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INFLUENCE OF THE ANESTHETIC 2,6-DIISOPROPYLPHENOL ON THE OXIDATIVE PHOSPHORYLATION OF ISOLATED RAT LIVER MITOCHONDRIA

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Abstract—Isolated rat liver mitochondria have been incubated in the presence of the general anesthetic 2,6-diisopropylphenol (0–100 μM) and the efficiency of oxidative phosphorylation has been evaluated by measuring the respiratory rates, the rates of ATP synthesis or hydrolysis and the magnitude of the transmembrane electrical potential. The results obtained indicate that: (a) in mitochondria energized either by succinate or by ATP, 2,6-diisopropylphenol decreased the transmembrane electrical potential and increased the rates of either electron transfer or ATP hydrolysis; (b) in succinate-energized mitochondria 2,6-diisopropylphenol, at concentrations causing substantial depression of the transmembrane electrical potential, did not modify either the rate of phosphorylation of added ADP or the rate of ADP-stimulated respiration; (c) in succinate-energized mitochondria 2,6-diisopropylphenol caused a concentration-dependent inhibition of the uncoupler-stimulated rate of succinate oxidation. These findings suggest that under the experimental conditions reported 2,6-diisopropylphenol affected the generation and/or maintenance of the transmembrane electrical potential while leaving unchanged the coupling between the electron flow in the respiratory chain and the synthesis of ATP.

General anesthetics have been reported to affect different processes of mitochondrial metabolism including the flow of electrons along the respiratory chain, the synthesis and hydrolysis of ATP, the generation and maintenance of the transmembrane proton electrochemical potential, the content of endogenous long-chain acylCoA and the efflux of calcium [1–3]. Most of these effects have been investigated using halogenated anesthetics. Both chloroform and halothane have been reported to inhibit the rate of ATP synthesis and to increase the rate of electron transfer in the respiratory chain without significantly affecting the transmembrane proton electrochemical potential [1]. Moreover it has been recently shown that in ATP-energized mitochondria the extent of the chloroform-induced increase of the rate of ATP hydrolysis is not justified by a concomitant increase of the proton conductance of the inner membrane [4]. Although these findings suggest that the interference of halogenated anesthetic with the mitochondrial energy-coupling processes is substantially different from that of classical protonophoric uncouplers, the mechanism underlying these effects is still debated [5].

Since general anesthetics are an extremely heterogeneous class of molecules, a comparison among the mitochondrial effects of structurally different anesthetics might be helpful both in clarifying the nature of the anesthetic–mitochondrion interactions and in recognizing common effects, if any, of general anesthetics.

In this paper we report and discuss the effects of 2,6-diisopropylphenol (DPP§), a general anesthetic structurally unrelated to halogenated anesthetics, on mitochondrial energy converting processes.

MATERIALS AND METHODS

Rat liver mitochondria were isolated in 0.25 M sucrose, 2.5 mM Na-HEPES, 0.25 mM EGTA (pH 7.4) as previously described [6], omitting EGTA in the final washing. Mitochondrial proteins were determined according to Gornall *et al.* [7].

All experiments were carried out at 30° using 1 mg of mitochondrial protein/mL of basal medium containing 100 mM sucrose, 50 mM KCl, 10 mM K-phosphate, 2 mM $MgSO_4$, 1 mM EDTA, 15 mM Tris–HCl (pH 7.4).

Mitochondrial respiratory rates were monitored with a Clark oxygen electrode (Yellow Springs Instruments Co.) in 2.5 mL of the basal medium supplemented with 5 mM K-succinate + 1.25 μM rotenone.

Mitochondrial transmembrane electrical potentials ($\Delta\Psi$) were measured by monitoring the concentration of TPP^+ in the extramitochondrial aqueous phase with a TPP^+-selective electrode [8] using 5 mL of the basal medium supplemented with either 5 mM K-succinate + 1.25 μM rotenone or 5 mM ATP + 1.8 μM antimycin A.

