Inhibition by dicyclohexylcarbodiimide of ATP synthesis in isolated rat hepatocytes

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Dicyclohexylcarbodiimide (DCCD) inhibits, by 50%, ATP synthesis in isolated hepatocytes. This inhibition is associated with DCCD-binding to a proteolipid fraction present in submitochondrial particles.

The oligomycin- and DCCD-sensitive ATPase complex catalyses the synthesis of ATP during mitochondrial respiration (1). At low concentrations, DCCD inhibits both the hydrolytic and synthetic activities of the ATPase with associated covalent modification of a low-molecular-weight subunit of the ATPase complex (2-4). DCCD-binding proteolipid has been isolated from both mitochondria (5), and the purified ATPase complex (3,6). Although the exact function of this protein in ATP synthesis is unknown it has been implicated in proton translocation in mitochondria. Support for this is provided by the finding that it is capable of proton transfer across lipid bilayers (7).

Modification of the β-subunit of the soluble F1-ATPase has also been reported (8). Under slightly acidic conditions, and using higher levels of DCCD, both the DCCD-binding proteolipid and the β-subunit of F1 are labelled; at alkaline pH only the proteolipid is modified.

In this paper we report the use of DCCD to inhibit ATP synthesis in isolated hepatocytes. At low concentrations of DCCD, ATP levels in rat hepatocytes fall by approximately 50% without apparent loss of cell viability; at higher concentrations ATP synthesis can be inhibited completely with concurrent increase in the rate of lactate dehydrogenase release in the cells. This inhibitory effect has been shown to be associated with DCCD binding to a proteolipid whose elution characteristics on thin-layer chromatography are comparable to the DCCD-binding protein present in submitochondrial particles.

Materials and Methods

Dicyclohexyl [14C]carbodiimide (14C-DCCD) was obtained from CEA (France). Collagenase was purchased from the Boehringer Corporation (London) Ltd. All other chemicals were obtained from Sigma Chemical Co. and were of the purest grade available.

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**Hepatocyte preparation**

Hepatocytes were prepared as described in (9). After isolation the cells were washed (x2) and resuspended in Krebs-Henseleit buffer (10) supplemented with 2% (w/v) bovine albumin, 0.1% glucose, and 25 mM Hepes buffer, pH 7.3. Cells were diluted to a density of 6 x 10^6 cells/ml with this buffer just prior to use.

**Cell incubations**

Incubations were in paired 250-ml (siliconized) Erlenmeyer flasks at 37°C, in a shaking water bath at a frequency of 140 strokes/min, using either 50 or 20 ml of cell suspension.

Reaction was initiated by addition of DCCD, dissolved in methanol, to the reaction flask. The final concentration of methanol in the reaction flask did not exceed 0.001% (v/v) in any experiment.

**Sampling**

At appropriate time intervals cells were recovered by centrifugation (50 g, 3 min) from aliquots (2 ml) of the incubating suspension. The supernatant was carefully removed and stored at -20°C, the cell pellet was quenched by addition of ice-cold 6% (w/v) perchloric acid (0.2 ml) or directly frozen unquenched, prior to storage at -20°C.

**Assays**

The activity of lactate dehydrogenase (EC 1.1.1.27; LDH) in the cell supernatant was determined as described in reference 11. ATP concentrations in the cell pellets were determined using the hexokinase (EC 2.7.1.1) method described in reference 12.

**Proteolipid isolation and analysis**

After incubation with 14C-DCCD (100 μM; 10 μmol/μCi; 60 min), cells from 50 ml of incubating suspension were recovered by centrifugation (50 g, 3 min) and the proteolipid extracted as described in reference 5. Analysis of the proteolipid mixture was by thin-layer chromatography, as described in reference 13.

**Scintillation counting**

Cell samples for scintillation counting were dissolved directly in a phase combining scintillation fluid (1 ml; PCS; Radiochemical Center, Amersham), prior to counting.

Bands from the thin-layer-chromatography plates were scraped off into a scintillation vial prior to addition of PCS and counting.

Counting was performed using a Packard Tri-Carb liquid-scintillation spectrometer, model 3375.

**DCCD labelling of submitochondrial particles**

These were prepared as described in reference 14.

