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THE EFFECT OF BENCYCLANE ON THE OXIDATIVE PHOSPHORYLATION IN ISOLATED MITOCHONDRIA OF HEART AND LIVER*

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Abstract—Our experiments were designed to localize the inhibitory influence of bencyclane† on the process of oxidative phosphorylation in isolated heart and liver mitochondria. The following results were obtained: (1) The state-3-respiration of rat liver and rabbit heart mitochondria was inhibited by bencyclane. This inhibition was dependent on the substrate used as energy donator, being much more pronounced with glutamate ($ED_{50} = 3.17 \times 10^{-8}$ or 1.85×10^{-7} moles/mg of protein, respectively) than with succinate ($ED_{50} = 3.4 \times 10^{-7}$ or 4.78×10^{-7} moles/mg of protein, respectively). Since the 2,4-dinitrophenol stimulated respiration was equally inhibited, and glutamate transfer through the mitochondrial membrane not influenced, we assume the NADH-coenzyme-Q-reductase to be the site of interaction at the molecular level. (2) Bencyclane stimulates the state-4-respiration of isolated mitochondria with concentrations $\geq 10^{-5}$ M. This effect depends on the molar bencyclane concentration of the incubation medium, and is not abolished by the addition of atractyloside, oligomycin or ruthenium red. Therefore, it is suggested that uncoupling of oxidative phosphorylation is the reason for this bencyclane effect. Theoretically, both of the described effects result in a reduction of the amount of ATP in the living cell. Possible consequences on myocardial function and the cardiovascular system are discussed in terms of previously published data in this field.

Bencyclane is a new vasodilator introduced for the treatment of disturbances in peripheral and central nervous system blood flow. In addition, Komlos and Petöcz [1] described a local anaesthetic action. In a previous publication [2] we demonstrated a cardiodepressive side effect with therapeutic doses of bencyclane, and an inhibition of the state-3-respiration and active calcium uptake of isolated mitochondria with concentrations $\geq 10^{-5}$ M. We therefore suggested that both drug effects had related properties. In the present paper we investigated its mechanism of action on the function of isolated rat liver and rabbit heart mitochondria.

MATERIALS AND METHODS

Rat liver mitochondria were isolated from Wistar albino rats. The liver was cut with scissors into small pieces in a refrigerated room and diluted (1 g/10 ml) with a solution (MST-solution) of the following composition: mannitol 210 mM, sucrose 80 mM and Tris–HCl, pH 7.4, 10 mM. The suspension was homogenized three times with an Ultra-Turrax, type 45 (Janke and Kunkel, West Germany), applying an electrical impulse of 0.5 sec duration by means of a special timing device, resulting in a constant total run of the homogenizer of 3 sec. Between each homogenization step an interval of 1 min was chosen. After differential centrifugation, the fraction obtained between 600 and 10,000 *g* was resuspended in the described MST-solution and stored at 0–1°. The protein content was determined by the Biuret method.

The mitochondrial preparations were found to be almost free from other cell contaminants by means of the electron microscope using the technique of negative staining. By checking the mitochondrial calcium uptake in the presence and absence of oligomycin according to Slater [3], it was shown that the preparation did not contain measurable amounts of Ca^{2+} accumulating particles different to mitochondria.

The isolation of rabbit heart mitochondria (white Newfoundlands) was done by the same procedure with the exception that the timing of the Ultra-Turrax was extended to 1 sec.

The estimation of the oxygen consumption was performed by incubating 4 mg of rat liver or 1.6 mg of rabbit heart mitochondria at 25° in 2.5 ml of a solution with the following composition: Tris-glutamate 10 mM, $MgCl_2$ 5 mM, Tris-phosphate 5 mM, mannitol 184.8 mM, sucrose 70.4 mM, Tris-buffer, pH 7.4, 8.8 mM.

The oxygen consumption was determined according to Potter and Chance [4, 5] by means of a Clark oxygen electrode (for further details see Noack [6]). 10 mM Tris-succinate was used instead of Tris-glutamate if indicated.

