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EFFECT OF THE GENERAL ANAESTHETIC, PROPANIDID ON OXIDATIVE PHOSPHORYLATION AND CALCIUM TRANSPORT IN RAT LIVER MITOCHONDRIA

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Abstract—The general anaesthetic, propanidid was found to have two major sites of action on mitochondrial oxidative phosphorylation. At low concentrations (<100 μM) the compound was an inhibitor of NAD^+-linked oxidations, acting at, or near, the rotenone-sensitive site on the NADH dehydrogenase enzyme system. High concentrations of propanidid (1–5 mM) stimulated both succinate oxidation and the mitochondrial ATPase: it was concluded that these effects were due to an uncoupling action. Mitochondrial Ca^{2+} transport was inhibited by propanidid when glutamate plus malate were used as substrate, but not when succinate replaced the NAD^+-linked substrates.

INTRODUCTION

A number of general anaesthetics have been shown to inhibit mitochondrial metabolism [1]. For example, inhalational anaesthetics such as halothane and intravenous anaesthetics such as the barbiturates and alphaxalone interact with the mitochondrial NADH dehydrogenase system [2–4]. Uncoupling activity has also been demonstrated for a variety of anaesthetics [5].

For a number of years, the proposal that the mechanism of action of general anaesthetics was mediated through their action on mitochondria has been under investigation. In particular, Krnjević and his coworkers have proposed that inhibition of mitochondrial oxidative phosphorylation by anaesthetics results in an impairment of Ca^{2+} transport across the mitochondrial membrane [6]. The consequence of this action would be an increase in cytoplasmic Ca^{2+}, which would lead to a modification of synaptic transmission in nerve cells. In support of this proposal, inhibition of mitochondrial Ca^{2+} transport has been demonstrated *in vivo*, after the administration of halothane, hexobarbitone or alphaxalone [7].

Because of the possible involvement of mitochondria in general anaesthesia, we have examined the effect of the short-acting, intravenous anaesthetic, propanidid on mitochondrial reactions, including substrate oxidation, adenosine triphosphatase and Ca^{2+} transport.

MATERIALS AND METHODS

Mitochondria. Tightly-coupled rat liver mitochondrial preparations were obtained by the method of Chappell and Hansford [8].

Enzyme activities. Oxygen consumption was measured polarographically using a Clark-type oxygen electrode (Rank Bros., Cambridge, U.K.). NADH oxidation was measured spectrophotometrically at 340 nm. NADH-ferricyanide reductase was measured by following the reduction of ferricyanide at 420 nm. ATPase activity was measured as described by Beechey [9], the inorganic phosphate released being determined by the method of Fiske and Subbarow [10]. The specific conditions employed in the measurement of the above reactions are given in the legends to the appropriate figures and table.

Protein. Protein was determined by the method of Gornall *et al.* [11], after solubilization of the mitochondria with sodium deoxycholate (0.16% w/v); bovine serum albumin was used as standard.

Chemicals. Analytical grade laboratory chemicals and biochemicals were purchased from British Drug Houses Ltd. (Poole, U.K. and Sigma Chemical Co., St. Louis, MO). Propanidid was provided by Bayer Pharmaceuticals Ltd. (Haywards Heath, U.K.). Propanidid was added to the reaction media as an ethanolic solution; controls carried out with equivalent amounts of ethanol showed that the solvent had no effect on the reactions under consideration.

RESULTS

Substrate oxidation

Figure 1 shows the effect of propanidid on the mitochondrial oxidation of two substrates, succinate and glutamate plus malate. In these experiments two distinct actions of propanidid on oxidative phosphorylation were discovered. First, the ADP-stimulated oxidation of glutamate plus malate (state 3) was progressively inhibited by low concentrations of

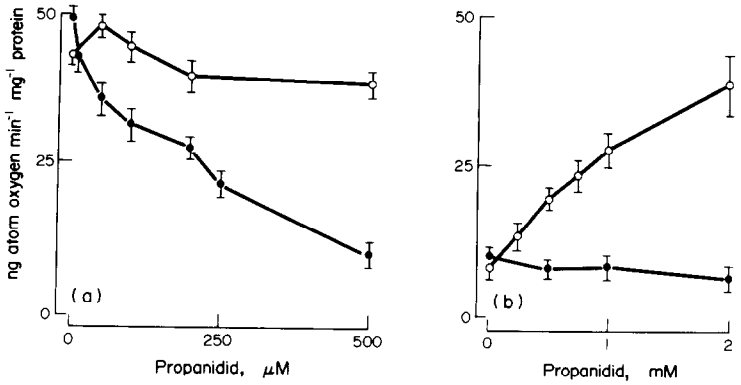

Fig. 1. Effect of propanidid on substrate oxidation in rat liver mitochondria. The reaction chamber of the oxygen electrode contained, 675 μmole sucrose, 9.2 μmole Tris–HCl buffer, pH 7.4, 10 μmole potassium phosphate buffer, pH 7.4, and either 10 μmole sodium succinate or 10 μmole sodium glutamate plus 10 μmole sodium malate. The reactions were initiated by the addition of rat liver mitochondria (5 mg protein) to the reaction chamber, followed 2 min later by the addition of 0.5 μmole ADP, to give state 3 conditions. Propanidid was added at the start of the experiment when the effect on state 3 respiration was measured, and 2 min after the addition of mitochondria when state 4 respiration was measured. The temperature was 30° and the reaction volume was 3 ml. (a) Effect of propanidid on state 3 respiration using succinate (upper trace) or glutamate plus malate (lower trace) as substrates. (b) Effect of propanidid on state 4 respiration using succinate (upper trace) or glutamate plus malate (lower trace) as substrates. Bars are the S.E. mean of 5 different experiments.

