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Effects of dibucaine on pyruvate and ketone-body transport in isolated rat heart mitochondria

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The local anaesthetics dibucaine (nupercaine), butacaine and tetracaine have been shown to inhibit substrate-dependent oxygen utilization in isolated rat heart mitochondria [1]. Pyruvate-dependent oxygen utilization, measured in the presence of low concentrations of α-cyano-3-hydroxycinnamate, an inhibitor of pyruvate transport [2], was most susceptible to inhibition. It was proposed that under the conditions employed, pyruvate transport across the inner mitochondrial membrane is the rate-limiting step in the sequence of reactions leading from pyruvate entry to oxygen utilization, and is sensitive to inhibition by low concentrations of dibucaine [1].

Recently, Pande and Parvin [3] have provided evidence which supports the proposal that pyruvate can enter mitochondria by two separate processes. These are proposed to be an α-cyano-3-hydroxycinnamate-sensitive, carrier-mediated transporter, which has a high affinity for the pyruvate anion, and simple diffusion which is insensitive to inhibition by α-cyano-3-hydroxycinnamate, does not transport the pyruvate anion but has a low affinity for the undissociated acid (reviewed in Refs. 3 and 4). The aim of the present work was to determine whether dibucaine has similar effects on these two proposed transport systems. As it has been proposed that 3-hydroxybutyrate, acetoacetate and pyruvate enter mitochondria by similar mechanisms [3–6], the effects of dibucaine on the oxidation of ketone bodies were also studied.

The isolation of mitochondria from rat hearts and measurement of the respiratory control ratio (the ratio of ADP-stimulated : ADP-depleted rates of oxygen utilization) and protein content of mitochondrial preparations were performed as described previously [1, 7]. Rates of oxygen utilization were measured at 30° as described previously [1]. Acetoacetate and 3-hydroxybutyrate were purchased from the Sigma Chemical Co, St. Louis, MO, U.S.A. Other chemicals were purchased from the sources described previously [1].

Pyruvate entry into mitochondria by the proposed α-cyano-3-hydroxycinnamate-insensitive process of simple diffusion was measured by employing 50 mM pyruvate, and 50 or 100 μM α-cyano-3-hydroxycinnamate to inhibit pyruvate entry catalysed by the pyruvate carrier [3]. Under these conditions, low concentrations of dibucaine were found to stimulate pyruvate-dependent oxygen utilization about 2-fold, with 50 per cent stimulation observed at 0.06 ± 0.01 (mean ± S.E.M., N = 5) mM dibucaine (Fig. 1a). Concentrations of dibucaine greater than 0.1 mM inhibited oxygen utilization. Although some variation in the rates of pyruvate-dependent oxygen utilization in the absence of dibucaine was observed, the degree of stimulation by dibucaine and the shape of the curves (Fig. 1a) were similar. In the absence of exogenous pyruvate, dibucaine caused a small inhibition of oxygen utilization (Fig. 1a).

No stimulation by dibucaine of pyruvate-dependent oxygen utilization was observed (Fig. 1b) at 2 mM pyruvate in the presence of 1.5 μM α-cyano-3-hydroxycinnamate (added to inhibit the rate of oxygen utilization by about 50 per cent and make the entry of pyruvate by the pyruvate carrier the rate-limiting step in the oxidation of pyruvate [1]). As reported previously [1], this reaction was markedly inhibited by low concentrations of dibucaine (Fig. 1b).

In the presence of pyruvate and malate, concentrations of dibucaine between 40 and 120 μM did not alter the respiratory control ratio of the mitochondria (results not shown). When measured at different concentrations of α-cyano-3-hydroxycinnamate, the degree of stimulation by dibucaine of pyruvate-dependent oxygen utilization (measured at 50 mM pyruvate) was found to be slightly greater at 200 and 100 μM α-cyano-3-hydroxycinnamate than that observed at 10 μM inhibitor (results not shown). These

