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EFFECT OF ALPHAXALONE, A NEW INTRAVENOUS ANAESTHETIC, ON OXIDATIVE PHOSPHORYLATION IN RABBIT BRAIN MITOCHONDRIA

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Abstract—The effects of alphaxalone, a new intravenous anaesthetic, on mitochondrial reactions are described. At low concentrations (20–100 μM) the compound was found to be an inhibitor of the oxidation of NAD^+-linked substrates in rabbit brain mitochondria. It was concluded that the site or action was at, or near, the rotenone-sensitive site on the NADH dehydrogenase enzyme system. When succinate was substrate, and when higher concentrations (300 μM) were used, alphaxalone was found to release respiratory control, stimulate state 4 respiration, relieve oligomycin-inhibited state 3 respiration and stimulate the mitochondrial ATPase. It was concluded that this second effect was due to an uncoupling action.

ALTHESIN, a new intravenous anaesthetic agent, is a mixture of two steroid molecules, alphaxalone (3α-hydroxy-5α-pregnane-11,20-dione) and alphadolone acetate (21-acetoxy-3α-hydroxy-5α-pregnane-11,20-dione). Both molecules possess anaesthetic properties but alphaxalone, which constitutes the major component of the mixture, is more potent than alphadolone.[1]

Many anaesthetics[2] and steroids[3] have been shown to impair mitochondrial function, and as a result it has been suggested that maintainance of the anaesthetic state could be due to depression of mitochondrial ATP synthesis. As a part of our investigations into the possible mode of action of alphaxalone we have tested the effects of the compound on mitochondrial reactions. In the past the majority of the studies concerning the effects of anaesthetics on mitochondrial function have been carried out using mitochondria prepared from non-nervous tissues such as liver. We feel that it is more relevant to use brain mitochondria, and so in the present studies we have used a rabbit brain preparation that is capable of carrying out tightly-coupled respiration with a variety of substrates. The effects of alphaxalone on mitochondrial reactions have not been previously reported.

MATERIALS AND METHODS

Mitochondria. Tightly-coupled rabbit brain mitochondrial preparations were obtained by the method of Clark and Nicklas,[4] as modified by Smith.[5]

Enzyme activities. Oxygen consumption was measured polarographically using a Clark-type oxygen electrode (Rank Bros., Bottisham, Cambridge). NADH-ferricyanide reductase was measured spectrophotometrically by following the reduction of ferricyanide at 420 nm. ATPase activity was measured as described by Beechey.[6]

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Inorganic phosphate was estimated by the method of Fiske and Subbarow.[7] The specific conditions employed in the measurement of the above reactions are included in the legends to the appropriate figures and tables.

Protein. Protein was determined by the method of Gornall *et al.*[8] after solubilization of the mitochondria with deoxycholate (0·16% w/v); bovine serum albumin was used as standard.

Chemicals. Rotenone was supplied by the Aldrich Chemical Co., Milwaukee, Wis., U.S.A. Analytical grade laboratory chemicals and biochemicals were purchased from British Drug Houses Ltd, Poole, Dorset, and Sigma Chemical Co., St. Louis, Mo., U.S.A. Alphaxalone was provided by Glaxo Laboratories Ltd, Greenford, Middlesex. Alphaxalone was added to the reaction media as a solution in ethanol; controls carried out with equivalent amounts of ethanol showed that the solvent had no effect on the reactions under consideration.