The transfer of L-glutamate across the inner mitochondrial membrane of rat liver mitochondria has been studied by swelling technique [7] and by direct measurement of L-[^{14}C]glutamic acid penetration, taking the distribution space of tritiated water as a measure of total mitochondrial water [8]. The pro-

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† Bencyclane = *N*-3-(1-benzene-cycloheptyl-oxi)-propyl-*N*,*N*-dimethyl-ammonium hydrogen fumarate.

cedure was checked with 1-fluoro-2,4-dinitrobenzene which inhibited the glutamate penetration with a concentration as low as 0.1 mM.

Substances used. Bencyclane: *N*-3-(1-benzene-cycloheptyl-oxi)-propyl-*N*,*N*-dimethyl-ammonium hydrogen fumarate (Dr. Thiemann GmbH, Lünen, West Germany). Atractyloside, potassium salt (Calbiochem., Los Angeles, U.S.A.). Oligomycin and 1-fluoro-2,4-dinitrobenzene (SERVA Feinbiochemica, Heidelberg, West Germany). Ruthenium red (Fluka AG, Switzerland). 2,4-dinitrophenol, L-glutamic acid, succinic acid, mannitol, sucrose and Tris-(hydroxymethyl)-aminomethan (Merck Chem. Co., Darmstadt, West Germany). Adenosine 5′ diphosphate disodium salt (C. F. Boehringer, Mannheim, West Germany). L-[U-^{14}C]glutamic acid (specific activity 276 mCi/m-mole) was bought from Amersham Buchler, Braunschweig, West Germany, and tritiated water (1 mCi/g) from NEN Chemicals GmbH, Dreieichenhain, West Germany.

RESULTS

In our studies with isolated mitochondria using bencyclane, all experimental conditions displayed a decrease of the RC-factor, which amounts to about 3.5 in control experiments. According to the definition of the RC-factor, the reason for the drug mediated inhibition of this parameter may be due to the alteration of the state-4- or state-3-respiration or even both. We studied the two factors separately in order to clarify this point of question.

The influence of bencyclane on state-3-respiration. The effect of bencyclane on the state-3-respiration of isolated rat liver and rabbit heart mitochondria is shown in Fig. 1. It is evident that in the presence

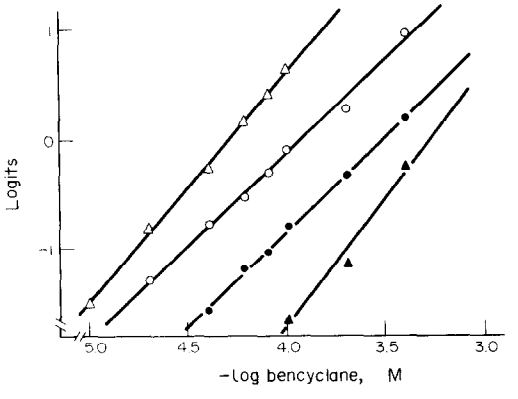

Fig. 1. Logit transformation of the percental inhibition of the rate of state-3-respiration. Rat liver mitochondria: △----△ glutamate as substrate; respiration in the absence of inhibitor: 224.2 nmoles O_2/sec/g protein, ED_{50}: 5.07×10^{-5} M bencyclane, $y = 2.11\ x - 1.49$, $r = 0.99$; $n = 6$. ▲----▲ succinate as substrate, control 227.6 nmoles O_2/sec/g protein, ED_{50}: 5.44×10^{-4} M bencyclane. $y = 2.31\ x - 4.01$, $r = 0.99$; $n = 3$. Rabbit heart mitochondria: O----O glutamate as substrate, respiration in the absence of inhibitor: 1251.2 nmoles O_2/sec/g protein, ED_{50}: 1.18×10^{-4} M bencyclane, $y = 1.69$, $x - 1.81$, $r = 0.99$, $n = 7$. ●----● succinate as substrate; respiration in the absence of inhibitor 1237.5 nmoles O_2/sec/g protein, ED_{50}: 3.06×10^{-4} M bencyclane, $y = 1.74\ x - 2.58$, $r = 0.99$, $n = 6$. For further details see method section.