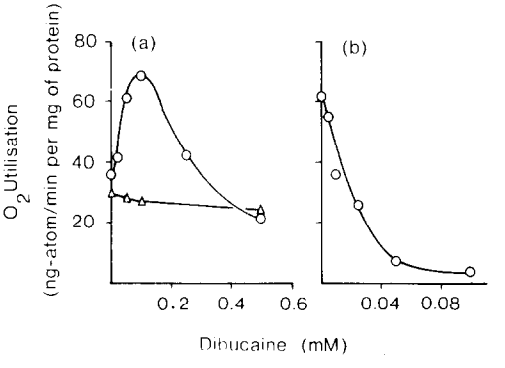

Fig. 1. Effects of increasing concentrations of dibucaine on the rate of pyruvate-dependent oxygen utilization measured in the presence of 50 mM pyruvate and 50 μM α-cyano-3-hydroxycinnamate (a) and 2 mM pyruvate and 1.5 μM α-cyano-3-hydroxycinnamate (b). Rates of pyruvate-dependent oxygen utilization (○) and oxygen utilization in the absence of added pyruvate (△) were measured at 30° as described previously [1]. The reaction media (pH 7.4, 2.0 ml total vol.) contained (a) 83 mM KCl, 13 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes)–KOH, 2 mM potassium phosphate, 1 mM potassium malate, 50 mM sodium pyruvate, 0.75 mM ADP, 50 μM or 100 μM α-cyano-3-hydroxycinnamate and 1 mg of mitochondrial protein per ml, for the measurement of pyruvate-dependent oxygen utilization at 50 mM pyruvate; or (b) 125 mM KCl, 20 mM Hepes–KOH, 2 mM potassium phosphate, 1 mM potassium malate, 2 mM sodium pyruvate, 0.75 mM ADP, 1.5 μM α-cyano-3-hydroxycinnamate and 1 mg of mitochondrial protein per ml for the measurement of pyruvate-dependent oxygen utilization at 2 mM pyruvate. For measurements at 50 mM pyruvate, the mitochondria were equilibrated with reaction medium for 2 min (in the presence of pyruvate) before the addition of ADP, and the rate of oxygen utilization in the presence of ADP measured. In experiments conducted at 2 mM pyruvate, mitochondria were equilibrated with reaction medium for 2 min (in the presence of ADP) before the addition of pyruvate, and the rate of oxygen utilization in the presence of pyruvate measured. In each case, rates of oxygen utilization in the absence of pyruvate (control) were measured, and the rate of oxygen utilization which was dependent on the presence of pyruvate (pyruvate-dependent oxygen utilization) calculated. The data shown are those for one out of five experiments which gave similar results.

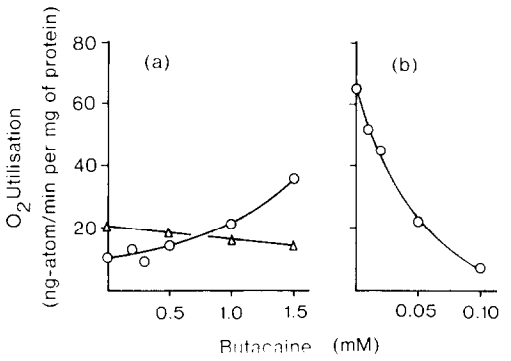

Fig. 2. Effect of increasing concentrations of butacaine on the rate of pyruvate-dependent oxygen utilization measured in the presence of 50 mM pyruvate and 100 μM α-cyano-3-hydroxycinnamate (a) and 2 mM pyruvate and 1.5 μM α-cyano-3-hydroxycinnamate (b). Rates of pyruvate-dependent oxygen utilization (○) and oxygen utilization in the absence of added pyruvate (△), were measured as described in the legend of Fig. 1. For the measurements conducted at 2 mM pyruvate, the medium contained 2.5 μM carbonyl cyanide *m*-chlorophenyl hydrazone in place of ADP, and the concentration of malate was 0.5 mM. The data shown are those for one out of three experiments which gave similar results.