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§ Abbreviations: DPP, 2,6-diisopropylphenol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid; EGTA, ethyleneglycol-bis-(β-aminoethylether)*N*,*N*′-tetraacetic acid; TPP^+, tetraphenylphosphonium; CCCP, carbonylcyanide-*m*-chlorphenylhydrazone; $\Delta\Psi$, transmembrane electrical potential.

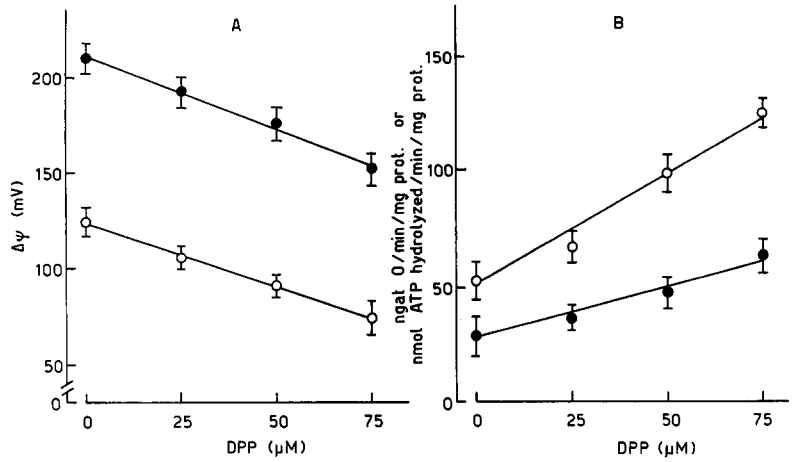

Fig. 1. Effects of DPP on the rates of respiration or ATP hydrolysis and on the transmembrane electrical potentials of isolated mitochondria. The transmembrane electrical potentials (A) and the rates of oxygen consumption or ATP hydrolysis (B) have been measured in parallel in mitochondria incubated in the presence of either succinate + rotenone (●) or ATP + antimycin A (○). Each point represents the mean of 12 experimental values ± SD.

The rates of ATP synthesis and hydrolysis were evaluated following the rates of proton consumption or production [9] by means of a combined pH electrode (Ingold Messtechnik AG) in 2.5 mL of the basal medium from which Tris–HCl was omitted. The incubation medium was supplemented with succinate + rotenone or with ATP + antimycin A (at the concentrations reported above) for ATP synthesis or hydrolysis measurements, respectively. The rates reported in the figures are the differences between the rates obtained in the absence and in the presence of 1 μg oligomycin/mL.

RESULTS

The results reported in this paper have been obtained with isolated mitochondria incubated in the presence of DPP concentrations not exceeding 100 μM. It is relevant to note that these concentrations are comparable to the plasmatic levels of DPP measured in man during the induction and the maintenance of general anesthesia (60–70 and 20–40 μM DPP, respectively) [10, 11].

Figure 1 summarizes the effects of DPP on the transmembrane electrical potential and the rates of either oxygen consumption or ATP hydrolysis measured in parallel in isolated mitochondria under steady-state conditions. As shown in Fig. 1A DPP depressed the ΔΨ maintained in mitochondria either by succinate oxidation in the presence of rotenone or by ATP hydrolysis in the absence of exogenous oxidizable substrates and in the presence of antimycin A. The decrease of ΔΨ was linearly dependent on the concentration of DPP in the 0–75 μM range and its extent (45–50 mV at 75 μM DPP) was very similar in succinate- or ATP-energized mitochondria. Correspondingly, DPP induced a concentration-dependent stimulation of the rates of both succinate oxidation and, to a larger extent, ATP hydrolysis (Fig. 1B). These results indicate that DPP (up to 75 μM) affected the maintenance of the transmembrane electrical potential generated either by the respiratory chain or by the ATPase proton-pumps without inhibiting either mitochondrial respiration or ATP hydrolysis. At concentrations higher than 75 μM (data not shown) DPP caused a sharp decrease of ΔΨ, which collapsed to less then 30 mV at approximately 100 μM DPP both in succinate-oxidizing and in ATP-hydrolysing mitochondria. This ΔΨ reduction was accompanied either by a limited increase of the respiratory rate or by total inhibition of the rate of ATP hydrolysis in succinate- or ATP-energized mitochondria, respectively.

