INTERACTION OF OLEFICIN WITH THE INNER MEMBRANE OF RAT LIVER MITOCHONDRIA

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The effects of oleficin, a polyene antibiotic of the nonmacrolide type, on isolated rat liver mitochondria were studied. Oleficin at a concentration of about 10 nmoles/mg protein increases both the rate of state 4 respiration and the "basal" ATPase activity of mitochondria. In contrast to this it inhibits the rate of both state 3 and uncoupled respiration and the DNP-stimulated ATPase activity. These inhibitions can be prevented by low concentrations (2~5 mM) of magnesium ions.

Oleficin induces a high amplitude swelling of non-respiring mitochondria in the isoosmotic nitrate and chloride solutions of K^+, Na^+, $Tris^+$, Tea^+ or Mg^{2+}. In contrast to that it does not induce swelling of mitochondria treated with ruthenium red in isoosmotic calcium acetate. Indirect evidence suggests that oleficin increases also the proton permeability of the inner membrane. The swelling observed in the isoosmotic solutions of monovalent cations can be prevented by low concentration (2~5 mM) of Mg^{2+}. In the presence of the antibiotic Mg^{2+} and Ca^{2+} but not K^+ and Na^+, are transferred from an aqueous phase into a butanol-toluene bulk phase. Oleficin depletes Mg^{2+} and Ca^{2+} from mitochondria in a concentration dependent manner. Complete depletion of Mg^{2+} occurs only in the presence of EDTA, while that of Ca^{2+} does not need the chelator.

It is concluded that the effects of oleficin on mitochondrial functions can be explained on the basis of an increase of the inner membrane permeability as the consequence of the depletion of Mg^{2+} from mitochondria caused by the antibiotic.

Oleficin was isolated from *Streptomyces parvulus* and was found to be effective against Gram-positive bacteria and YOSHIDA sarcoma by GYIMESI *et al.*[1]. Its chemical structure was established by HORVÁTH *et al.*[2] and GYIMESI *et al.*[3] They drew attention to the structural similarity existing between oleficin and other antibiotics which contain the tetramic acid residue, first of all the polyene lipomycin[4,5] and erythroskyrin[6~8].

In this paper we report the effect of oleficin on the inner membrane of rat liver mitochondria. Oleficin belongs to the group of the non-macrolide type polyene antibiotics containing tetramic acid residue, which group has not yet been examined with respect of its possible membrane effects.

Materials and Methods

Rat liver mitochondria were prepared according to JOHNSON and LARDY[9]. Each experiment was carried out at 25°C in 3 ml volume. The composition of media used and other experimental details are given in the legends to the Figures and Table. Oxygen uptake, ATPase activity and osmotic

Abbreviations: Tris, Tris(hydroxymethyl)aminomethane; Tea, triethanolamine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol bis(aminoethylether)-N, N′-tetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid; DNP, 2,4-dinitrophenol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

swelling of mitochondria were determined as described earlier[10].

The chemicals used were the purest commercially available except the sucrose, which was further purified by recrystallization in the presence of EDTA. Oleficin was a kind gift of Dr. J. GYIMESI (Institute for Drug Research, Budapest, Hungary) and dissolved in methanol. In this form the antibiotic could be stored at 0~4°C at least for a month without any sign of decomposition. The greatest amount of solvent used was without any effect on mitochondria.

The Mg^{2+} and Ca^{2+} content of mitochondria was determined according to REED and LARDY[11] by the following way: after the incubation time indicated in the figures the mitochondria were cooled to 0°C and separated from the medium by centrifugation (2 minutes 16,000×g). The mitochondria were washed in the incubation medium and recentrifuged. The pellet was extracted with a solution containing 10% trichloroacetic acid and 1% $LaCl_3$ (this latter to eliminate phosphate) and was centrifuged again. The cation content of the supernatant was determined by atomic absorption spectrometry (Beckman-485).