propanidid, whereas when ADP-stimulated succinate oxidation was measured, although there was evidence of a loss of respiratory control, the reaction was virtually unaffected by propanidid over the same concentration range (Fig. 1a). Second, when state 4 respiration was measured (i.e. ADP absent, but substrate and oxygen in excess), propanidid elicited a concentration-dependent stimulation of respiration in the presence of succinate, but was without effect when glutamate plus malate was used as substrate (Fig. 1b). These results indicated the possibility of two types of action; an inhibition of the mitochondrial respiratory chain when glutamate plus malate was the substrate, and an uncoupling action in the presence of succinate.

Respiratory chain inhibition

Uncoupling concentrations of 2,4-dinitrophenol did not restore the respiration rates to control levels in mitochondria incubated with inhibitory amounts of propanidid, in the presence of glutamate plus malate, confirming that the anaesthetic was acting as a respiratory chain inhibitor. In support of this conclusion, the measurement of mitochondrial difference spectra showed that whereas the mitochondrial cytochromes were reduced by the addition of glutamate plus malate, there was no evidence of cytochrome reduction when the experiment was repeated in the presence of propanidid. It was concluded that propanidid was interacting with a component of the respiratory chain located on the substrate side of cytochrome *b*.

The results of experiments designed to locate the site of action of propanidid more precisely are summarized in Table 1. NADH oxidation was inhibited by propanidid, rotenone and *p*-hydroxymercuriben-

Table 1. Effect of propanidid of NADH oxidation and ferricyanide reduction in rat liver mitochondria

Additions	NADH oxidised	Ferricyanide reduced
	(μmoles min^{-1} mg^{-1} mitochondrial protein)	
Control	0.78	0.75
Propanidid (250 μM)	0.03	0.77
Rotenone (15 nmoles)	0	0.84
p-Hydroxymercuribenzoate (1 μmole)	0.25	0.19

NADH oxidation was measured spectrophotometrically at 340 nm. Blank and experimental cuvettes contained 670 μmole sucrose, 27 μmole Tris–HCl buffer, pH 7.4, 2 mg rat liver mitochondrial protein and 500 μg deoxycholate. The reaction was initiated by the addition of 0.4 μmole NADH to the experimental cuvette. Ferricyanide reduction was measured spectrophotometrically at 420 nm. Blank and experimental cuvettes contained 670 μmole sucrose, 30 μmole potassium phosphate buffer, pH 7.4, 2 μmole potassium ferricyanide, 10 μg antimycin A, 500 μg sodium deoxycholate and 2 mg rat liver mitochondrial protein. The reaction was initiated by the addition of 0.5 μmole NADH to the experimental cuvette. For both reactions, inhibitors were added 2 min before the addition of NADH; the reaction temperature was 30° and the final vol. 3 ml.

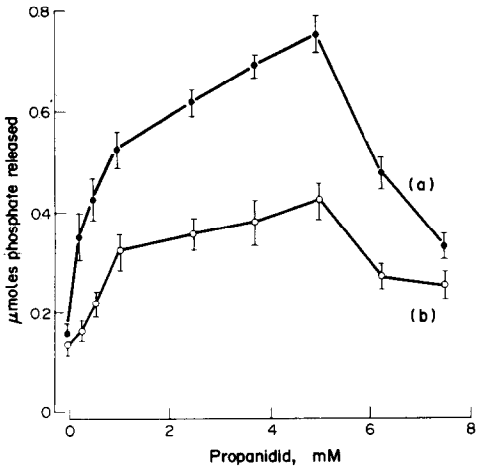

Fig. 2. Effect of propanidid on rat liver mitochondrial adenosine triphosphatase. At zero time 0.1 ml mitochondrial suspension (1 mg protein) was added to 0.9 ml reaction medium containing, 0.125 M sucrose, 63 mM Tris–HCl buffer, pH 7.4 and 2.5 mM ATP. Propanidid was included in the reaction medium at the concentrations shown. After 10 min incubation at 30°, the reaction was terminated by the addition of 0.1 ml 40 per cent (w/v) trichloracetic acid, and the inorganic phosphate liberated was estimated by the method of Fiske and Subbarow [10]. (a) Effect of range of concentrations of propanidid; (b) effect range of concentrations of propanidid in the presence of oligomycin (1 μg). Bars are the S.E. mean of 5 different experiments.