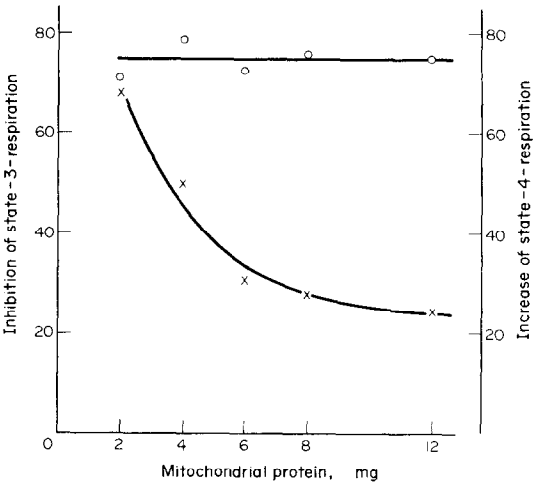

Fig. 2. The influence of 6×10^{-5} M bencyclane on the state-3- (×----×) and state-4-respiration (O----O) of isolated rat liver mitochondria in the presence of different mitochondrial protein concentrations.

of glutamate as substrate the half-maximal inhibitory concentration in rat liver mitochondria is about ten times lower ($ED_{50} = 5.07 \times 10^{-5}$ M) than in the presence of succinate (5.44×10^{-4} M). This finding is the same in our experiments in rabbit heart mitochondria, where the ED_{50} for glutamate as substrate is lower (1.18×10^{-4} M) than with succinate (3.06×10^{-4} M). The extent of the described inhibition is dependent on the absolute amount of mitochondrial protein used in the experiment as shown in Fig. 2 for rat liver mitochondria. The bencyclane concentration of Fig. 1 was recalculated according to this result and expressed in moles bencyclane/mg mitochondrial protein, as shown in Fig. 3. Thus, in the presence of succinate as substrate there is no longer any difference between the drug effect on liver and heart mitochondria. Instead with glutamate the sensitivity of liver mitochondria is about 5.8 times more pronounced than that of heart mitochondria. We obtained the following ED_{50}-values, expressed as 10^{-8} moles of bencyclane/mg of protein: liver mitochondria, glutamate: 3.17; succinate: 34.0. Heart mitochondria, glutamate: 18.5, succinate: 47.8.

The ratio of ADP to extra oxygen consumption

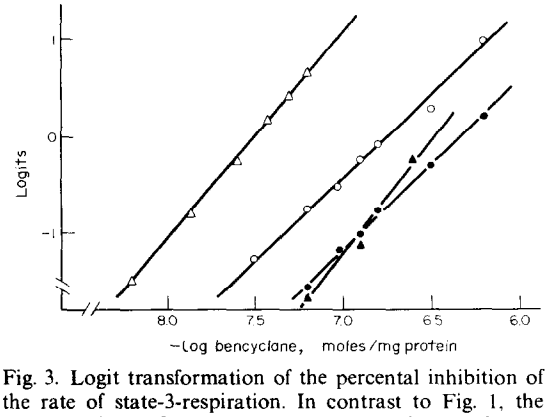

Fig. 3. Logit transformation of the percental inhibition of the rate of state-3-respiration. In contrast to Fig. 1, the concentrations of bencyclane are expressed as moles/mg protein. For further details see Fig. 1.

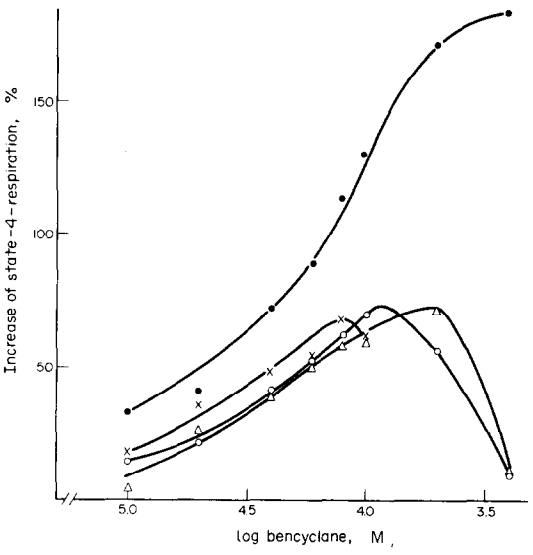

Fig. 4. Semilogarithmic plot between the increase of the rate of state-4-respiration in percent of control values and the concentration of bencyclane. Rat liver mitochondria: ×----× glutamate as substrate, control 61.2 nmoles O_2/sec/g protein, ●----● succinate as substrate, control 69 nmoles O_2/sec/g protein; rabbit heart mitochondria: O----O glutamate as substrate, control 219.5 nmoles O_2/sec/g protein, △----△ succinate as substrate, control 348.3 nmoles O_2/sec/g protein. For incubation conditions see method section.

