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PROTECTION OF OXIDATIVE PHOSPHORYLATION BY BENCYCLANE AGAINST THE DAMAGING EFFECT OF MITOCHONDRIAL SWELLING*

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Abstract—The effect of bencyclane on the relationship between the energy-dependent swelling and oxidative phosphorylation was studied in isolated rabbit heart mitochondria. The oxygen consumption and the osmotic volume changes of mitochondria as well as the extramitochondrial proton concentration were simultaneously measured in the same sample. In sucrose medium the oxidative phosphorylation was uncoupled and the electron transport chain inhibited by bencyclane concentrations higher than 10 μM. In a medium of high potassium concentration the swelling of mitochondria was accompanied both by a decrease rate and efficiency of oxidative phosphorylation. Both swelling and impairment of oxidative phosphorylation were inhibited by 2.5–10 μM bencyclane concentrations. The relation of these effects to the myocardial function was discussed.

INTRODUCTION

The antispasmodic agent bencyclane which was introduced for the treatment of cerebral blood flow disturbances has been shown to possess a pronounced antianginal action [1, 13, 14, 15]. During the past few years considerable efforts were made to explain its therapeutic effect by an influence exerted on the cellular energetic processes. In experiments carried out on isolated mitochondria prepared from beef heart [2], rat liver and rabbit heart [3], uncoupling of oxidative phosphorylation and inhibition of the respiratory chain have been demonstrated. On the basis of these observations it was suggested that the vasodilation caused by bencyclane is the result of the reduction of the intracellular ATP stores and the accompanying production of the free adenosine.

In the present paper the effect of bencyclane on the relationship between the energy-dependent swelling and oxidative phosphorylation was studied in rabbit heart mitochondria.

MATERIALS AND METHODS

Mitochondria were isolated from the rabbit heart according to the method of Sordahl *et al.* [4]. The isolation medium contained 0.18 M KCl, 0.01 M EDTA and 0.5 % bovine serum albumin; its pH was adjusted to 7.40 at 4° by the addition of Tris base. The final mitochondrial pellet obtained was resuspended in the same medium and the protein concentration was determined by the biuret reagent [5].

Oxygen consumption, proton concentration and osmotic volume changes were simultaneously measured in the same mitochondrial suspension. Both Clarke type and glass oxygen electrodes as well as reference electrodes were immersed into a water-jacketed cuvette developed in our laboratory. Through the quartz windows of the same cuvette the swelling–shrinkage cycles of mitochondria were determined photometrically. He Ne laser, λ = 6328 Å (CW Radiation Inc. model LS05) served as a light source. The light intensity was measured by a selenium photoelectric cell. The content of the cuvette was thermostabilized at 37° and magnetically stirred. The electrodes were attached to an oxygraph (Gilson Medical Electronics) and to a Radiometer PHM 64 Research pH meter, respectively. All changes were simultaneously recorded by a four channels recorder (MTA KUTESZ type 175, Hungary).

The 4 ml sample contained about 4–5 mg mitochondrial protein. Other additions are indicated on the figures. The exact concentration of the ADP solution was enzymatically determined using Boehringer ADP/AMP test combinations. The ADP:O ratio was calculated according to Estabrook [6].

The chemicals used were Bencyclane, *N*-[3-(1-benzylcycloheptyl - oxy) - propyl] - *N*,*N* - dimethylammoniumhydrogenfumarate (EGYT Budapest), adenosine-5'-diphosphoric acid cryst. and ADP/AMP test combination (Boehringer), 2-4-dinitrophenol (Reanal, Budapest).

RESULTS

In the first part of the experiments the function of mitochondria was studied in sucrose medium (Fig. 1). After addition of mitochondria to this medium, state 4 respiration, negligible swelling of the mitochondria and nearly unchanged proton concentration in the medium could be recorded (Fig. 1, control experiment).

The addition of external ADP induced state 3 respiration accompanied by a moderate shrinkage of the mitochondria and by a considerable decrease in the proton concentration of the medium. When all the added ADP was phosphorylated to ATP, the mitochondria returned to the state 4 respiration, i.e.