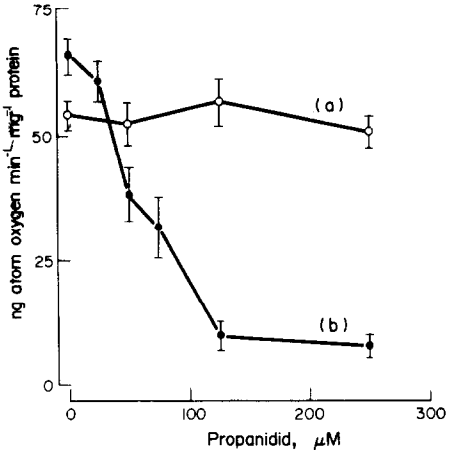

Fig. 3. Effect or propanidid on Ca^{2+}-stimulated respiration in rat liver mitochondria. Oxygen uptake was measured as described in the legend to Fig. 1, except that ADP was replaced with $CaCl_2$ (1 μmole). (a) Succinate as substrate; (b) glutamate plus malate as substrate. Bars are the S.E. mean of 5 different experiments.

zoate at the same concentrations that affected glutamate plus malate oxidation; this excludes either glutamic or malic dehydrogenases as possible targets for the inhibitors. Since ferricyanide accepts electrons from the mitochondrial NADH dehydrogenase, effectively splitting the overall reaction into two parts, ferricyanide reduction can be used to determine whether an inhibitor acts between NADH and the flavoprotein system, or between the flavoprotein and cytochrome *b*. Table 1 shows that *p*-hydroxymercuribenzoate blocked the reduction of ferricyanide, but both propanidid and rotenone were without effect: in fact, a variable stimulation of up to 30 per cent was usually observed. These results indicate that low concentrations of propanidid act on the mitochondrial NADH dehydrogenase at, or near, the rotenone-sensitive site.

Uncoupling action

When succinate was used as substrate, both propanidid and the uncoupling agent, 2,4-dinitrophenol released the inhibition of ADP-stimulated respiration brought about by the energy-transfer inhibitor, oligomycin. Propanidid stimulated the mitochondrial ATPase over the same concentration range that produced stimulation of state 3 respiration; further increases in the concentration of propanidid gave a concentration-dependent inhibition of the stimulated reaction (Fig. 2). The propanidid-stimulated ATPase was inhibited by oligomycin (Fig. 2). Similar results were obtained with 2,4-dinitrophenol.

Ca^{2+}-stimulated respiration

The addition of Ca^{2+} to tightly-coupled mitochondria, in the presence of a permenant anion such as phosphate or acetate, results in the rapid uptake of the cation; this uptake is accompanied by a stimulation of respiration, which returns to control levels when the Ca^{2+} has been sequestered by the mitochondria. Ca^{2+}-stimulated respiration can therefore be used as an index of mitochondrial Ca^{2+}uptake. Propanidid inhibited the stimulation of respiration elicited by Ca^{2+} when glutamate plus malate were used as substrate, but not when the mitochondria were incubated in the presence of succinate (Fig. 3). A similar pattern of activities was found when phosphate was replaced with acetate.

DISCUSSION

The results presented in this paper show that propanidid has two main effects on mitochondrial ATP synthesis. At low concentrations, propanidid inhibited electron transport by an interaction with the NADH dehydrogenase system. The second action, uncoupling of oxidative phosphorylation, became evident when NAD^+-linked substrates were replaced with succinate, and when the concentration of propanidid was increased. We consider that, in view of the non-specific nature of the uncoupling action, it is unlikely to play a role in anaesthesia.

Krnjević has suggested that one important consequence of an action of general anaesthetics on ATP synthesis would be a blockade of Ca^{2+} transport across the mitochondrial membrane [12], but little direct evidence has been presented in support of his proposal. Thus, although many general anaesthetics inhibit ATP synthesis [1–5], only halothane, hexobarbitone and alphaxalone have been shown to inhibit Ca^{2+} transport [7, 13]. The present work demonstrates that propanidid is a potent inhibitor of Ca^{2+} transport, since Ca^{2+}-stimulated respiration was blocked by low concentrations of the anaesthetic, in the presence of glutamate plus malate. The site of action of propanidid on Ca^{2+} transport is likely to be the NADH dehydrogenase. Two lines of evi-

dence support this conclusion: inhibition of Ca^{2+}-stimulated respiration, in the presence of glutamate plus malate, and inhibition of glutamate plus malate oxidation required the same concentrations of propanidid; and Ca^{2+}-stimulated respiration was not inhibited when succinate was used as substrate. The failure of propanidid to block Ca^{2+} transport in the presence of succinate excludes the possibility of a direct action of the anaesthetic on the Ca^{2+} transport system.

Since one of the important postulates of the Krnjević hypothesis for the mechanism of general anaesthesia is that anaesthetics should be effective inhibitors of mitochondrial Ca^{2+} uptake, it is important to determine the action of anaesthetic molecules on this process. In the present paper, the intravenous anaesthetic, propanidid has been found to be a potent inhibitor of mitochondrial ATP synthesis and Ca^{2+} transport.

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