amounted to 2.76 and 1.87 in the presence of glutamate and succinate, respectively. It was not affected by bencyclane. If, however, the ratio of ADP added and the amount of oxygen consumption during the whole period of state-3-respiration is calculated according to Chance and Williams [9], we find a distinct lowering of these values, which is due to the stimulation of state-4-respiration in the presence of bencyclane.

The 2,4-dinitrophenol induced uncoupling of oxidative phosphorylation, which makes a stimulation of oxygen consumption comparable to the ADP-stimulated extra oxygen consumption, is inhibited by bencyclane to the same extent (ED_{50} in liver mitochondria, glutamate: 3.36×10^{-8} moles/mg of protein, heart mitochondria, glutamate: 20.1×10^{-8} moles/mg of protein). The values for the median effective dose (= ED_{50}) were calculated by means of the logit-transformation. According to Hafner *et al.* [10], the gradient of the logit-dose regression line directly indicates the number of drug molecules inter-acting with one receptor site. The mean value for the gradient was determined to be 1.95 ± 0.10 with $n = 6$.

Additional experiments showed that the glutamate transfer through the inner mitochondrial membrane was not influenced by bencyclane in concentrations up to 4×10^{-4} M.

The influence of bencyclane on state-4-respiration. A concentration as low as 10^{-5} M of bencyclane induces an increase of state-4-respiration of isolated mitochondria under all experimental conditions tested (Fig. 4). It is remarkable to note that the increase of state-4-respiration by bencyclane always reaches a maximal effect, after the state-3-respiration is already inhibited by more than 50 per cent (see also Fig. 1). This finding explains why we did not get a maximal effect of the stimulated state-4-respiration with liver mitochondria and succinate as substrate in the concentration range tested. In contrast to the results which we presented for the state-3-respiration, the stimulation of state-4-respiration was not correlated with the absolute amount of mitochondrial protein present. Figure 2 shows that the effect runs parallel with the concentration of bencyclane in the incubation medium.

The observed increase of state-4-respiration in the presence of bencyclane may be due to an enlarged intra- or extra-mitochondrial splitting and rephosphorylation of adenine nucleotides, or to an increased efflux of, e.g. calcium ions which are then transported by the mitochondria in an energy-dependent uptake process. The underlying mechanism was clarified by means of inhibitors of oxidative phosphorylation or active calcium transport. We found that neither atractyloside (30 μM) nor oligomycin (7 μM), which in control experiments both blocked the ADP-stimulated respiration without influencing the active calcium uptake, did have any effect on the bencyclane-stimulated state-4-respiration. Furthermore, in the presence of 27 μM ruthenium red, which totally blocked the active calcium transport into mitochondria without affecting the ADP-stimulated state-3-respiration, there was no change of the respiratory activity (see Table 1).

Table 1. Increase of the state-4-respiration as caused by bencyclane in the presence of atractyloside, oligomycin or ruthenium red. 4 mg of rat liver mitochondria per experiment. The rates of respiration are given in nmoles O_2/sec/g protein. The data represent the means $\bar{x} \pm s\bar{x}$ of six replicate determinations. For further details see Method section.

	State-4-respiration		State-3-respiration in the presence of			
			500 nmoles ADP		500 nmoles Ca^{2+}	
	control	bencyclane 6×10^{-5} M	control	bencyclane 6×10^{-5} M	control	bencyclane 6×10^{-5} M
Control	65.6 ± 1.7	124.1 ± 3.0	262.3 ± 8.4	141.3 ± 6.9	192.3 ± 5.6	108.5 ± 3.5
+ Atractyloside 3×10^{-5} M	63.1 ± 1.9	118.5 ± 3.2	0.0	0.0	195.6 ± 6.2	111.3 ± 2.9
+ Oligomycin 7×10^{-6} M	66.1 ± 3.4	123.1 ± 2.2	0.0	0.0	194.5 ± 4.8	110.8 ± 3.2
+ Ruthenium red 2.7×10^{-5} M	68.6 ± 2.0	123.5 ± 2.7	258.4 ± 9.2	143.4 ± 3.6	0.0	0.0