The cation transport ability of oleficin into a bulk organic phase was studied according to REED and LARDY[11]. To the aqueous phase buffered at pH 7.2 containing the metal salts and the antibiotic the butanol-toluene (30~70 v/v %) mixture was added, and the two phases were vigorously mixed in a Vortex mixer for 10 minutes at 25°C. After separation of the two phases by centrifugation the cation content of the organic phase was determined by atomic absorption following elimination of the organic solvents by evaporation and digestion with concentrated H_2O_2 plus HNO_3.

The protein content of mitochondrial preparations was determined as described by SCHACTERLE and POLLACK[12] using bovine serum albumin (Sigma) as standard.

Results

Effect of Oleficin on Mitochondrial Respiration and ATPase Activity

The effect of oleficin on the respiration of mitochondria depends upon the composition of media used. At high KCl concentration (80 mM) oleficin was found to inhibit the state 3 (Fig. 1d and e) and the uncoupled (Fig. 1f and g) respiration, but to increase the rate of the state 4 respiration (Fig. 1b). The stimulatory effect of the antibiotic is smaller than that of typical uncouplers as DNP or CCCP, and decreases gradually in time. Addition of Mg^{2+} (2~5 mM) prevents both the inhibition of state 3 and uncoupled respiration (not shown) and the increase of state 4 respiration induced by oleficin (Fig. 1c).

At a low potassium concentration, however, the stimulatory effect of oleficin on state 4 respiration is greater and permanent (Fig. 1a). This effect cannot be prevented by addition of Mg^{2+}. Added Mg^{2+} increases even further the rate of respiration if more than 10 nmoles/mg protein of oleficin was present (Fig. 2).

As it is shown in Fig. 2 the stimulation of state 4 respiration induced by the antibiotic is diminished but not completely abolished by either EDTA, EGTA—chelators of bivalent cations—or ruthenium red, an inhibitor of the natural Ca-carrier of the mitochondrial inner membrane. The stimulation of respiration is abolished only if a high concentration of the antibiotic was applied and EDTA added.

In accordance with its stimulatory effect on respiration oleficin also activates the "basal" ATPase activity of mitochondria (Fig. 3). However, maximal stimulation requires added Mg^{2+}. Under these conditions the ATPase activity is practically the same as that measured in the presence of an uncoupler. As it can also be seen in Fig. 3 oleficin inhibits the DNP-stimulated ATPase activity. This inhibition can be completely prevented by addition of Mg^{2+}.

From the findings that oleficin increases both the rate of state 4 respiration and the "basal" ATPase activity, it seemed to be likely that the antibiotic acts rendering the mitochondrial inner mem-

brane more permeable.

Oleficin Induced Swelling of Mitochondria

As it is shown in Fig. 4a oleficin induces a high amplitude passive swelling of non-respiring mitochondria in isoosmotic KNO_3, indicating that the inner membrane permeability to K^+ was greatly increased. The induced influx of K^+ could be prevented or inhibited by adding 2~5 mM Mg^{2+} to the medium (Fig. 4a and b). Similar

Fig. 1. Effect of oleficin on mitochondrial respiration.

The medium contained 140 mM sucrose, 120 mM mannitol, 2mM HEPES in exp. a and 90mM sucrose, 80 mM KCl, 5 mM HEPES in exp. b~g. Other ingredients were 3.3 mM $MgCl_2$ in exp. c and 5 mM K-phosphate in exp. d~g. Further additions indicated by arrows were the followings: mitochondria (RLM 4.2 mg in exp. a and 4.0 mg in exp. b~g), ADP (2.5 mM), DNP (0.1 mM) and oleficin (7.8 in exp. a and 8.2 nmoles/mg protein in exp. b, c, e, g). Each experiment was carried out in the presence of K-succinate (3 mM) and rotenone (3 μM). The pH was adjusted to 7.4 with KOH.