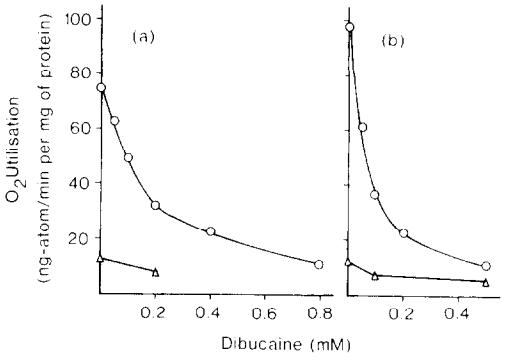

Fig. 3. Effects of increasing concentrations of dibucaine on the rate of 3-hydroxybutyrate-(a) and acetoacetate-(b)-dependent oxygen utilization measured in the presence of a high concentration of α-cyano-3-hydroxycinnamate. Rates of ketone-body-dependent oxygen utilization (○) and oxygen utilization in the absence of exogenous substrate (△), were measured as described in the text. The composition of the reaction media (pH 7.4) was 125 mM KCl, 20 mM Hepes–KOH, 2 mM potassium phosphate, 10 mM potassium 3-hydroxybutyrate, 0.75 mM ADP, 100 μM α-cyano-3-hydroxycinnamate and 1 mg of mitochondrial protein per ml (a); and 83 mM KCl, 13 mM Hepes–KOH, 2 mM potassium phosphate, 2 mM potassium acetoacetate, 0.75 mM ADP, 500 μM α-cyano-3-hydroxycinnamate and 1 mg of mitochondrial protein per ml (b). In each case, the reaction was initiated by addition of mitochondria. Rates of oxygen utilization in the absence of ketone bodies (control) were measured and used to calculate the rate of ketone-body-dependent oxygen utilization. The data shown are those for one out of two experiments which gave similar results.

observations indicate that the stimulation of pyruvate-dependent oxygen utilization by dibucaine (Fig. 1a) is not due to an uncoupling of oxidative phosphorylation or to the displacement of α-cyano-3-hydroxycinnamate from the pyruvate carrier. Further support for this argument comes from the data of Fig. 1b. If low concentrations of dibucaine displaced α-cyano-3-hydroxycinnamate, an increase in pyruvate-dependent oxygen utilization should also have been observed at the much lower concentration of α-cyano-3-hydroxycinnamate (1.5 μM) used in this experiment.

Butacaine (0.2–1.5 mM) was also found to stimulate pyruvate-dependent oxygen utilization at 50 mM pyruvate and 100 μM α-cyano-3-hydroxycinnamate (Fig. 2a), while at low concentrations of pyruvate and α-cyano-3-hydroxycinnamate this parameter was inhibited (Fig. 2b). A small stimulation of pyruvate-dependent oxygen utilization, measured under conditions similar to those described in Fig. 1a, was observed in the presence of 1–2 mM tetracaine (data not shown).

The rate of pyruvate-dependent oxygen utilization measured at 50 mM pyruvate and 50 μM α-cyano-3-hydroxycinnamate in the absence of a local anaesthetic is much lower than both the maximum capacity of the mitochondria to oxidize pyruvate (about 200 ng atom O_2/min per mg of protein) and the rate of pyruvate-dependent oxygen utilization observed at 2 mM pyruvate. Therefore, pyruvate entry to the mitochondria is likely to be the rate-limiting step in the metabolism of pyruvate at high pyruvate and α-cyano-3-hydroxycinnamate concentrations. It is concluded that the stimulation of pyruvate-dependent oxygen utilization by dibucaine observed at 50 mM pyruvate represents a stimulation of the diffusion of pyruvate across the mitochondrial inner membrane via an α-cyano-3-hydroxycinnamate-insensitive pathway. The range of dibucaine concentrations which stimulate pyruvate-dependent oxygen utilization is similar to that which inhibits oxygen utilization under conditions designed to make pyruvate entry by the α-cyano-3-hydroxycinnamate-sensitive pathway the rate-limiting step in the metabolism of pyruvate. These observations suggest that the two different effects of dibucaine on pyruvate-dependent oxygen utilization may arise from a common perturbation by dibucaine of the inner mitochondrial membrane, and are consistent with the presence of two transport systems for pyruvate [3, 4].

The observation that dibucaine influences the diffusion of pyruvate across the mitochondrial membrane is consistent with the proposal that the perturbation induced by the drug results from an interaction with the phospholipid component of the inner membrane, as proposed for the action of local anaesthetics on other mitochondrial anion transport systems [1, 8, 9]. The present results can be compared with those of Feinstein *et al.* [10], who showed that dibucaine increases the permeability of erythrocyte plasma membranes to lipophilic compounds which may cross the membrane without the aid of a carrier protein, whereas the transport of urea, which may be catalysed by a pore or channel, is slightly inhibited by dibucaine.

