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# INHIBITION BY VALPROIC ACID OF PYRUVATE UPTAKE BY BRAIN MITOCHONDRIA

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**Abstract**—The anticonvulsive drug, valproic acid, inhibits competitively the pyruvate carrier in rat brain and liver mitochondria. Due to this inhibition the oxygen consumption supported by pyruvate oxidation is also affected. In our experimental conditions, pyruvate oxidation is partially inhibited by VPA concentration as low as 0.05 mM. Valproic acid, however, is unable, even at 10 mM, to fully inhibit pyruvate oxidation. Concentrations of VPA higher than 1 mM have an uncoupling effect on mitochondrial respiration. The oxidation of other mitochondrial substrates such as isocitrate, 2-ketoglutarate, DL-3-hydroxybutyrate and succinate is uncoupled but not inhibited by VPA. The effects of VPA on mitochondrial metabolism may be related to the therapeutic and/or toxicologic properties of this drug.

Valproic acid (di-n-propylacetic acid, VPA) is the only antiepileptic drug with an aliphatic structure, containing no nitrogen. The therapeutic effects of VPA have been attributed to its ability to raise the $\gamma$-amino butyric acid (GABA) levels in the CNS [1, 2], due to the inhibition of GABA degradative enzymes [3, 4]. Since GABA is an inhibitory neurotransmitter, higher levels of this compound can prevent the seizures [5]. Plasma levels of glycine, another inhibitory neurotransmitter [6], are also raised in patients and in rats treated with VPA [7, 8].

The results presented in this paper provided evidence of the VPA inhibitory effect on pyruvate metabolism, due to the inhibition of pyruvate mitochondrial carrier. This inhibition is discussed in relation to the therapeutic effect of VPA.

## MATERIALS AND METHODS

Mitochondria were isolated from adult male rats of the Wistar strain weighing 200–300 g. Brain mitochondria were prepared by the method of Clark and Nicklas [9]; liver mitochondria were prepared according to Schneider [10] and then resuspended in 0.32 M sucrose at a concentration of 50 mg protein/ml. For some experiments, mitochondria were osmotically disrupted by dilution in 10 vol. of ice cold 5 mM Tris–HCl, pH 7.4; after 30 min, the mitochondrial suspension was centrifuged at 8000 *g* for 10 min and the pellet was resuspended at a concentration of 50 mg protein/ml in 0.32 M sucrose. Protein was determined by the fluorimetric method of Resch *et al.* [11], calibration carried out by the method of Lowry *et al.* [12] with bovine serum albumin as standard.

[1-$^{14}$C]Pyruvate uptake was measured by a filtration technique. Aliquots of 10 $\mu$l of mitochondrial suspension (about 0.5 mg mitochondrial protein) were preincubated at 27° in 100 $\mu$l of a solution of 120 mM KCl, 20 mM Tris–HCl pH 7.4, 5 mM succinate, 2 mM arsenite and 10 $\mu$M rotenone pH 7.4. After 2 min, [1-$^{14}$C]pyruvate (1 $\mu$Ci/$\mu$mole) and VPA were added in a final volume of 10 $\mu$l. The uptake was terminated 30 sec later by diluting in 5 ml of ice cold incubation medium and immediately filtering in a vacuum assembly through moistened Millipore filters (0.45 $\mu$m, pore size). The filters were rinsed twice with 5 ml of ice cold incubation medium. The dilution, filtration and washing procedures were carried out within 15 sec. Radioactivity in the filters was measured by liquid scintillation counting. All the experiments were corrected with a control obtained by diluting the mitochondrial incubation mixture before adding the radioactive substrate. No $^{14}CO_2$ was evolved during the uptake experiment. Oxygen utilization was measured in a 2 ml temperature controlled cuvette with a Clark-type electrode, connected to a Hitachi model 1002 recorder.

[1-$^{14}$C]-Pyruvate was purchased from Radiochemical Centre (Amersham, U.K.). Valproic acid was a gift from Laboratorios Labaz (Barcelona, Spain). All the other reactives were of reagent grade and were utilized without further purification.

