AVENACIOLIDE INHIBITION OF ANION TRANSPORT IN MITOCHONDRIA

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Received 12 July 1974

1. Introduction

McGivan and Chappell reported in 1970 that avenaciolide has a specific inhibitory effect on glutamate transport in rat liver mitochondria [1]. They observed 50% inhibition of glutamate oxidation on the addition of 47 μM avenaciolide [2] while oxidation of succinate, 3-hydroxybutyrate, citrate or malate was not inhibited by avenaciolide. They suggested that this inhibition was due to an analogy of structure between glutamate and avenaciolide and proposed to use this antibiotic to block glutamate transport specifically during investigations of the movements of other anions.

On the basis of their conclusion, this antibiotic has been used for this purpose in many recent works [3–7]. The present paper, shows that avenaciolide inhibits not only glutamate entry into mitochondria, but also entry of other dicarboxylic anions: for example, avenaciolide inhibits malate penetration when measured by the rate of reduction of endogenous pyridine nucleotide with a K_i lower than that measured for glutamate entry into rat liver mitochondria or pig heart mitochondria.

2. Materials and methods

Glutamate dehydrogenase activity was determined according to Julliard and Gautheron [8]. Preparation of rat liver mitochondria, pig heart mitochondria and determination of their protein content have been described previously [9,10] changes in the oxido–reduction state of intramitochondrial pyridine nucleotides were monitored by double beam, two wavelength spectrophotometry at 340–374 nm according to Chappell and Haarhoff [11]. Cardiolipin micelles were prepared and estimated as previously [12].

Avenaciolide was a generous gift either from the Northern Utilization Research Laboratories, Peoria, Illinois or from Dr. W. B. Turner, ICI United Kingdom. The uncoupler, 3,5-di-tert-butyl-4-hydroxybenzylidenemalonitrile, was a gift from Sumitomo Chemical Co. Ltd., Japan.

3. Results and discussion

As reported by McGivan and Chappell [1], the penetration of glutamate into rat liver mitochondria as measured by the reduction of endogenous pyridine nucleotides was inhibited by avenaciolide addition. However, table 1 shows that the concentration of avenaciolide corresponding to half maximum inhibition did not depend on glutamate concentration which did not support the idea of a direct competition between avenaciolide and glutamate. Moreover avenaciolide inhibition could also be observed in the same range of concentrations when pyridine nucleotides reduction was reduced by addition of malate or succinate. Similar results have been observed with pig heart mitochondria instead of rat liver and FCCP as uncoupler as well. Similar results were obtained whatever the avenaciolide origin was.

Avenaciolide (fig. 1) [2] contains a hydrocarbon chain and a polar part. It bears in position 11 a methylene group activated by an adjacent carbonyl and can react with sulfhydryl groups: when avenaciolide is added to reduced glutathione, it prevents the latter from reacting with Ellman's reagent. Its structure also

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Table 1
Avenaciolide inhibition of endogenous pyridine nucleotides reduction by different substrates

Substrate	Concentration (mM)	Avenaciolide concentration at half maximum rate (μM)
Glutamate*	0.33	12.6
	0.67	12.5
	1.67	10
	3.33	12
Malate*	1.67	4
	6.67	3.8
	16.7	3.5
Succinate**	1.67	7.3
	8.3	6.7

* Rat liver mitochondria (4.5 mg) were suspended in a medium containing 120 mM KCl, 20 mM Tris–chloride, 5 mM Tris–phosphate and avenaciolide, pH 7.4, in the cuvette of a double beam spectrophotometer. The total volume was 3 ml. 0.01 μM uncoupler, 3,5-di-tert-butyl-4-hydroxybenzyl idenemalononitrile, was added and after a 5 min incubation at 30°C, this was followed by 1.5 μg antimycin. The rate of reduction of pyridine nucleotides was followed at 340–374 nm after addition of glutamate or malate.