As reported by others [3, 4, 11], high concentrations (100–500 μM) of α-cyano-3-hydroxycinnamate caused only a small inhibition (about 15 per cent) of 3-hydroxybutyrate (10 mM)- and acetoacetate (2 mM)-dependent oxygen utilization (results not shown). No stimulation by dibucaine of 3-hydroxybutyrate- or acetoacetate-dependent oxygen utilization, measured in the presence of high concentrations of α-cyano-3-hydroxycinnamate, was observed (Fig. 3) at concentrations of the local anaesthetic which were found to stimulate the oxidation of 50 mM pyruvate. Instead, an inhibition was observed, with 50 per cent inhibition at 0.14 ± 0.02 and 0.14 ± 0.05 mM dibucaine (mean ± range, N = 2) for 3-hydroxybutyrate- and acetoacetate-dependent oxygen utilization, respectively (Fig. 3). The local anaesthetic had little effect on oxygen utilization measured in the absence of exogenous substrate.

It is concluded that either the movement of 3-hydroxybutyrate or acetoacetate across the mitochondrial mem-

brane, possibly by simple diffusion [3, 4], is not the rate-limiting step in the oxidation of these metabolites under the conditions used, or that the transport of these compounds by simple diffusion is not stimulated by dibucaine. These observations indicate that the properties of the transport systems for 3-hydroxybutyrate and acetoacetate differ in some respects from those of the system which transports pyruvate.

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Triton WR-1339 induced changes in the fatty acid composition of serum lipids in rats

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Hyperlipidemia induced by Triton WR-1339 is a frequently used experimental model when studying hypolipidemic agents. According to Garattini *et al.* [1], hepatic cholesterol biosynthesis may be enhanced by activating hydroxymethylglutaryl-CoA-reductase with intravenously administered triton. Triton treatment results in considerably elevated serum triglyceride concentrations due to the protective coating developed on the surface of the very low density (VLD) lipoproteins. Accordingly, they are shielded from hydrolytic cleavage by lipoprotein lipase [2]. It has also been reported [3] that triton, in addition to increasing cholesterol biosynthesis, augments cell membrane permeability and inhibits the postheparin clearing factor. In Illingworth's [4] studies triton proved to inhibit the LCAT activity in squirrel monkeys. As the triton-induced hyperlipidemic model has been frequently used in our studies [5] we examined Triton WR-1339 for its effect on the esterification of cholesterol as well as on the fatty acid composition of different lipid fractions.

Methods. CFY rats of 180–200 g body wt were treated intravenously with single 200 mg/kg doses of Triton WR-1339. Ten animals per group were killed at 6-, 24-, 48- and 72-hr intervals. Blood samples were centrifuged and serum lipid extracted according to Carlson [6] and total serum cholesterol, free cholesterol [7], triglyceride [8] and phospholipid [9] levels assessed.

In the second stage of the experiment, lipids were separated by thin-layer chromatography in a mixture of *n*-hexane–diethylether–formic acid (80:20:2). Following spraying with Rhodamine 6G, cholesterol ester, triglyceride and phospholipid fractions were identified under a u.v. lamp, and subsequently removed together with the coating. The fatty acid composition of individual lipid fractions was measured after treatment with methanol–hydrochloric acid, with a Hewlett–Packard gas chromatograph, type 5830A, at 195° (glass column 1.6 m × 4 mm, stationary phase Sp 2340 Chromosorb W, AW 80/100). The fatty acids were identified using a set of standard fatty acid methyl esters (KD Applied Science Laboratories, State College, Pennsylvania).

Statistical evaluation was performed by applying Student's *t*-test. In cases of *F*-test positivity, the *d*-test was used.

Results. Maximum concentration of total serum cholesterol was observed in the sixth hour after Triton WR-1339 administration, thereafter it decreased reaching normal level by the 72nd hour. Cholesterol esterification characterized by the cholesterol ester/free cholesterol ratio showed, however, opposite changes (Table 1). This ratio fell to minimum in the first 6 hr after triton injection, thereafter increased progressively and by the 72nd hour became identical with that of the control. Both serum triglyceride and phospholipid levels showed an abrupt rise during the first 6 hr and were found to have returned to the normal level by the 48th hour.

The fatty acid composition of the cholesterol ester fraction is represented in Table 2. The 6th hour and even more the 24th hour after triton administration were characterized by decreased proportions of both linoleic acid (18:2) and arachidonic acid (20:4), in contrast to the rise observed in the proportion of oleic acid (18:1). A moderate rise could also be noted in the percentage of stearic acid (18:0). After 72 hr, however, the above values proved to be identical with those of the controls. In these experiments no change could be detected in the proportions of palmitic (16:0) and palmitoleic (16:1) acids.