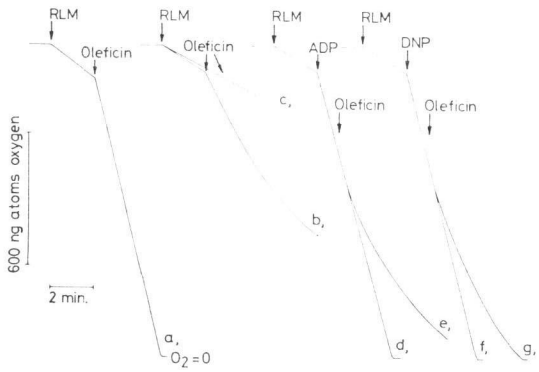

Fig. 2. Effect of EDTA, EGTA, ruthenium red and added Mg^{2+} on the oleficin stimulated respiration.

The composition of media and the amount of mitochondrial protein were the same as that of Fig. 1a.

Further additions were: ×—× none; ○—○ 0.5 mM EDTA; ●—● 0.5 mM EGTA; △—△ 6.0 nmoles ruthenium red; ▲—▲ 3.3 mM $MgCl_2$.

The experimental points were calculated from the initial rate values of oxygen uptake.

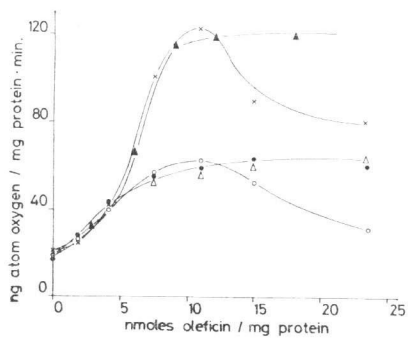

Fig. 3. Effect of oleficin on the ATPase activity of mitochondria.

The media contained 130 mM mannitol, 40 mM sucrose, 30 mM KCl, 10 mM Tea and 10 mM ATP. The pH was adjusted to 7.4. The reaction was started with mitochondria (3.6 mg) and was stopped after 5 minutes by the addition of ice cold trichloroacetic acid.

Other additions were: ●—● none; ○—○ 1.0 mM $MgCl_2$; ▲—▲ 0.15 mM DNP; ×—× DNP+$MgCl_2$.

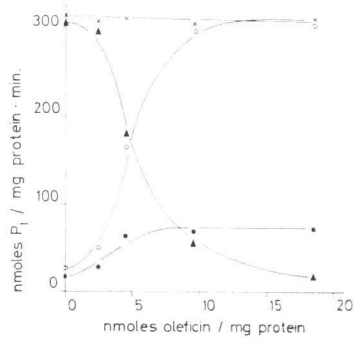

Fig. 4. Passive swelling of mitochondria induced by oleficin.

The medium contained 140 mM KNO_3 in a, b and 100 mM $Mg(NO_3)_2$ in c. 5 mM Tea(NO_3) and 3 μM rotenone was also present in each experiment. The pH was adjusted to 7.4.

Further additions were at the arrows: oleficin (18 nmoles/mg protein) and 3.3 mM $MgCl_2$. The amount of mitochondrial protein used was 3.9 mg in each experiment.

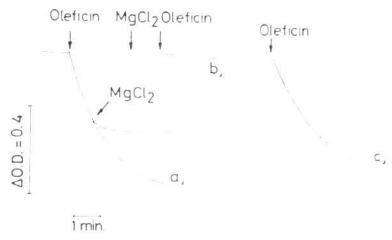

Fig. 5. Swelling of mitochondria in isoosmotic calcium acetate. Effect of oleficin.

The medium contained 100 mM $Ca(CH_3COO)_2$, 3 μM rotenone. The pH was adjusted to 7.4 with acetic acid. The amount of mitochondrial protein was 3.8 mg in each experiment.

Other additions at the arrows were: CCCP (3 μM), ruthenium red (RR 6.0 nmoles) and oleficin (18.0 nmoles/mg protein).

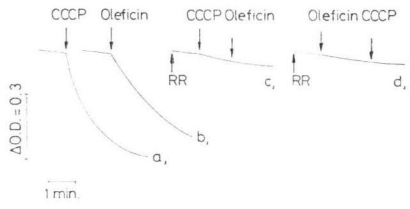

Fig. 6. Mg^{2+} and Ca^{2+} efflux induced by oleficin. Time dependency.

