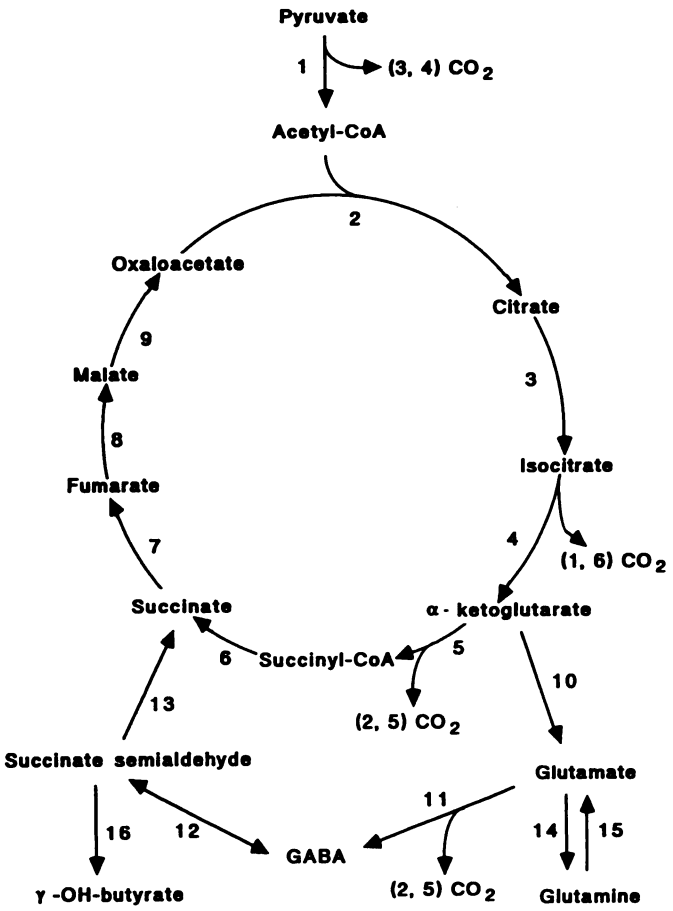

Fig. 1. Relationship between the tricarboxylic acid cycle and the GABA shunt. Numbers in parentheses designate the position of the ^{14}C label in glucose that decarboxylates to CO_2. Enzymes are designated as follows: *1*, pyruvate dehydrogenase (EC 1.2.4.1); *2*, citrate synthase (EC 4.1.3.7); *3*, aconitase (EC 4.2.1.3); *4*, isocitrate dehydrogenase-NAD$^+$ (EC 1.1.1.41); *5*, α-ketoglutarate dehydrogenase (EC 1.2.4.2); *6*, succinyl-CoA synthetase (EC 6.2.1.4); *7*, succinate dehydrogenase (EC 1.3.99.1); *8*, fumarase (EC 4.2.1.2); *9*, malate dehydrogenase-NAD$^+$ (EC 1.1.1.37); *10*, glutamate dehydrogenase (EC 1.4.1.2); *11*, GAD (EC 4.1.1.15); *12*, GABA aminotransferase (EC 2.6.1.19); *13*, succinate-semialdehyde dehydrogenase (EC 1.2.1.24); *14*, glutamine synthetase (EC 6.3.1.2); *15*, glutaminase (EC 3.5.1.2); *16*, aldehyde reductase (EC 1.1.1.2).

drugs is not a consequence of a putative increase in overall glucose utilization (7), because the flux through the pyruvate dehydrogenase (EC 1.2.4.1)-catalyzed reaction, as measured by the rate of [3,4-^{14}C]glucose oxidation (23) (Fig. 1), was not changed by the presence of VPA or *E*-Δ^2-VPA (Table 1). In addition, the rate of [6-^{14}C]glucose oxidation was not modified by the presence of these drugs (Table 1), suggesting that the activity of the tricarboxylic acid cycle is not changed under these circumstances. These results are in agreement with those of Chapman *et al.* (8), who reported no change in the rate of cortical glucose utilization in VPA-treated rats, and are consistent with the idea that VPA does not decrease the cerebral energy metabolism of the rat (26). It has been suggested that VPA might enhance the GABA shunt by inhibiting α-ketoglutarate dehydrogenase (11), which may divert α-ketoglutarate from the tricarboxylic acid cycle to the GABA shunt (Fig. 1). However, the presence of VPA or *E*-Δ^2-VPA did not decrease [6-^{14}C]glucose and [2-^{14}C]glucose oxidation (Table 1), which would be expected if VPA inhibited the α-ketoglutarate dehydrogenase-catalyzed reaction. Instead, our results are consist-

TABLE 1

Effect of VPA, *E*-Δ²-VPA, MSO, and GHB on the rate of glucose oxidation through different metabolic pathways in neonatal rat brain

Brain slices were incubated in phosphate-buffered saline (pH 7.4) containing D-glucose (5.4 mM) and 0.5 μCi of glucose labeled at the positions indicated, in the absence (control) or in the presence of 1 mM VPA, *E*-Δ²-VPA, MSO, or GHB. The rate of oxidation to CO_2 was determined as described in Materials and Methods. Results are means ± standard errors for six to 10 neonates from five or six different litters.

