RESPIRATION AND PREGNENOLONE SYNTHESIS IN BOVINE ADRENAL CORTEX MITOCHONDRIA

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1. Introduction

Mitochondria from the adrenal cortex contain enzymes which transfer electrons to cytochrome P450, one species of which converts deoxycorticosterone to corticosterone, and another which cleaves the side chain of cholesterol to produce pregnenolone [1–3]. Cytochrome P450 competes for reducing equivalents with the mitochondrial electron transport chain which acts to phosphorylate ADP [4,5].

Whilst respiratory control ratios [6] of 6–10 are routine for liver mitochondria, several workers [4,7–10] have not obtained ratios >4 in adrenal cortex mitochondria. In a report on corticosterone synthesis, it was proposed [4] that the low respiratory controls reflected a somewhat inhibited state 3 [11] respiratory rate, due to a restriction upon ATPase activity. The supply of NADPH for cytochrome P450 was considered an energy-dependent process proceeding at the expense of ATP synthesis. Pregnenolone production has also been suggested [12–14] to compete with ATP synthesis for the energy derived from electron transport; we have now investigated the relationship between respiratory rate and pregnenolone production in adrenal cortex mitochondria.

Abbreviations: CCFP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; corticosterone, 11β,21-dihydroxy-4-pregnene-3-20-dione; deoxycorticosterone, 21-hydroxy-4-pregnene-3-20-dione; DNP, 2,4-dinitrophenol; pregnenolone, 3β-hydroxy-5-pregnene-20-one; trilostane, 2α-cyano-4α,5-epoxy-17β-hydroxy-androstan-3-one; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine

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2. Methods

Bovine adrenal cortex mitochondria were prepared as in [10] and the standard incubation medium was 100 mM sucrose, 10 mM PO_4 (K^+-salt, pH 7.0), 5 mM $MgCl_2$ and 10 μM trilostane (in acetone to 0.3% final conc. (v/v)); the latter inhibits pregnenolone metabolism (see [10]). Respiratory rates, ADP:O ratios, protein concentration and mitochondrial pregnenolone synthesis were determined as in [10]. Mitochondrial ATPase was quantified by a method [15] that assumes 1 mol enzyme binds 1 mol oligomycin. Cytochrome *a* was determined by difference spectrophotometry [16] and no significant effect was observed during these determinations if ADP was substituted by CCFP. All incubations were stirred and maintained at 30°C. Student's paired *t*-test was employed for statistical analyses.

CCFP, DNP and oligomycin (Sigma, Kingston-Upon-Thames) were added in ethanol (final conc. <0.4% (v/v)). ADP and TMPD were also supplied by Sigma. Trilostane was a generous gift from Sterling-Winthrop (Surrey); stock solutions of this inhibitor were maintained at −20°C and were used within 2 h of their preparation (see [10]).

3. Results and discussion

As increasing concentrations of uncouplers are added to mitochondria, the respiratory rate is at first stimulated, then reaches a maximum, before exhibiting inhibition at the higher uncoupler concentrations [17,18]. Bovine adrenal cortex mitochondria (0.6–0.7 mg protein/ml) were incubated in the standard medium plus either 5 mM K^+-succinate or 10 mM K^+-malate. CCFP maximally enhanced respiratory

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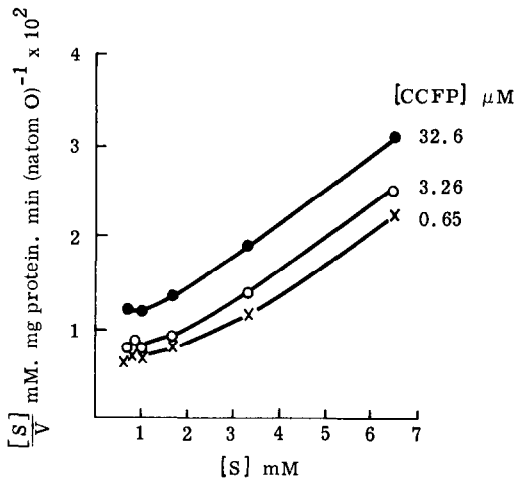

Fig.1. The relationship between concentration of respiratory substrate *(S)* and rate of oxygen consumption *(V)* by bovine adrenal cortex mitochondria incubated in the presence of CCFP. Incubation conditions: Standard medium plus K^+ succinate and CCFP as indicated; 0.5–0.8 mg mitochondrial protein/ml, final volume 3.25 ml. Points represent means of 2 preparations of mitochondria.

rate over 0.4–3.3 μM. The optimum DNP was 80–90 μM, but the maximum respiratory rate was 18–23% ($n = 3$) less than that induced by CCFP, which was therefore preferred in experiments described below.

The rate of CCFP-enhanced respiration *(V)* was dependent upon the concentration of oxidisable substrate *(S)*. The non-linearity of a plot of S/V against S verifies the complexity of the respiratory inhibition by CCFP (fig.1); in contrast, simple competitive inhibition by CCFP was deduced from linear plots of the kinetics of respiration of liver mitochondria [18]. Moreover, liver mitochondrial CCFP-dependent oxidation of the lowest substrate concentrations gradually decreased in rate as incubation time increased ([18]; our unpublished data), whilst our CCFP-treated adrenal mitochondria respired at a constant rate throughout the incubation. It appears that the interaction of CCFP with adrenal cortex mitochondria may be different from the effect of this compound upon liver mitochondria. Note that the above results were unaffected if trilostane was omitted from the incubations.

