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Effects of Sodium Valproate on Mitochondrial Membranes: Electron Paramagnetic Resonance and Transmembrane Protein Movement Studies

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SUMMARY

Sodium valproate (VPA), the salt of a branched short-chain fatty acid, is a major antiepileptic whose mode of action, as yet unclear, may involve effects on the organization of membranes. VPA was either injected into rats whose liver and kidney mitochondria were then isolated, or was preincubated with isolated mitochondria. First, liver and kidney mitochondria were studied with paramagnetic probes. The electron paramagnetic resonance spectra of proteins of VPA-treated mitochondria spin-labeled with 4-maleimido-2,2,6,6-tetramethyl-1-pyrrolidinoxyl showed that the ratio of weakly immobilized to strongly immobilized SH groups was reduced with respect to control mitochondria, more so in liver than in kidney mitochondria of VPA-injected rats, and more so in kidney than in liver mitochondria for VPA-incubated mitochondria. Spectra of mitochondrial lipids spin-labeled with 5-doxyl stearic methyl ester showed that VPA had no significant effect on order parameters *S*. Second, the transmembrane movement of aspartate aminotransferase was studied by incubating liver mitochondria in a sucrose-succinate medium and then fractionating them. The translocation of aspartate aminotransferase from mitoplasts, vesicles formed of inner membrane and matrix, to the intermembrane fluid, was significantly higher in VPA-treated than in control mitochondria. Thus, VPA, at concentrations in the range of those used therapeutically, interacted with membranes by modifying the structural organization of the internal mitochondrial membrane, essentially the membrane protein conformation.

VPA (2-propylpentanoate) is a major antiepileptic used in the treatment of generalized epileptic seizures, in particular, nonconvulsive seizures (1). Its mode of action, as yet unclear, has been ascribed to an increase of the inhibitory neuromediator GABA, to a decrease of excitatory neuromediators, especially aspartate, or to an effect on membranes (2). Besides acting on neurons, VPA modifies certain aspects of cellular metabolism. The drug inhibits respiration of rat liver mitochondria *in vivo* (3) and *in vitro* (4). In the rat, as in man, the intake of VPA invariably induces a hyperammonemia due to an increased release of ammonia from the kidney into the circulation; the ammonia metabolism of liver is not affected (5, 6). But in the kidney, mitochondria play a major role in the metabolism of ammonia. These effects suggest that VPA, an 8-carbon, branched-chain fatty acid, could modify the properties of mitochondrial membranes. Here we have studied the effects of VPA on the organization and structure of renal and hepatic mitochondria of rats and on the organization of lipids and proteins in the mitochondrial membrane. In one set of experiments, paramagnetic probes were used. In a second set, the localization of the mitochondrial enzyme AAT (EC 2.6.1.1) was studied as a function of the extramitochondrial environment (7, 8); this localization depends upon the structure and composition of the inner mitochondrial membrane.

Materials and Methods

Mitochondrial isolation and VPA treatment. Male Wistar rats weighing 250–300 g were fasted for 18 hr. Fifteen min before sacrifice by decapitation, one group was injected intraperitoneally with VPA (Labaz Laboratories, France) 200 mg/kg in 0.9% NaCl, pH 7.4, to provide "VPA *in vivo*" mitochondria; this dose of VPA protects against convulsions (2). A control group was injected with 0.9% NaCl alone, and their mitochondria were incubated with VPA at 10, 20, 50, 100, 500, or 10,000 μM for EPR experiments or with VPA at 20, 100, or 500 μM for the AAT translocation experiments; these were the "VPA *in vitro*" mitochondria. Liver mitochondria were isolated as described by

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ABBREVIATIONS: VPA, sodium valproate; AAT, mitochondrial aspartate aminotransferase; EPR, electron paramagnetic resonance; GABA, γ-aminobutyric acid; Hepes, *N*-2-hydroxoethylpiperazine-*N*-2-ethanesulfonic acid; IMF, intermembrane fluid; MSL, 4-maleimido-2,2,6,6-tetramethyl-1-pyrrolidinoxyl; NADH, reduced nicotinamide adenine dinucleotide; 5-NMS, 5 doxyl stearic methyl ester; *S*, strongly immobilized spin-labeled SH groups; *W*, weakly immobilized spin-labeled SH groups.