TABLE 1. THE EFFECT OF ALPHAXALONE ON NAD^+-LINKED SUBSTRATE OXIDATIONS IN RABBIT BRAIN MITOCHONDRIA

Alphaxalone concn (μM)	Oxygen consumption (ng atom min^{-1} mg^{-1} mitochondrial protein) Glutamate plus malate State 3	Glutamate plus malate State 4	Pyruvate plus malate State 3	Pyruvate plus malate State 4
Control	60·8	12·8	105	17·6
33	41	12·8	85·2	17·6
66	28·8	12·8	67·2	17·6
132	24	12·8	38·4	19·2
264	12·8	12·8	21	21

The reaction chamber of the oxygen electrode contained, 675 μmoles sucrose, 9·2 μmoles Tris–HCl buffer, pH 7·4, 10 μmoles potassium phosphate buffer, pH 7·4, and either 10 μmoles sodium glutamate plus 10 μmoles sodium malate or 10 μmoles sodium pyruvate plus 10 μmoles sodium malate. The reactions were initiated by the addition of rabbit brain mitochondria (5 mg protein) to the electrode chamber, followed 2 min later by 0·5 μmoles ADP. When present alphaxalone was added at the concentrations shown at the start of the experiment. The temperature was 30° and the reaction volume was 3 ml.

RESULTS

NAD-linked oxidations. Table 1 shows the effect of alphaxalone on the oxidation of NAD^+-linked substrates in rabbit brain mitochondria. State 3 respiration (i.e. ADP present with substrate and oxygen in excess) was inhibited by low concentrations of alphaxalone (20–100 μM) when either glutamate plus malate or pyruvate plus malate were used as substrates. State 4 respiration (i.e. ADP absent, but substrate and oxygen in excess) was virtually unaffected by alphaxalone. The inhibition of state 3 respiration was not released by the addition of uncoupling concentrations of 2,4-dinitrophenol.

Measurement of mitochondrial difference spectra, in the presence and absence of alphaxalone and rotenone, a well-documented inhibitor of mitochondrial NAD^+-linked oxidations,[9] showed that both compounds gave similar spectra (see Fig. 1). In control experiments all the cytochromes were reduced, but when the inhibitors were added there was no evidence of cytochrome reduction, showing that the sites of action were on the substrate side of cytochrome *b*.

Ferricyanide will accept electrons from the flavoprotein component of the NADH dehydrogenase enzyme system, provided that the mitochondria are supplied with a suitable NAD^+-linked substrate, such as pyruvate plus malate. Thus ferricyanide

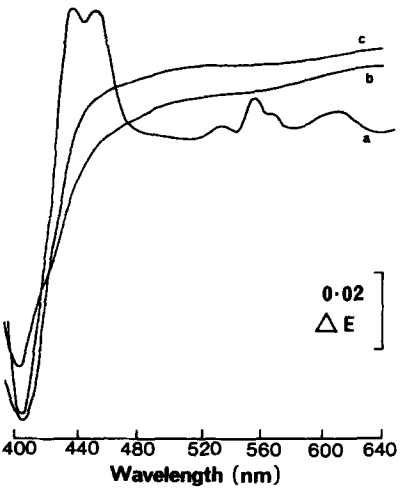

FIG. 1. Effect of alphaxalone and rotenone on difference spectra in rabbit brain mitochondria. Blank and experimental cuvettes of 1 cm light path contained 675 μmoles sucrose, 50 μmoles potassium chloride, 50 μmoles triethanolamine buffer, pH 7·4 and rabbit brain mitochondria (10 mg protein). The reaction was initiated by the addition of 10 μmoles sodium glutamate plus 10 μmoles sodium malate to the experimental cuvette. (a) Control experiment showing the mitochondrial difference spectra obtained in the presence of 10 μmoles potassium cyanide; note the cytochrome peaks; (b) 10 nmoles rotenone; (c) 260 μM alphaxalone.

allows the NADH dehydrogenase to be split into two parts, one between NADH and the flavoprotein, and one between the flavoprotein and cytochrome *b*. Table 2 shows the effects of alphaxalone and two NADH dehydrogenase inhibitors, rotenone and *p*-hydroxymercuribenzoate, on ferricyanide reducation in rabbit brain mitochondria. Both rotenone and alphaxalone failed to inhibit the reaction; in fact, a variable stimulation of between 25 and 50 per cent was usually observed. *p*-Hydroxymercuribenzoate, on the other hand, gave a marked inhibition of ferricyanide reduction. These results indicate that low concentrations of alphaxalone interact with the NADH dehydrogenase enzyme system at, or near, the rotenone-sensitive site.