## RESULTS

*Effects of VPA on the oxidation of several respiratory substrates by brain mitochondria.* Table 1 shows the effect of VPA on the oxidation by brain mitochondria of pyruvate, three tricarboxylic acid cycle intermediates (isocitrate, 2-ketoglutarate and succinate) and DL-3-hydroxybutyrate. VPA at 2.5 mM concentration produced a significant inhibition of the oxidation of pyruvate only. This effect was not due to the inhibition of tricarboxylic acid cycle since the oxidation of its intermediates was not affected. The oxidation of DL-3-hydroxybutyrate, which enters the cycle at the level of acetyl-CoA, was not affected

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Table 1. Effect of VPA on the oxidation of several substrates by brain mitochondria

| Addition | Oxygen consumption (natom/min/mg protein) No VPA State 4 | No VPA State 3 | 2.5 mM VPA State 4 | 2.5 mM VPA State 3 |
|---|---|---|---|---|
| 2 mM Pyruvate +2 mM malate | 36 | 179 | 73 | 73 |
| 2 mM Isocitrate +2 mM malate | 35 | 90 | 80 | 96 |
| 2 mM 2-Ketoglutarate +2 mM malate | 32 | 85 | 75 | 91 |
| 4 mM DL-3-Hydroxybutyrate +2 mM malate | 16 | 65 | 59 | 72 |
| 5 mM Succinate +10 μM rotenone | 51 | 122 | 130 | 130 |

Mitochondria (0.5 mg protein) were incubated at 27° in 2 ml 120 mM KCl, 20 mM Tris–HCl and 2 mM potassium phosphate, pH 7.4. To induce State 3, 200 nmole ADP was added. State 4 was measured after the ADP stimulation of oxygen consumption was finished. Each value is the mean of 3 experiments, with a S.E. of less than 5% in all the cases.

either. Furthermore, VPA had no inhibitory effect on the electron transport process of the respiratory chain since the oxidation of NADH by osmotically disrupted mitochondria was not affected by this compound (Table 2).

*Effect of VPA on the pyruvate oxidation.* The effect of several VPA concentrations on the pyruvate oxidation by brain mitochondria was tested (Table 3). State 3 respiration was inhibited by VPA, with 50% inhibition between 0.25 and 0.5 mM VPA. This inhibitory effect was not reversed by 5 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (a proton ionophore), indicating that the VPA effect was not on the oxidative phosphorylation (results not shown). In our experimental conditions, the inhibition by VPA of the pyruvate oxidation was, however, never greater than 65%, even at high VPA concentrations (10 mM).

*Uncoupling effect of VPA.* VPA stimulated the State 4 respiration of all the substrates tested (Tables 1 and 3). This uncoupling ability could be due to the fatty acid structure of VPA, allowing it to enter the mitochondria with a proton, causing the pH gradient to collapse. Due to this characteristic, VPA lowered the respiratory control ratio of all the substrates tested. Figure 1 shows the values of the respiratory control ratio for the oxidation of pyruvate and succinate at several VPA concentrations. When 3 mM VPA was present, the mitochondria became fully uncoupled (respiratory control ratio = 1). Oligomycin, a $H^+$-ATPase inhibitor, could not revert the stimulation of the State 4 respiration by VPA. This uncoupling effect has been previously reported by Ciesielski *et al.* [13] in the oxidation of pyruvate by brain mitochondria.

Table 2. Effect of VPA on the oxidation of several substrates by osmotically disrupted brain mitochondria

| Addition | Oxygen consumption (natom/min/mg protein) No VPA | 2.5 mM VPA |
|---|---|---|
| 0.1 mM NADH | 78 | 79 |
| 2 mM Pyruvate +2 mM malate | 165 | 162 |

Osmotically disrupted mitochondria (0.5 mg protein) were incubated at 27° in 2 ml 120 mM KCl, 20 mM Tris–HCl and 2 mM potassium phosphate, pH 7.4. Each value is the mean of 3 experiments with a S.E. of less than 5%.