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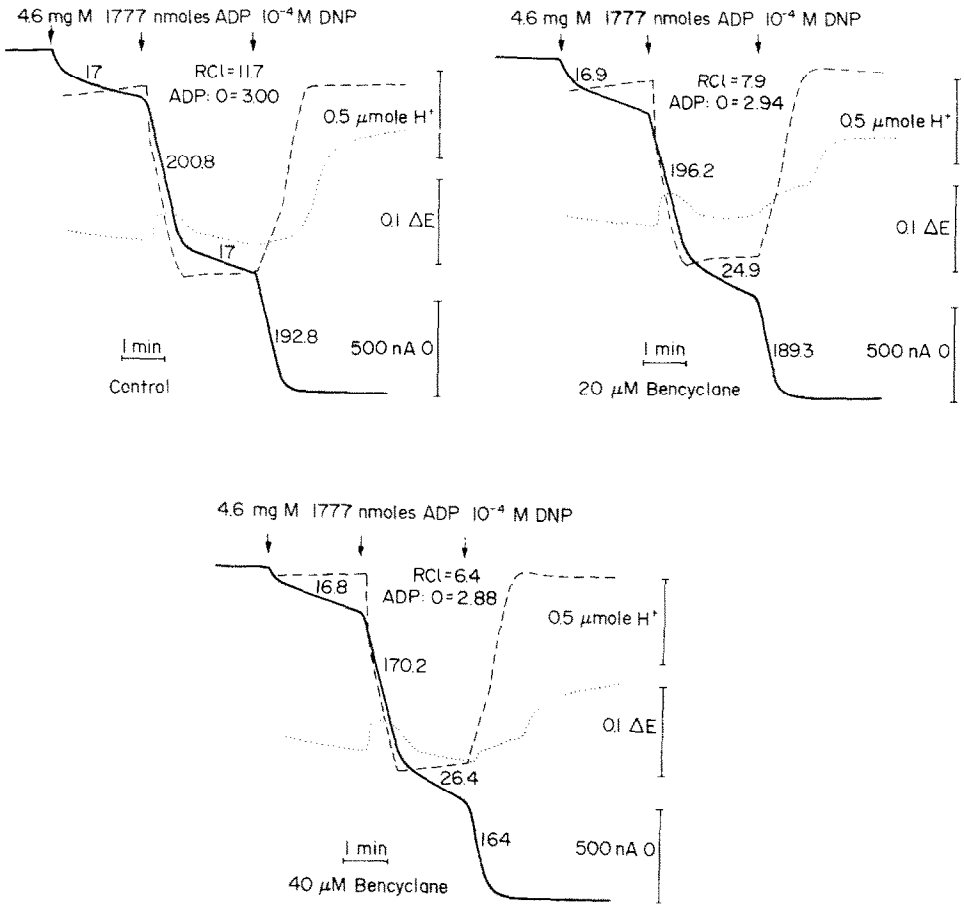

Fig. 1. Effects of bencyclane in sucrose medium. The experimental medium consisted of 250 mM sucrose, 1 mM sodium pyruvate and 5 mM potassium phosphate buffer, pH 7.00, at 37°. In 4 ml medium 4.6 mg mitochondrial protein (M) was used. Additions are indicated by the arrows. Solid lines represent the oxygen consumption of mitochondria, broken lines indicate the proton concentration changes in the medium and the dotted lines represent the osmotic volume changes of mitochondria. The rate of oxygen consumption was expressed in nanoatom oxygen min^{-1} mg mitochondrial protein $^{-1}$.

the oxygen consumption was slowed down, moderate swelling and nearly constant extramitochondrial proton concentration were measured. The subsequent addition of DNP greatly stimulated the respiration, induced a slow but definite shrinkage of the mitochondria and fast increase of proton concentration in the medium. When all the oxygen content of the sample was used, the generation of protons under the influence of DNP became even faster than under aerobic conditions. And finally when the proton concentration in the medium returned to about the level measured before the addition of ADP, shrinkage of the mitochondria was even pronounced. The calculated ADP:O ratio was 3.00 and the respiratory control index (RCI) 11.7, both characteristics of functionally intact mitochondria.

Bencyclane (20 μM) slightly increased the rate of the state 4 respiration measured after ADP induced state 3 respiration and decreased the RCI to 7.9. Furthermore, the passive shrinkage of mitochondria observed under anaerobic conditions was also reduced by bencyclane, but the other parameters were not influenced (Fig. 1). Higher concentrations of bencyclane (40 μM) produced a moderate inhibition of both state 3 and DNP-stimulated respiration and resulted in a further decrease of the RCI (Fig. 1).

In the second part of our experiments the function of mitochondria was studied in an experimental medium containing similarly high concentration of potassium as can be found in the intracellular space. The effects of both inorganic phosphate and bencyclane were studied in this condition (Table 1). The values of state 3 respiration and ADP:O ratio were slightly lower than those measured in the sucrose medium even in the case when only 2 mM inorganic phosphate was present. Furthermore, the elevation of phosphate concentration from 2 to 4 mM induced a significant depression of these parameters. The presence of 10 μM bencyclane was able to prevent the decrease in the state 3 respiration and significantly protected against the decrease in the ADP:O

Table 1. Effects of inorganic phosphate (Pi) and bencyclane on the oxidative phosphorylation of isolated heart mitochondria in an experimental medium containing high K^+ concentration*