TABLE 2. THE EFFECT OF ALPHAXALONE ON FERRICYANIDE REDUCTION IN RABBIT BRAIN MITOCHONDRIA

Additions	Ferricyanide reduced (μmoles min^{-1} mg^{-1} mitochondrial protein)
Control	1·0
Alphaxalone (100 μM)	1·05
Alphaxalone (200 μM)	1·50
Alphaxalone (300 μM)	1·57
Rotenone (15 nmole)	1·25
p-Hydroxymercuribenzoate (1 μmole)	0·18

The enzyme activity was measured spectrophotometrically by following the reduction of ferricyanide at 420 nm. Blank and experimental cuvettes of 1 cm light path contained 675 μmoles sucrose, 30 μmoles potassium phosphate buffer, pH 7·4, 60 μmoles potassium chloride, 60 μmoles triethanolamine buffer, pH 7·4, 2 μmoles potassium ferricyanide, 10 μg antimycin A. 500 μg sodium deoxycholate and rabbit brain mitochondria (2 mg protein). The reaction was initiated by the addition of 0·5 μmoles NADH to the experimental cuvette. The temperature was 30° and the final volume was 3 ml. Inhibitors were added at the concentrations shown at the start of the reaction.

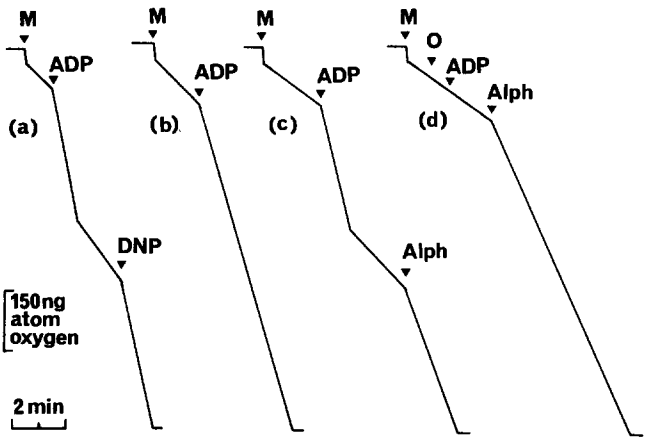

FIG. 2. The effect of alphaxalone on succinate oxidation in rabbit brain mitochondria. The experimental conditions were the same as described in the legend to Table 1, expect that sodium succinate (10 μmoles) was the substrate in all cases. Additions were made as follows; M, rabbit brain mitochondria (5 mg protein); ADP, 0·5 μmoles ADP; DNP, 30 nmoles 2,4-dinitrophenol; Alph, 1 μmoles alphaxalone; 0, 3 μg oligomycin. (a) Control showing effect of 2,4-dinitrophenol; (b) loss of respiratory control in the presence of 330 μM alphaxalone; (c) stimulation of state 4 respiration by alphaxalone; (d) release of oligomycin-inhibited state 3 respiration by alphaxalone.

Succinate oxidation. A different pattern emerged when the NAD^+-linked substrates were replaced with succinate. State 3 respiration was inhibited only after treatment with high concentrations of alphaxalone (maximum inhibition 20 per cent at 264 μM alphaxalone). This slight inhibition was accompanied by a progressive increase in state 4 respiration, until eventually the rate of state 3 respiration was equal to that of state 4 (Fig. 2b). In other words, when succinate was substrate alphaxalone was found to induce a loss of respiratory control. Addition of alphaxalone to mitochondria in state 4 produced a stimulation of respiration similar to that induced by the uncoupling agent 2,4-dinitrophenol (Fig. 2a and c). Figure 2d shows that alphaxalone released oligomycin-inhibited state 3 respiration: this effect has been shown with other uncoupling agents.[10]