** Conditions were identical except that inhibitors were omitted and succinate was added after 10 min incubation at 30°C.

gives it the properties of a detergent. Other non ionic detergents such as Lubrol WX or Triton X-100 or ionic detergents such as sodium cholate or sodium dodecyl sulfate inhibited the reduction of endogenous nucleotides. Moreover avenaciolide increased the rate of reduction of externally added NAD in the presence of glutamate (fig. 2). This activation can be explained if avenaciolide damages the mitochondrial membrane inducing glutamate dehydrogenase leakage from the mitochondria. Avenaciolide did not inhibit glutamate dehydrogenase activity when measured on the purified enzyme in solution with widely varying NAD and glutamate concentrations. Inhibition by avenaciolide of the rate of glutamate entry into mitochondria was dependent on mitochondrial concentration. In the

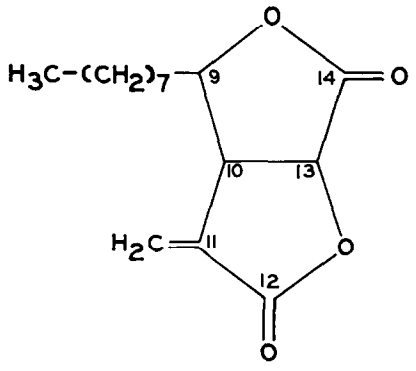

Fig. 1. Avenaciolide.

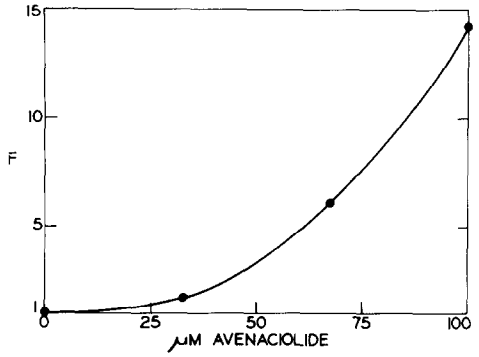

Fig. 2. Effect of avenaciolide on reduction of added NAD by glutamate in rat liver mitochondria. Conditions as described in table 1. After reduction or endogenous NAD in the presence of 1.7 mM glutamate, 0.33 mM external NAD was added and its rate of reduction measured. F = factor of multiplication of the rate of reduction of added NAD. Control rate = 7 nmoles NADH produced/min/mg mitochondrial protein.

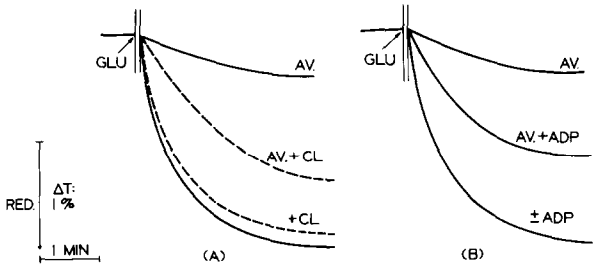

Fig. 3. Release of avenaciolide inhibition of mitochondrial pyridine nucleotides reduction by cardiolipin and ADP. Conditions are as in table 1. 33 μM avenaciolide, 80 nmoles cardiolipin micelles and 0.2 mM ADP were added (when indicated) 1 min before glutamate (1.67 mM) addition and 1 min after antimycin addition (not shown). Each assay contained 4 mg mitochondrial protein in a volume of 3 ml.

presence of 1.5 mg/ml mitochondrial protein 16.6 μM avenaciolide gives 60% inhibition; with 3 mg/ml of mitochondrial protein/ml, inhibition was only 20%.

In a previous paper [12], it has been shown that glutamate dehydrogenase can form aggregates with cardiolipin micelles and that ADP can increase the amount of glutamate dehydrogenase bound per mole of cardiolipin. Fig. 3 shows that addition of cardiolipin to mitochondria partly prevented avenaciolide inhibition when endogenous pyridine nucleotide reduction was induced by glutamate addition; ADP is also able to prevent partly avenaciolide inhibition in mitochondrial pyridine nucleotide reduction experiments. Therefore conditions that increased glutamate dehydrogenase association to cardiolipin also released avenaciolide inhibition.

4. Conclusions

The aim of the present paper is to point out the fact that great care should be taken in using avenaciolide as a *specific* inhibitor of glutamate translocation into mitochondria. Specificity may be only a matter of experimental conditions since inhibition of malate entry into mitochondria may be observed at avenaciolide concentrations lower than the one used in glutamate penetration inhibition. Harris and coworkers [6,13] already suggested that avenaciolide may act by freeing membrane-bound divalent cations inducing a lessened adsorption of anions such as glutamate. This paper shows that this decreased adsorption is not limited to glutamate. Our results are in favour of a detergent-like effect of avenaciolide as were the experiments made with analogs of avenaciolide [4] and do not exclude the reaction of avenaciolide with mitochondrial thiol groups as suggested previously [4, 14].

Acknowledgements

This work was partly realized in Dr. H. A. Lardy's laboratory. The author is very grateful to him for helpful advice and also thanks Dr. Gautheron for stimulating discussions.

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