	State 3 respiration	ADP:O ratio
2 mM Pi	145 ± 6.3	2.50 ± 0.08
4 mM Pi	124 ± 4.4†	1.83 ± 0.18†
4 mM Pi + 10 μM Bencyclane	164 ± 11.3‡	2.24 ± 0.15‡

* The experimental medium consisted of 150 mM KCl, 1 mM sodium pyruvate and different concentration of potassium phosphate buffer, pH: 7.0 at 37°. Where indicated, 10 μM bencyclane was also present. In 4 ml medium 4–5 mg mitochondrial protein was used. State 3 respiration was initiated by the addition of 1.4 μmoles ADP. The rate of oxygen consumption was expressed in nanoatom oxygen min^{-1} mg mitochondrial $protein^{-1}$. Values are the mean ± S.E. of eight independent experiments. The results were statistically analysed by the Student *t*-test.

† Statistically different from 2 mM Pi ($P < 0.05$).

‡ Statistically different from 4 mM Pi ($P < 0.05$).

ratio induced by the elevation of the extramitochondrial phosphate concentration. Figure 2 demonstrates the effects of bencyclane on the function of mitochondria in high potassium medium containing 4 mM inorganic phosphate. The experimental results displayed by the figure were from the same mitochondrial preparation. The rate of oxygen consumption of mitochondria suspended in this medium (state 4 respiration) was about twice as high as that of mitochondria suspended in sucrose medium. At the same time a considerable swelling of the mitochondria and a moderate increase in the extramitochondrial proton concentration was measured (Fig. 2, control experiment). The addition of ADP stimulated the respiration, stopped the swelling of the mitochondria (causing even a negligible shrinkage) and reduced the proton concentration of the medium. When the oxidative phosphorylation was stopped by the absence of ADP, the rate of oxygen consumption was slowed down. At the same time further swelling of the mitochondria and a small increase in the proton concentration of the medium could be recorded. Under anaerobic conditions swelling of the mitochondria stopped and an increase in the proton concentration of the medium was observed. The ADP:O was 1.25 and RCI 4.3, both characteristics of a greatly impaired function of the mitochondria. Under the influence of 10 μM bencyclane in the same medium the following significant changes were observed: the swelling of mitochondria measured before the addition of ADP was reduced, the rate of state 3 respiration and the accompanying consumption of protons from the extramitochondrial space were stimulated, the ADP:O ratio increased by 40 per cent and the RCI by 23 per cent (Fig. 2).

DISCUSSION

The oxidative phosphorylation of isolated rabbit heart mitochondria was measured both by polarographic determination of the ADP:O ratio and by the direct measurement of the scalar proton consumption coupled to the mitochondrial ATP synthesis. The net ion transport of mitochondria, in our conditions presumably the potassium phosphate transport, was measured in the same sample by the photometric determination of the osmotic volume changes. This type of experimental setting made the study of the relationship between oxidative phosphorylation and the energy-dependent ion transport of mitochondria possible.

The function of mitochondria and the influence of bencyclane were studied in two different experimental conditions. In sucrose medium the function of mitochondria is characterized by a high RCI and ADP:O ratio showing the intact oxidative phosphorylation of mitochondria. The relatively small osmotic volume changes of mitochondria measured during the aerobic phase of these experiments indicate that if the extramitochondrial potassium concentration is low, no significant net ion transport can be observed. Increase of the state 4 respiration and decrease of the RCI in the sucrose medium by 20 μM and higher bencyclane concentrations indicate the uncoupling effect of the drug. Decrease of the state 3 and the DNP stimulated respiration by bencyclane concentrations higher than 20 μM are the result of the inhibition of the mitochondrial electron transport chain [3].

In the medium containing high potassium concentration similar to that of intracellular space, the function of mitochondria is characterized by decreased rate and efficiency of oxidative phosphorylation. The impairment of oxidative phosphorylation largely depends on the extramitochondrial concentration of inorganic phosphate. The increase of the phosphate concentration, which can be found also in the ischemic myocardial tissue, induced osmotic swelling of mitochondria and resulted in the inhibition and uncoupling of oxidative phosphorylation. The osmotic swelling of mitochondria was the result of ion accumulation, in our conditions mainly due to the uptake of potassium phosphate. In the same medium lower bencyclane concentrations (2.5–10 μM) having neither uncoupling nor electron transport inhibitory effects, considerably inhibited the swelling of mitochondria. At the same time state 3 respiration and the ADP:O ratio were increased. The consumption of scalar protons was also stimulated by bencyclane, indicat-