TABLE 3. THE EFFECT OF ALPHAXALONE ON RABBIT BRAIN MITOCHONDRIAL ATPASE

Additions	ATPase (μmoles inorganic phosphate released 10 min^{-1} mg^{-1} mitochondrial protein)
Control	0·3
Alphaxalone (84 μM)	0·43
Alphaxalone (166 μM)	0·5
Alphaxalone (330 μM)	0·95
Alphaxalone (500 μM)	0·53
Alphaxalone (660 μM)	0·23
Alphaxalone (330 μM) plus oligomycin (1 μg)	0·17

At zero time 0·1 ml mitochondrial suspension (1 mg protein) was added to 0·9 ml reaction medium, which contained 0·125 M sucrose, 63 mM Tris–HCl buffer, pH 7·4, and 2·5 mM ATP. The reaction was maintained at 30° for 10 min, when it was terminated by the addition of 0·1 ml 40% (w/v) trichloroacetic acid. The inorganic phosphate liberated was estimated by the method of Fiske and Subbarow.[7]

ATPase. The results described above suggested that, at high concentrations, alphaxalone was acting as an uncoupling agent. To test this hypothesis the effect of alphaxalone on the mitochondrial ATPase was examined. Table 3 shows that the steroid elicited a 3-to 4-fold stimulation of the ATPase reaction. Maximum stimulation occurred at an alphaxalone concentration of 330 μM; further increases in concentration resulted in a concentration-dependent inhibition of the stimulated reaction. Similar results were obtained with 2,4-dinitrophenol. Table 3 also shows that the energy-transfer inhibitor oligomycin prevented the stimulation of the ATPase produced in control experiments by 330 μM alphaxalone.

DISCUSSION

Many anaesthetics have been shown to interfere with mitochondrial function, and the present studies show that alphaxalone can be included in the same category. At low concentrations alphaxalone is an inhibitor of NAD^+-linked oxidations. We have concluded that the site of action of alphaxalone is between the NADH dehydrogenase flavoprotein and cytochrome *b*, at a locus that may be either identical to, or close to the rotenone-sensitive site. This conclusion is based on the finding that both alphaxalone and rotenone inhibited cytochrome reduction and failed to prevent ferricyanide reduction. The ability of alphaxalone to inhibit NAD^+-linked oxidations is a property shared with other general anaesthetics such as barbiturates,[11] halothane[12] and forane,[13] and may suggest a common mechanism of action for these compounds.

When succinate was used as substrate alphaxalone did not inhibit respiration. Even when high concentrations of the anaesthetic were employed the maximum inhibition obtained was only 20 per cent. These high concentrations of alphaxalone did, however, result in a loss of respiratory control. This indicated to us that the compound possessed the ability to uncouple oxidative phosphorylation. The following observations confirmed the suggestion. (1) State 4 succinate oxidation was stimulated by alphaxalone; (2) oligomycin inhibition of state 3 respiration was relieved by alphaxalone; (3) alphaxalone stimulated the mitochondrial ATPase reaction in a concentration-dependent manner; (4) the alphaxalone-stimulated ATPase was sensitive to low concentrations of oligomycin. Uncoupling of oxidative phosphorylation has also been shown to occur with other steroids, e.g. progesterone.[14]

Thus there are two sites of action of alphaxalone on mitochondrial reactions. First, electron transport is blocked, at low concentrations, by an interaction with the NADH dehydrogenase, and second, at high concentrations the compound uncouples oxidative phosphorylation. Both these actions result in impaired ATP synthesis *in vitro*. It is not possible at present to relate these findings to intact organisms undergoing anaesthesia. However, experiments are in progress to determine whether there is a correlation between our *in vitro* work on brain mitochondria and the *in vivo* situation. Supression of brain mitochondrial ATP synthesis could, for example, lead to an impairment of some of the energy-linked functions of the mitochondrion such as ion transport reactions. This could, in turn, lead to a modification of impulse conduction in brain neurones.

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