Waksman and co-workers (9, 10), and kidney mitochondria were isolated by the method of Adam and Simpson (11). These and further steps were caried out at 4° unless otherwise noted.

EPR experiments. Whole mitochondria from liver and kidney were suspended in a medium containing sucrose, 70 mM, mannitol, 220 mM, Hepes, 2 mM, bovine serum albumin, 0.5 g/liter, pH 7.4.

Proteins were marked with the spin-label MSL (Syva, Palo Alto, CA), which reacts specifically with SH groups (12). The number of SH groups was determined before and after labeling (13). Since MSL can also label NH_2 groups in certain lipids (14), the number of NH_2 was also measured before and after labeling. A 2-ml mitochondrial mixture containing 50 mg of protein was incubated with MSL, 5×10^{-4} M, for 45 min at 0° with constant shaking, centrifuged at 35,000 × *g* for 15 min, and washed until no free label could be detected in the supernatant; three washes sufficed. The final protein concentration was 50 mg/ml.

For each spectrum, the *W/S* ratio was measured, that is, the ratio of weakly immobilized spin-labeled SH groups (*W*) to strongly immobilized spin-labeled SH groups (*S*).

Lipids of whole mitochondria were marked with the spin-label 5-NMS (Syva). The label solvent, ethanol, was evaporated in a stream of nitrogen gas, and the label was suspended in the incubation medium and sonicated for 5 min. The suspension was then incubated for 1 hr at 4° with the mitochondrial mixture containing 6 mg of protein in a final volume of 1 ml, and 1 μg of spin-label/mg of mitochondrial protein. The membranes were then centrifuged at 35,000 × *g* for 15 min at 4°, and washed three times. The order parameter, *S*, which is in this case an evaluation of the fluidity of the environment of fatty acids, was calculated for each spectrum from the outer and inner hyperfine splittings, 2 $T_\parallel$ and 2 $T_\perp$ (15).

EPR spectra were measured at 4° on a Brucker ER 420 apparatus equipped with an NMR gaussmeter, a frequency meter, and a thermocouple placed in the resonant cavity for temperature measurement. Samples were temperature-equilibrated for 5 min before the spectra were measured.

AAT transmembrane movement. To liver mitochondria suspended in 0.25 M sucrose and containing 50 mg of protein/ml, sodium succinate at concentrations ranging from 0 to 50 mM in 0.25 M sucrose was added in order to release AAT from the inner membrane and matrix into the IMF (9). The mixture was incubated for 5 min at 37° with constant shaking and the mitochondria were then fractionated: IMF was separated from mitoplasts, the vesicles formed by the inner membrane and matrix, by the digitonin technique (9), using 1 mg of digitonin/10 mg of mitochondrial protein. The Lubrol method was used to separate the mitoplasts into inner membrane and matrix (10).

AAT activity was measured spectrophotometrically at 340 nm (16, 17) in the presence of 0.5% Triton X-100. Specific activity of AAT was expressed as μmol of NADH oxidized/min/mg of protein. Protein concentrations were determined by the method of Lowry *et al.* (18). Statistical calculations were performed using an analysis of variance applied to two factor-experiments with repeated measures on one factor.

Results

Tissular VPA concentrations. Total VPA concentrations, measured by a gas chromatographic method, were: for the serum of rats injected with VPA, 250 ± 30 mg/liter (mean ± SD; three measurements); for the liver, 3.00 ± 0.20 μg/mg of protein; for the kidney, 2.52 ± 0.38 μg/mg of protein; and for the hepatic and renal mitochondria, 0.30 and 0.29 μg/mg of protein, respectively.

EPR studies. The protein spin-label MSL did not mark lipid NH_2 groups in our conditions. The EPR spectra of liver and kidney mitochondria were similar: they are the superposition of two spectra, one corresponding to a population of strongly immobilized spin-labels (*S*), and the other to a population of weakly immobilized spin-labels (*W*). In "VPA *in vivo*" mitochondria compared to controls (Table 1), *W/S* was reduced, more for liver than for kidney mitochondria. In "VPA *in vitro*" mitochondria, there was also a decrease of *W/S*, but more for kidney than for liver mitochondria (Fig. 1). This effect appeared already at low VPA concentration. At a high concentration, 10 mM, the effect was inverse, insofar as *W/S* increased. Detergent treatments have yielded similar results,[1] suggesting that VPA at high concentration acts like a detergent.