**Results and Discussion**

Data for the uptake and DCCD-binding to a suspension of isolated hepatocytes are shown in Fig. 1A. There was an immediate association of DCCD with the hepatocytes, followed by a slow (transient)
Fig. 1. Effects of dicyclohexylcarbodiimide on isolated rat hepatocyte suspensions. (A) Uptake of $^{14}$C-DCCD by hepatocyte suspensions. Methods are described in the text. (B) Effect of various concentrations of DCCD on hepatocyte ATP concentration. ATP measurements are described in ref. 12. (C) Rate of lactate dehydrogenase release in the presence and absence of DCCD. Lactate dehydrogenase activity was determined as in reference 11.

increase and then a rapid loss of labelled DCCD from the cell fraction after 20 min. Over the same time period, measurements of cell ATP concentrations were made (Fig. 1B). Starting ATP (4.2 nmol/mg) concentrations were consistent with published values (15). In control incubations, after an initial fall in cell ATP levels, there was a rapid recovery to starting values. In the presence of DCCD there was a rapid fall in ATP levels. At the two lowest DCCD concentrations tested (30 and 100 μM) there was no difference between experimental and control ATP values for the first 20 min of incubation. After this time there was a rapid fall in ATP concentration in the cells incubated in the presence of DCCD. At 50 μM DCCD the ATP concentration fell to approx. 40% of control values while with 100 μM DCCD there was a complete loss of ATP. At the highest DCCD concentration (250 μM) there was complete loss of ATP within 40 min of incubation.

Since hepatic ATP concentrations are a sensitive index of cell viability (15) the ATP losses observed could have been the result of DCCD-induced viability loss. As an independent assessment of cell viability, measurement of LDH was made coincident with DCCD treatment (Fig. 1C). At 50 M DCCD, inhibition of ATP synthesis was not accompanied by an increase in the rate of LDH release, as compared to the controls. No significant departure of LDH values from the control was observed at the higher DCCD concentrations until complete loss of ATP had occurred. This would be expected on the grounds that cells devoid of ATP are by definition nonviable.

In order to determine the nature of the DCCD inhibition effect, and more specifically to ascertain whether the DCCD-sensitive protein
of the ATPase complex was involved, the hepatocyte proteolipid protein was extracted and analysed by thin-layer chromatography (Fig. 2 A-C). There was a rapid labelling of a fast-moving component (Rf = 0.86) which showed a loss of label as the incubation proceeded. This fraction quantitatively contained the largest fraction of total hepatocyte-associated label. There was also a time-dependent labelling of a slower-moving component (Rf = 0.4) which appears to correspond to the DCCD-binding protein of the ATPase associated with submitochondrial particles (Fig. 2D). The slight differences in mobility can be accounted for by the viability inherent in this system. No fast-moving component was found in the submitochondrial particles to correspond with that in the hepatocytes.

![Graphs showing labelling of hepatocyte proteolipid protein by DCCD](image)

Fig. 2. Labelling of the hepatocyte proteolipid protein by DCCD. Hepatocyte suspensions were incubated with $^{14}$C-DCCD for 60 min prior to proteolipid extraction and thin-layer-chromatography analysis as described in references 5 and 13. Bands from the thin-layer plate were recovered and counted, as described in the text, the $^{14}$C-distribution data over the first 40 min of incubation are shown in A to C. Also shown in D is the result of proteolipid analysis of DCCD-labelled submitochondrial particles. A major peak is found in fraction 6 (Rf = 0.57), associated with the DCCD-binding protein.
These findings, taken in conjunction with the DCCD uptake and ATP measurements, suggest a possible scheme for inhibition of hepatocyte ATP production. On addition of DCCD to the hepatocyte suspension there is a rapid and presumably nonspecific association of DCCD with the hepatocyte. This may account for the rapidly labelled fast-moving component. This would be followed by the slow uptake of DCCD and its conveyance to the mitochondria, an event consistent with the delay prior to the fall in the cell ATP concentrations after DCCD addition. At the mitochondrion the DCCD forms a complex with a specific protein of the ATPase complex (the DCCD-binding protein), resulting in a rapid loss of ATP, since the hepatocyte ATP pool (and nucleotide pool in general) is in a constant and rapid state of flux. At the same time there is presumably a rapid degradation or dissociation of DCCD (perhaps by equilibration of DCCD with the bovine albumin in the incubation buffer).

Although inhibitory effects of DCCD on glycolytically mediated membrane transport have been noted in bacteria (16) and yeast (17), no attempts have been made previously to determine whether the inhibition in intact cells resulted from nonspecific interaction of the highly reactive DCCD with several essential cell constituents or specifically with the mitochondrial proteolipid. The temporal correlation of decline of ATP levels with labelling of proteolipid, but not of other bands, favors the latter interpretation, and also shows that at low concentrations of DCCD, the cellular ATP levels can be reduced effectively without loss of cell viability. Such controlled perturbation is useful for simulating the ATP changes in aging (18), hypoxia, and ischemic cell death. In myocardial ischemia cell death, for example, whether irreversible cell injury results from ATP loss, lactic acid accumulation (19), or palmityl-carnitine accumulation (20) is an unsettled issue.

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