The continuous lines in Fig. 2 show the effects of increasing concentrations of DPP on the rates of respiration and ATP synthesis induced in succinate-energized mitochondria by the addition of ADP; the dashed line refers to the uncoupler-stimulated oxygen consumption rates measured under the same experimental conditions. The presence of DPP up to 75 μM in the incubation medium did not significantly modify either the ADP-stimulated respiration or the rate of ATP synthesis. On the contrary, concentrations of DPP higher than 75 μM caused a sharp decrease of both the ATP synthesis, which vanished at 100 μM DPP, and the ADP-stimulated respiration, which was reduced to approximately 60% of the control at 100 μM DPP. Measurements of the respiratory rates of CCCP-uncoupled mitochondria in the presence of DPP (dashed line) revealed that increasing concentrations of DPP caused a progressive inhibition of the uncoupled respiration. As a result of this inhibition, when the concentration of DPP in the incubation medium was increased up to 75 μM the rate of the ADP-stimulated respiration (which remained unchanged) gradually approached and finally

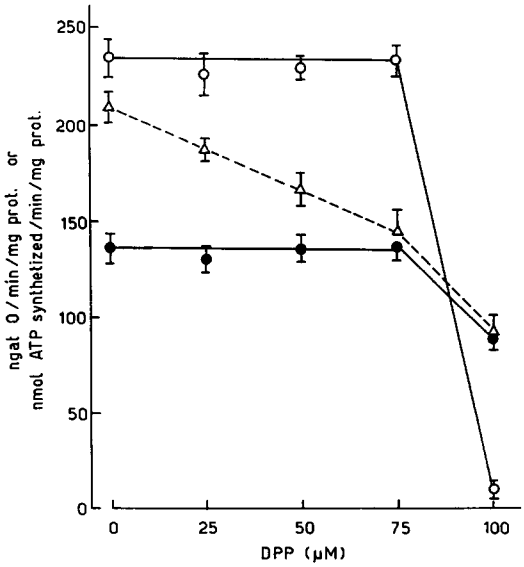

Fig. 2. Effects of DPP on the rates of ATP synthesis, of ADP-stimulated respiration and of uncoupler-stimulated respiration in isolated mitochondria. The rates of ADP-stimulated respiration (●) and of ATP synthesis (○) have been measured in parallel in isolated mitochondria incubated in the presence of succinate + rotenone and 150 μM ADP; the rates of uncoupled respiration (△) have been monitored in the presence of succinate + rotenone and 1.6 μM CCCP. Each point represents the mean of 10 experimental values ± SD.

matched that of uncoupler-stimulated respiration; over 75 μM DPP the inhibitory effect on the maximal respiratory rate was enhanced and was accompanied by a marked reduction of both the ADP-stimulated respiration and the rate of ATP synthesis.

DISCUSSION

The results of the experiments presented in this paper show that in mitochondria incubated in the presence of DPP concentrations up to 75 μM the ATP synthesis was not affected regardless of the significant decrease of the steady state $\Delta\Psi$.

At concentrations higher than 75 μM DPP caused relevant damage to mitochondria, as demonstrated by the collapse of $\Delta\Psi$ and by the total inhibition of ATP synthesis and hydrolysis. It appears then likely that in the 75–100 μM range DPP induced a severe disarrangement of the inner mitochondrial membrane.