	Glucose incorporated into CO_2				
	Control	VPA	*E*-Δ²-VPA	MSO	GHB
	μmol/hr/g of wet weight				
[1-^{14}C]Glucose	0.734 ± 0.025	0.728 ± 0.057	0.635 ± 0.033[a]	0.866 ± 0.023[b]	0.760 ± 0.036
[6-^{14}C]Glucose	0.425 ± 0.009	0.458 ± 0.009	0.431 ± 0.035	0.410 ± 0.042	0.415 ± 0.016
[2-^{14}C]Glucose	1.136 ± 0.038	1.364 ± 0.069[b]	1.782 ± 0.184[b]	1.186 ± 0.044	1.242 ± 0.079
[3,4-^{14}C]Glucose	2.955 ± 0.20	2.755 ± 0.144	3.173 ± 0.122		
Pathways estimates[c]					
PPP	0.309 ± 0.027	0.270 ± 0.051	0.204 ± 0.048[a]	0.456 ± 0.013[d]	0.345 ± 0.01
GABA shunt	0.505 ± 0.039	0.726 ± 0.053[b]	1.215 ± 0.187[d]	0.472 ± 0.192	0.597 ± 0.086

[a] $p < 0.05$ versus the control value.
[b] $p < 0.01$ versus the control value.
[c] Calculated as PPP = $^{14}CO_2$ (C_1) − $^{14}CO_2$ (C_6) and GABA shunt = [$^{14}CO_2$ (C_2 − C_6)] − 2/3[$^{14}CO_2$ (C_1 − C_6)], where subscripts designate the position of the ^{14}C label in glucose (see text and Refs. 23–25).
[d] $p < 0.001$ versus the control value.

ent with the idea that the effect of VPA in stimulating the GABA shunt is exerted on some reaction within the GABA shunt itself. It should be mentioned that the inhibition of GABA aminotransferase and succinate-semialdehyde dehydrogenase by VPA has been reported (9) as a putative mode of action of the drug. Thus, the increase in GABA concentrations presumably accomplished by the inhibition of these enzymes would result in the antiepileptic effect. However, the concentrations of VPA used by those authors (9) were much higher than those reached in blood with therapeutic doses (1–3). Nevertheless, the inhibition of these enzymes cannot explain the enhancement of [2-^{14}C]glucose decarboxylation caused by VPA or *E*-Δ²-VPA observed in our experiments (Table 1). Additionally, the activation of the GABA shunt by VPA and by *E*-Δ²-VPA observed in our experiments does not seem to be a consequence of the elevation of glutamate concentrations (1) presumably brought about by the inhibition of glutamine synthetase (EC 6.3.1.2) by VPA (Fig. 1). Thus, the activity of the GABA shunt was not changed by the presence of MSO (Table 1), a known inhibitor of glutamine synthetase (27, 28). Finally, the effect of VPA on the GABA shunt seems to be unrelated to the accumulation of GHB caused by administration of the drug (29, 30), because the presence of GHB (Table 1) did not change GABA shunt activity.

Alternatively, the enhancement of [2-^{14}C]glucose oxidation caused by these drugs (Table 1) suggests that the glutamate decarboxylase-catalyzed reaction might be the target for the effect of VPA, because this enzyme specifically decarboxylated the carbon at the 2-position (Fig. 1). In agreement with this suggestion, we observed an increase of GAD activity (12.8%; $p < 0.001$; three experiments) at 30 min after *in vivo* administration of a therapeutic dose of VPA (200 mg/kg, intraperitoneally). In addition, GAD activity was significantly enhanced (17.5%; $p < 0.005$; three experiments) when brain slices were incubated for 2 hr in the presence of 1 mM VPA under the experimental conditions used for the estimation of GABA shunt activity. However, the presence of 1 mM VPA in the reaction medium had no effect on GAD activity measured with either crude or partially purified enzymes from neonatal rat brain. Neither the K_m nor the V_{max} of GAD were changed by the presence of the drug (results not shown). Consequently, these results suggest that VPA activates GAD by an indirect, long term mechanism.

In conclusion, our results suggest that VPA and *E*-Δ²-VPA increase GABA concentrations in neonatal rat brain by stimulating GABA synthesis, rather than by inhibiting GABA degradation. The results are also consistent with the idea that these drugs activate the GAD-catalyzed reaction, thus accounting for their mechanism of action.

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