*Effect of VPA on the pyruvate uptake by brain mitochondria.* The inhibitory effect of VPA on pyruvate oxidation disappeared when mitochondria were osmotically disrupted (Table 2). For this reason, it is possible that the effect of VPA was at the level of a pyruvate carrier. Pyruvate enters the liver [14] and brain [15] mitochondria by a carrier mediated process. VPA inhibited the uptake of pyruvate in brain mitochondria (Fig. 1, inset) with similar quantitative effects as that on pyruvate respiration (see Table 2).

The kinetic characteristics of this inhibition has been studied (Fig. 1). In our experimental conditions, pyruvate uptake by brain mitochondria had a $V_{max}$ of 30.3 nmole/min per mg protein and a $K_m$ of 0.46 mM. VPA inhibited the uptake in a competitive manner ($V_{max}$ remained unchanged) and with apparent $K_m$ of 1.53 mM (0.25 mM VPA) and 2.56 mM (0.5 mM VPA). From these data the $K_i$ calculated for VPA was 0.106 mM.

VPA never fully inhibited pyruvate uptake (Fig. 1 inset), or the pyruvate oxidation (Table 3). In our experimental conditions, the inhibitory effect on the carrier was never greater than 65% and was reached at 1 mM VPA. This may be due to the fact that VPA is unable to fully inhibit the carrier, or that some pyruvate can enter the mitochondria by a carrier independent process. The effect of VPA on pyruvate uptake by liver mitochondria has also been tested. In this case, a competitive inhibition was also found with a $K_i$ for VPA of 0.17 mM. VPA also had similar inhibitory and uncoupling effects on the oxidation of pyruvate by liver mitochondria (results not shown).

Table 3. Effect of several VPA concentrations on pyruvate and succinate oxidation by rat brain mitochondria

| VPA concn (mM) | Oxygen consumption (natom/min/mg protein) in the presence of 2 mM pyruvate + 2 mM malate | | |
|---|---|---|---|
| | State 4 | State 3 | Respiratory control ratio |
| — | 36 | 179 | 4.9 |
| 0.05 | 38 | 137 | 3.6 |
| 0.1 | 40 | 116 | 2.9 |
| 0.25 | 39 | 102 | 2.6 |
| 0.5 | 41 | 71 | 1.7 |
| 1 | 68 | 68 | 1 |
| 2 | 71 | 71 | 1 |
| 5 | 68 | 69 | 1 |

| VPA concn (mM) | Oxygen consumption (natom/min/mg protein) in the presence of 5 mM succinate + 10 μM rotenone | | |
|---|---|---|---|
| | State 4 | State 3 | Respiratory control ratio |
| — | 51 | 121 | 2.4 |
| 0.25 | 64 | 121 | 1.9 |
| 0.5 | 73 | 124 | 1.7 |
| 1 | 91 | 127 | 1.4 |
| 2 | 115 | 127 | 1.1 |
| 5 | 130 | 130 | 1.0 |

Mitochondria (0.5 mg protein) were incubated at 27° in 2 ml 120 mM KCl, 20 mM Tris–HCl, 2 mM potassium phosphate, pH 7.4. To induce State 3, 200 nmole ADP was added. State 4 was measured after the ADP stimulation of oxygen consumption was finished. Each value is the mean of 3 experiments with a S.E. of less than 5% in all the cases.

## DISCUSSION

The results allow us to conclude that VPA has an inhibitory effect on pyruvate oxidation by brain mitochondria, by interfering with the carrier for pyruvate.

VPA inhibits the pyruvate carrier in a competitive manner. This fact suggests that it should compete for the pyruvate binding site in the carrier. However, from these results it cannot be concluded that VPA utilizes the pyruvate carrier to enter the mitochondria, even though this possibility remains open.