The fact that in succinate-energized mitochondria the depression of $\Delta\Psi$ caused by DPP up to 75 μM was accompanied by a stimulation, rather than by an inhibition, of the rate of oxygen consumption (Fig. 1) demonstrates that this effect was not a consequence of an inhibition of the respiratory chain. This was confirmed by the ability of DPP to decrease $\Delta\Psi$ in ATP-energized mitochondria also. Indeed the decrease of the rate of uncoupled respiration induced by DPP at concentrations lower than 75 μM (Fig. 2) indicates that this compound might also inhibit the electron transport chain. However this inhibitory effect of DPP was only observed in CCCP-uncoupled mitochondria suggesting that either it affected only the maximal rate of succinate oxidation or it resulted from the concomitant interaction of the inner mitochondrial membrane with two membrane-damaging agents (CCCP and DPP).

The concentration-dependent decrease of $\Delta\Psi$ and the corresponding stimulation of either succinate oxidation or ATP hydrolysis induced by DPP up to 75 μM appear consistent with an increase of the proton backflux across the inner mitochondrial membrane. These results suggest that DPP, as other substituted phenols [12, 13], might act as a protonophoric agent increasing the proton influx across the inner mitochondrial membrane; alternatively the same phenomena might be caused by an increase of the intrinsic uncoupling of the respiratory chain and ATPase proton pumps, which would transfer some electrons or hydrolyse some ATP molecules without translocating protons from the matrix to the external medium [5]. Finally the possibility exists that DPP, being a lipophilic molecule, caused a disarrangement of the membrane assembly resulting in an alteration of the permeability to protons.

However, according to the chemiosmotic hypothesis, any decrease of the net proton extrusion by mitochondria is expected to reduce, besides the steady-state transmembrane electrochemical proton gradient, the rate of ATP synthesis. In fact if the delocalized transmembrane proton electrochemical gradient (i.e. that existing between the intra- and extra-mitochondrial aqueous phases, which is measured by the TPP^+-selective electrode) is assumed to be the only coupling intermediate in oxidative phosphorylation, and hence the only driving force for ATP synthesis, its decrease should be accompanied not only by a stimulation of the electron flow in the respiratory chain, but also by a depression of the synthesis of ATP. Thus the observation that in succinate-energized mitochondria DPP concentrations up to 75 μM, which caused a significant decrease of $\Delta\Psi$ in the absence of added ADP, did not affect the rate of ATP synthesis (Fig. 2) cannot be explained by the classical chemiosmotic hypothesis.

On the other hand it has been widely demonstrated that some aspects of free-energy coupling cannot be readily explained by a straightforward application of the chemiosmotic concepts proposed by Mitchell. These discrepancies have led to the proposal of alternative or additional coupling mechanisms which, although recognizing the fundamental role of classical delocalized proton coupling, hypothesize the existence of localized coupling pathways [14–16]. According to these views energized protons might flow directly from the redox to the ATPase proton pumps within membrane-controlled spaces, without being released in the extramembrane aqueous phases. The existence of such a direct interaction between the respiratory chain and the ATP synthetase has been proposed to account for the observation that in mitochondria, as well as in bacteria and chloroplasts, the rates of ATP synthesis appear to be correlated with the rates of electron

transfer rather than with the magnitude of the delocalized transmembrane proton gradient [17, 18]. The finding that in isolated mitochondria incubated with DPP concentrations up to 75 μM both the rate of ATP synthesis and that of ADP-stimulated respiration did not significantly differ from those of control mitochondria while the transmembrane electrical gradient appeared significantly lower might then indicate that DPP, although affecting the maintenance of the delocalized transmembrane proton gradient by any of the mechanisms discussed above, did not interfere with the proposed direct interaction between the respiratory chain and the ATP synthetase.

Finally the ability of DPP (up to 75 μM) to depress $\Delta\Psi$ without affecting the ATP synthesis suggests that its effects on mitochondria substantially differ from those of halogenated anesthetics, which have been reported to inhibit the synthesis of ATP without collapsing the transmembrane proton electrochemical gradient [1, 2, 4]. Thus the study of the effects of structurally different anesthetics, such as DPP and halothane or chloroform, on mitochondrial oxidative phosphorylation does not allow, at this time, recognition of a specific behaviour common to general anesthetics.

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