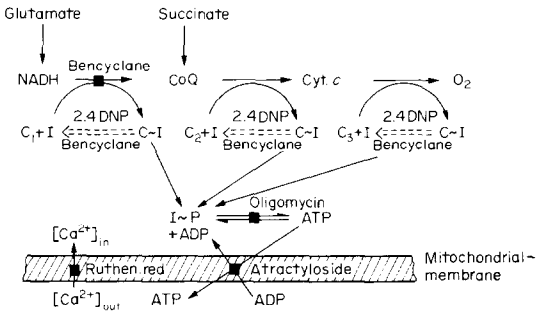

Fig. 5. Hypothetical scheme of the mitochondrial energy transfer system linked to the electron transport chain and the sites of bencyclane action. The sites of inhibitor interaction are indicated by the bars. The dotted arrows indicate the reaction step which is activated by bencyclane causing the uncoupling of oxidative phosphorylation.

DISCUSSION

In the present paper bencyclane was shown to exhibit two different effects on the respiratory function of isolated heart and liver mitochondria: on the one hand it causes an inhibition of the state-3-respiration, and on the other a stimulation of the state-4-respiration. The finding that the inhibition of the state-3-respiration by bencyclane is dependent on the amount of bencyclane per mg of mitochondrial protein and not only on the molar inhibitory concentration favours the idea that the drug interacts with a special receptor site. In addition, according to Hafner *et al.* [10] it is possible to demonstrate by means of the logit transformation that 2 bencyclane molecules interact with one receptor binding site. Since the state-3-respiratory activity is much more influenced by bencyclane in the presence of glutamate than by succinate as a respiratory substrate, and the glutamate transfer through the inner mitochondrial membrane is not inhibited by bencyclane, we suppose that the main site of interaction of bencyclane is located at the NADH-coenzyme-Q-reductase. This conclusion is in agreement with the observation that the 2,4-dinitrophenol-stimulated respiration is inhibited to the same extent as the state-3-respiration (see Fig. 5).

It is interesting to note that the described stimulation of the state-4-respiration is neither inhibited by atractyloside, oligomycin nor by ruthenium red. Therefore, an additional energy consuming calcium transport process or an additional exchange and synthesis of adenine nucleotides may be excluded as a reason for the observed effect. Thus the uncoupling of oxidative phosphorylation is supposed to be the mechanism of action for this effect (see also Fig. 5).

The drug interaction described in this report may well be related to the *in vivo* effects of bencyclane, since the concentrations used in our mitochondrial studies are comparable to those which produce a negative inotropic effect on isolated heart preparations or are obtained after therapeutic application. Köhler *et al.* [11] found a half-maximal inhibition of contraction force in isolated left guinea-pig atria at 2×10^{-5} g/ml = 4.9×10^{-5} M of bencyclane. In isolated mitochondria this drug concentration produces an increase of 50 per cent over the state-4-respiration (in liver mitochondria it already amounts to 80 per cent) and a pronounced inhibition of the state-3-respiration (by 50 per cent in liver and by 20 per cent in heart mitochondria in the presence of glutamate as a substrate). In addition the oral bencyclane dosage of 600 mg/day (single dose 200 mg) recommended by the manufacturer for therapeutic use, which is described to have a bio-availability of almost 100 per cent [12] or the intra-arterial infusion of 500 mg bencyclane during a 30 min period, theoretically leads to a tissue concentration of more than 10^{-5} M of bencyclane.

The effects of bencyclane, i.e. inhibition of state-3-respiration and uncoupling of state-4-respiration in isolated mitochondria, are supposed to cause a reduction of high energy phosphate (ATP) stores of the cell. Therefore, the cardiodepressive side effect of bencyclane may be explained by an intramyocardial lack of energy. At the same time it may be expected that the vasodilation is due to a decreased rate of synthesis of adenine nucleotides leading to an increased production of free adenosine, which may then function as a potent vasodilator.

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