In lipid spin-labeling with 5-NMS, VPA *in vivo* (Table 1) or *in vitro* did not significantly affect order parameters *S* of liver or kidney mitochondria.

AAT translocation in liver mitochondria. Fig. 2 shows the values of AAT specific activity of liver mitochondrial fractions in 0.25 M sucrose and in the sucrose medium containing successively higher concentrations of succinate. For control mitochondria, the presence of succinate reduced AAT activity in mitoplasts and increased it significantly ($p < 0.001$) in IMF; AAT was translocated from its normal position on the internal side of the inner membrane to the intermembrane fluid.

"VPA *in vivo*" mitochondrial fractions in sucrose medium showed the same AAT activity as controls. But in the presence of increasing succinate concentrations, significantly more AAT was translocated from mitoplasts to IMF than in controls ($p < 0.001$). The change in bulk protein localization followed a pattern comparable to that of AAT except that the phenomenon was less pronounced ($p < 0.01$).

The same effects were observed with "VPA *in vitro*" mitochondria. Fig. 2 shows the values of AAT specific activity for fractions of whole mitochondria preincubated with VPA, 20, 100, or 500 μM, a concentration similar to that used therapeutically, and the effects of progressively higher concentrations of succinate on the localization of AAT activity.

Discussion

The two different experiments described demonstrate that VPA interacts with mitochondrial membranes. EPR studies are a method for examining the structural state of membranes. The use of a protein probe and the measurement of *W/S* changes, a sensitive indication of conformational changes in protein (19), showed that VPA, in reducing *W/S* ratios, affected the conformation of mitochondrial membrane proteins. EPR spectra reflect the superposition of structural perturbations arising in different proteins and protein domains. VPA acted as if to change the microenvironment of protein SH groups but, since the distribution of spin-labeled SH groups is unknown, the *W/S* changes observed cannot be assigned to a particular type of protein. As the decreased *W/S* ratios differed with the organ and the mode of VPA administration, VPA may have acted not only directly, but also indirectly, through a metabolite of the drug.

VPA had no significant effect, however, on the mitochondrial lipid matrix in our experimental conditions. Fatty acid spin-labels are not uniformly distributed in membrane lipids (20) but are localized in the most fluid domains. Either the lipid domains marked by the 5-NMS probe were not involved in the VPA-induced membrane changes, or VPA did not induce changes in the lipid domain. In mouse synaptosomal membranes, VPA only weakly modifies the order parameters *S*

[1] Unpublished results.

TABLE 1

W/S ratio and order parameter S of control and "VPA-in vivo" mitochondria of liver and kidney

Rats were injected 15 min before sacrifice with saline or with VPA (200 mg/kg) in saline. *W/S* ratio was measured from the weakly immobilized spin-labeled SH groups (*W*) to strongly immobilized spin-labeled SH groups (*S*). Order parameter *S* was calculated from the outer and inner hyperfine splittings of EPR spectra. Statistical analysis was performed using Student's *t* test. Values are means ± standard deviation.

Mitochondria	*W/S* ratio		*S*	
	Control	VPA	Control	VPA
Liver ($n = 6$)	12.30 ± 3.13	6.84 ± 2.21[a]	0.850 ± 0.001	0.864 ± 0.001
Kidney ($n = 5$)	7.91 ± 0.97	6.51 ± 0.89[b]	0.863 ± 0.001	0.849 ± 0.001

[a] $p < 0.01$.
[b] $p < 0.05$.

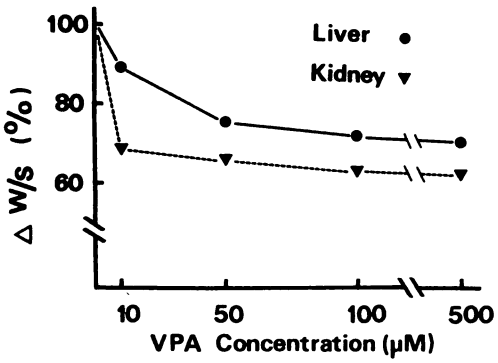

Fig. 1. In liver and kidney mitocondria preincubated with VPA and probed with the protein spin-label MSL, *W/S* derived from EPR spectra versus VPA concentration in the preincubation medium. *W/S* was calculated as the ratio of weakly to strongly immobilized SH groups of "VPA *in vitro*" mitochondria over the ratio of weakly to strongly immobilized SH groups of control mitochondria.

determined by EPR measurements (21), an effect confirmed by fluorescence polarization of diphenyl hexatriene. Lipid-domain membrane disordering is correlated with fatty acid chain length and antiepileptic efficacy (22, 23).