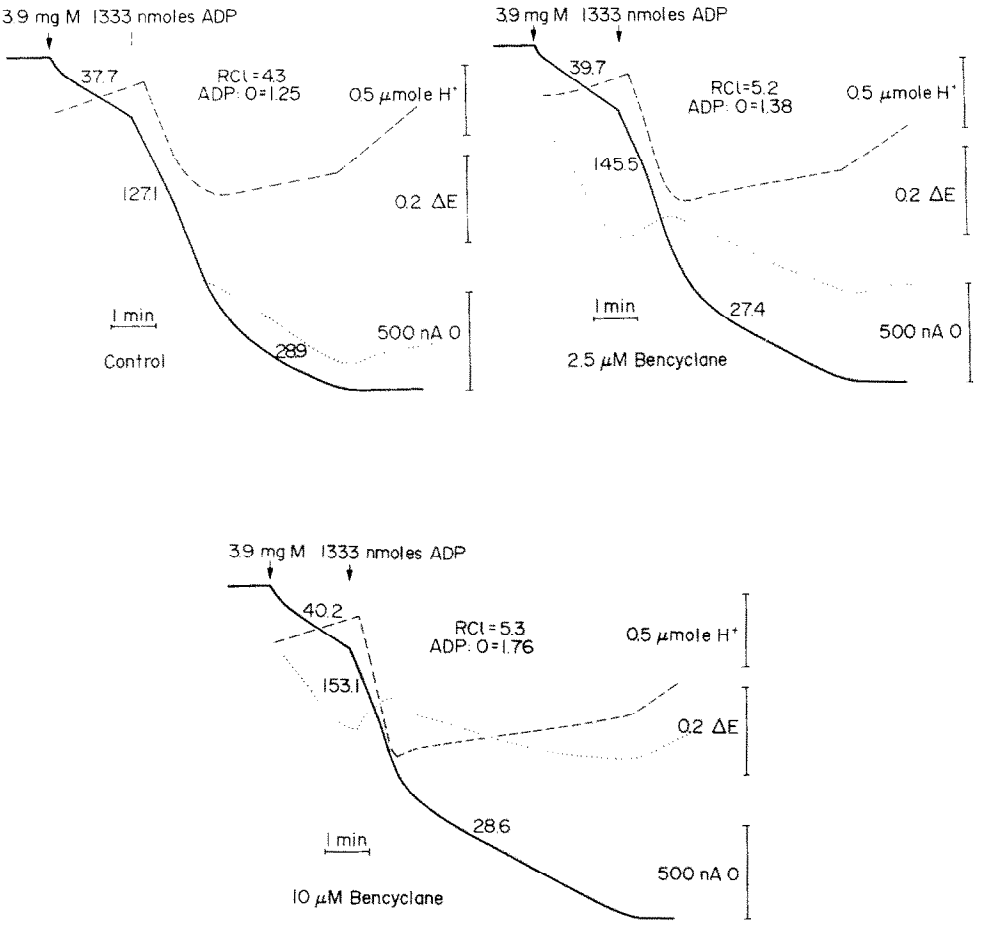

Fig. 2. Effects of bencyclane in a medium containing high potassium concentration. The experimental medium consisted of 150 mM potassium chloride, 1 mM sodium pyruvate and 4 mM potassium phosphate buffer, pH 7.00, at 37°. In 4 ml medium 3.9 mg mitochondrial protein (M) was used. For symbols and other details see Fig. 1.

ing that the rate of ATP synthesis was increased under the influence of the drug. Accordingly, bencyclane inhibited the energy-dependent uptake of ions into the mitochondria by an action independent of its uncoupling and electron transport inhibitory effects. It was recently suggested that a cardiodepressive side effect of bencyclane is the result of the reduction of high energy phosphate stores of the cells caused by an electron transport inhibitory and uncoupling actions. At the same time the beneficial vasodilation was explained by another consequence of these effects, i.e. by an increased production of free adenosine [3]. On the contrary, the increased anoxia tolerance observed under the influence of bencyclane [7] is rather difficult to imagine if the substance would represent an uncoupling agent in the concentrations increasing anoxia tolerance. It has also been demonstrated that in the ischemic myocardium both the structure and function of mitochondria are considerably impaired [8, 9, 10]. One of the main causes of this impairment could be the energy-wasting uptake of potassium phosphate into the mitochondria from the intracellular space as a result of the elevated intracellular inorganic phosphate concentration under ischemic conditions. This latter is supported by the observations showing that the accumulation of potassium by the mitochondria is greatly stimulated by the inorganic phosphate [11, 12]. The uptake of ions results in changes demonstrated in the ischemic myocardium [9, 10] and in our experiments (Fig. 2, control), namely swelling of the mitochondria and impairment of oxidative phosphorylation. Bencyclane was found to be capable of inhibiting swelling of the mitochondria preventing thereby the impairment of oxidative phosphorylation. We assume that this effect of bencyclane is related to its antianginal action. The uncoupling effect exerted only at higher concentrations may be related to the side effects of this substance.

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