Land *et al.* [15] found that D(−)-3-hydroxybutyrate enters the brain mitochondria by a carrier mediated process, as indicated by the inhibitory effect of phenylpyruvate and 2-ketoisocaproate. The fact that

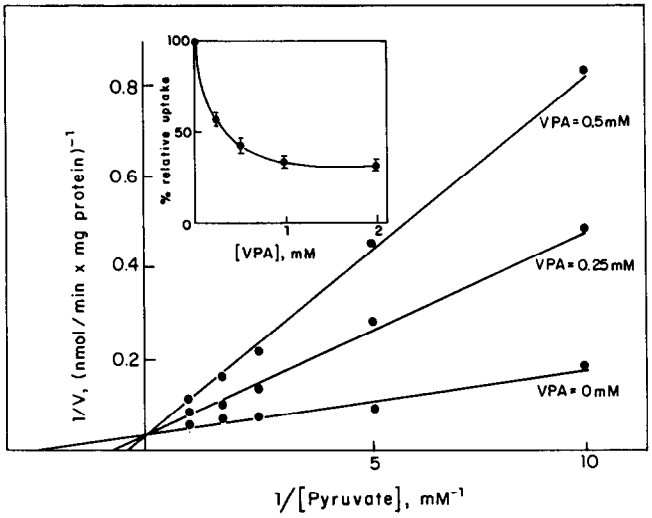


Fig. 1. Pyruvate uptake inhibition by VPA. Lineweaver–Burk plot of the VPA effect: brain mitochondria were incubated in the presence of several concentrations of [1-$^{14}$C]pyruvate and VPA. Each point represents the mean of 5 experiments. Lines were calculated by the least squares method. Correlation coefficients were higher than 0.96 in the three cases. Inset: effect of several concentrations of VPA on the uptake of 2 mM [1-$^{14}$C]pyruvate by brain mitochondria. 100% represents the uptake in absence of VPA. Each point represents the mean ± S.E. of 5 experiments.

VPA, which inhibits the pyruvate carrier, does not inhibit the DL-3-hydroxybutyrate oxidation suggests that this compound must enter mitochondria by a different way from the pyruvate carrier. On the other hand, the specificity and competitivity of the inhibition by VPA of pyruvate oxidation indicate a direct effect on the carrier rather than through collapsing the mitochondrial pH gradient, since in this case an inhibition of DL-3-hydroxybutyrate oxidation would be expected.

In the adult brain almost all metabolic energy is generated by the oxidation of pyruvate [16]. Thus, the inhibition of pyruvate carrier can interfere with energy production. The fact that VPA produces similar effect on the oxidation and on the transport of VPA, indicates that the carrier may be the rate-limiting step in the oxidation of pyruvate by brain mitochondria, as has been previously suggested by Mowbray [17] and Halestrap [14] for liver mitochondria.

Perhaps the degree of inhibitory activity of VPA is not enough to affect the glycolytic flux in normal physiological conditions, but when a high rate of energy production is needed, for example in convulsive periods, the carrier activity may become a limiting path for aerobic glycolysis, so that the rate of ATP synthesis will not be enough to support the seizures. This is supported by the work of Eymard *et al.* [18] who found that the administration of VPA (200 mg/kg) lowers the oxygen consumption in rats by 30%. The effect of VPA on GABA metabolism in relation to the prevention of seizures [2, 3, 5] is also important in explaining its therapeutic properties.

Some toxic effects of VPA have been described [19, 20]. These effects have been attributed to its interaction with other anticonvulsant drugs such as barbiturates. The possibility that they are also related to the effect of VPA on pyruvate carrier remains open, since, as some authors have suggested, neurological damage in phenylketonuria and maple syrup urine disease may be attributed to the inhibition of the pyruvate carrier by phenylpyruvate and 2-keto-isocaproate, respectively [15, 21]. The concentration of VPA found in the rat brain (0.3 μmolc/g wet wt, 1 hr after the intraperitoneal injection of 200 mg/kg body wt of VPA, results not published from our laboratory) is in the range of the VPA concentrations which inhibit pyruvate oxidation. It is difficult, given the uneven distribution of VPA in the brain [13] to quantitate this inhibition in *in vivo* conditions. The fact that VPA cannot fully inhibit pyruvate oxidation makes it unlikely that toxic effects due to this inhibition could be produced. However, in the presence of barbiturates, which inhibit the mitochondrial respiration [22], the VPA inhibition of pyruvate carrier may lead to toxic effects.

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