The AAT translocation experiments demonstrate that VPA modified mitochondrial transmembrane protein movement, a movement which depends on the structure and composition of the inner mitochondrial membrane (7, 8). The AAT, localized on the internal face of the inner membrane, can be translocated to the IMF in the presence of succinate. In the absence of succinate, the distribution of AAT in control and VPA mitochondria does not differ. In succinate medium, more enzyme is translocated in VPA *in vivo* and VPA *in vitro* mitochondria than in controls. This suggests, first, that, in the VPA *in vivo* mitochondria, the effect of VPA is direct and not due to a metabolite of the drug, and this with tissular concentration comparable to some tissue values of VPA injected at therapeutic doses (1, 2). Second, in the VPA *in vivo* mitochondria, VPA was closely associated with mitochondria; if not, VPA would have disappeared during mitochondrial isolation. In support of this interpretation, intraperitoneally injected ^{14}C-labeled VPA is preferentially bound to the mitochondrial fraction in kidney and liver (24).

Although the EPR studies and the AAT translocation experiments demonstrate that VPA acts on mitochondrial membranes, the results cannot be correlated, since the two methods are quite different; EPR studies appear to be more sensitive at very low concentrations, concentrations comparable to some tissue values of injected VPA (2), a similarity rarely observed hitherto. On the contrary, at high concentration (10 mM), the mode of action of VPA appears to change. The VPA-induced

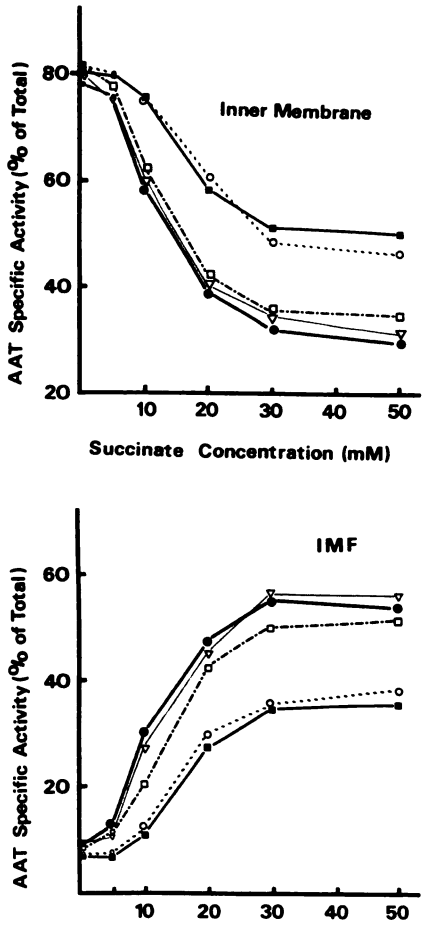

Fig. 2. AAT specific activity as percentage of total measured in control and VPA-treated rat liver mitochondrial fractions versus succinate concentration of incubation medium. *Top*, values for inner mitochondrial membrane; *bottom*, values for IMF, ■, controls; ●, "VPA *in vivo*" (see Materials and Methods). "VPA *in vitro*" (see Materials and Methods): ○, 20 μM; □, 100 μM; ▽, 500 μM.

modifications of the organization and movement of certain proteins could explain the drug's effect in inhibiting liver mitochondrial respiration (3, 4).

VPA can act on other biological membranes; applied to *Aplysia* neurons, it increases membrane potassium conductance and hyperpolarizes the resting membrane potential (25). VPA modifies the membrane potential of frog dorsal roots via an effect on the intra- and extracellular potassium concentration (26). VPA, which also potentiates the inhibitory effects of GABA (27–29), may act on GABAergic postsynaptic membranes (2, 30).

In conclusion, it has been shown with two different experimental approaches that VPA, a salt of a short branched-chain fatty acid, modifies the organization of liver and kidney mitochondrial membranes.

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