The maximum uncoupled respiratory rates significantly exceeded respiratory rates in state 3, irrespective of the source of reducing equivalents (table 1). A similar phenomenon was observed in brown adipose tissue mitochondria, and was attributed to a reduced state 3 respiratory rate due to a relatively low ratio of ATPase to respiratory enzymes [19]. Such an explanation does not apparently apply to our mitochondria since we measured 0.94 μmol ATPase/nmol cytochrome *a* (0.21 nmol ATPase/mg protein) which is approximately the proportion of ATPase in rat liver and heart mitochondria in which state 3 and uncoupled respiratory rates are similar [15]. However, ATPase activity in adrenal cortex mitochondria has been suggested to be relatively inhibited in comparison with liver mitochondria, which could limit state 3 respiratory rate [4]. Alternatively, we suggest that in view of the unusual interaction of CCFP with adrenal cortex mitochondria (see above), uncoupled respiration may be relatively enhanced in these organelles.

NADPH, when used for pregnenolone synthesis, may be produced by energy-dependent transhydrogenation between NADH and NADP [20]. Such a supply

Table 1
Respiratory parameters of bovine adrenal cortex mitochondria

Substrate	Respiratory rate (natoms O . mg protein^{-1} . min^{-1})				
	State 4 (before ADP)	State 3	State 4 (after ADP)	Uncoupled	ADP:O
(a) Malate	30.4 ± 3.7	54.6 ± 5.6	27.7 ± 4.2	89.4 ± 11.4[a]	2.55 ± 0.16
(b) Succinate	46.5 ± 1.7	102.1 ± 6.5	41.4 ± 3.5	225.8 ± 17.8[b]	1.75 ± 0.18
(c) TMPD	226 ± 14.6	299.5 ± 30	219.8 ± 15.7	431.8 ± 28[c]	0.69 ± 0.07

Incubation conditions: standard medium (see section 2) containing either (a) 10 mM K^+-malate, (b) 5 mM K^+ succinate or (c) 1 mM TMPD plus 10 mM ascorbic acid. 250 μM ADP was added at 2 min and uncoupled respiration was that stimulated by 0.6 μM CCFP. Mitochondria were present at 0.5–0.8 mg protein/ml. Final volume 3.25 ml. Means ± standard errors are from 4 or 5 preparations of mitochondria. [a] $p < 0.05$, [b] $p < 0.001$, [c] $p < 0.02$ for a comparison of uncoupled and corresponding state 3 respiratory rates

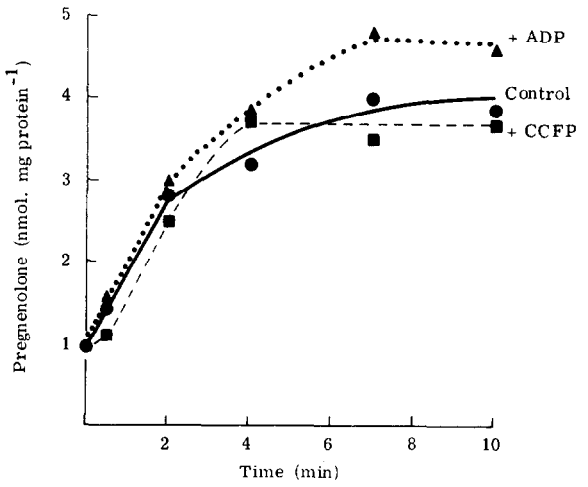

Fig.2. The effect of ADP and CCFP upon pregnenolone synthesis by bovine adrenal cortex mitochondria. Incubation conditions: Standard medium plus 10 mM K^+-malate (●——●), plus 2 mM ADP (▲. . .▲) or 0.6 μM CCFP (■– – –■); 0.4–0.8 mg mitochondrial protein/ml, final volume 5.3 ml. Points represent means of 3 preparations of mitochondria.

of NADPH may not be affected by ADP, if there is relatively little dissipation of the mitochondrial transmembrane electrochemical gradients by the ATPase, which might be expected if ATPase activity were somewhat inhibited. On the other hand CCFP, by minimising the size of the electrochemical gradients, should uncover any energy-dependence of pregnenolone synthesis. However, neither ADP or CCFP significantly affected the cholesterol side chain cleavage reaction (fig.2), so presumably sufficient NADPH for cytochrome P450 was generated by energy-independent sources such as malic enzyme (EC 1.1.1.40). Note that some earlier studies [21,22] have indicated that uncouplers inhibit pregnenolone synthesis by adrenal cortex mitochondria. Comparisons of the latter studies with our own are complicated by a number of differences in incubation conditions. For example, in [21] pregnenolone metabolism was not prevented; in [22] cyanide was also present, and this inhibits electron transport. Moreover, in our study, care was taken to employ fresh solutions of trilostane. Older preparations of this and other inhibitors of pregnenolone metabolism may inhibit electron transport [10] which may in turn itself affect pregnenolone synthesis [21,23].

In conclusion, our data are not consistent with proposals [12,13] that ATP production need compete with pregnenolone synthesis for the free energy change from electron transport in adrenal cortex mitochondria. In addition, under conditions where uncoupled respiratory rates significantly exceeded those of state 3, the energy-independence of pregnenolone synthesis indicates that even if state 3 respiration was somewhat inhibited [4], this was not essential for the maximisation of pregnenolone production.

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