The media were identical with that of Fig. 1a.

Further additions were: ▲—▲ none; △—△ 0.5 mM EDTA; ●—● oleficin (24.0 nmoles/mg protein); ○—○ oleficin+EDTA.

The reaction was started with mitochondria (5.4 mg) and was stopped with cooling the samples and rapid centrifugation. The bivalent cation content was determined as described in Materials and Methods.

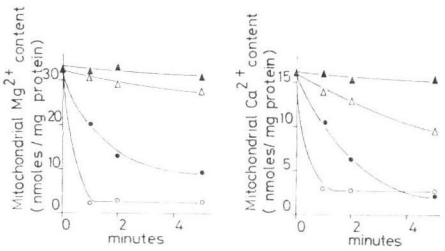

Fig. 7. Mg^{2+} and Ca^{2+} efflux induced by oleficin. Concentration dependency.

The composition of the media used and the amount of mitochondrial protein were the same as that of Fig. 6.

Further additions were: ●—● none; ○—○ 0.5 mM EDTA. The time of incubation was 5 minutes.

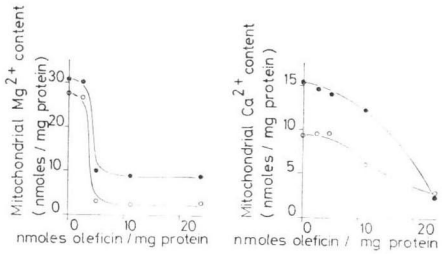

Table 1. Transport of metal cations by oleficin into a bulk organic phase

	Cation transported into the organic phase in μmoles			
	K^+	Na^+	Ca^{2+}	Mg^{2+}
Control	0.01	0.01	0.03	0.02
+2.14 μmoles oleficin	0.02	0.01	0.38	0.40

One ml of aqueous phase containing 20 mM metal-chloride and oleficin was buffered at pH 7.4 with 20 mM Tea-Cl and was vigorously mixed with 1.5 ml of a 30% 1-butanol - 70% toluene mixture for 10 minutes. Following centrifugation to separate the phases, the cation content of the organic phase was determined by atomic absorption spectrometry as described under Materials and Methods.

results were obtained if either K^+ was replaced by Na^+, $Tris^+$, Tea^+ or the freely permeable nitrate anion[13] was replaced by the otherwise non-penetrant chloride (not shown).

As Fig. 4c shows the antibiotic induces swelling of mitochondria also in isoosmotic $Mg(NO_3)_2$ indicating that it renders the inner membrane also permeable to Mg^{2+}.

Non-respiring mitochondria swell in isoosmotic calcium acetate in the presence of a protonophore (Fig. 5a). This swelling can be prevented by ruthenium red, which inhibits the Ca-translocator (Fig. 5c). Fig. 5c and d show that oleficin does not induce a swelling of ruthenium red treated mitochondria in isoosmotic calcium acetate in contrast to that what the synthetic Ca-ionophore found by CARONI *et al.*[14] does. Therefore, it can be concluded that oleficin does not render the inner membrane permeable to Ca^{2+}. It should be emphasized that ruthenium red does not influence at all the oleficin induced swelling of mitochondria in either magnesium or potassium nitrate (not shown).

Fig. 5b also shows that the uncoupler can be effectively substituted by oleficin in this system indicating an increase of proton permeability of the inner membrane induced by the antibiotic.

The question should be raised whether the non-selective permeability increase caused by oleficin is correlated with its ability to bind cations.

Cation Transport Ability of Oleficin into a Bulk Organic Phase

Table 1 shows that the antibiotic can transfer Mg^{2+} and Ca^{2+} from an aqueous phase into a bulk organic one. This does not occur with either K^+ or Na^+. The presence of the lipophilic thiocyanate anion does not increase the amount of cations transported (not shown). Thus, it can be concluded that the antibiotic can form an electroneutral complex with both bivalent cations, which complexes are able to enter the organic phase.

There are numerous data indicating a possible role of Mg^{2+} and Ca^{2+} in the control of the mitochondrial inner membrane permeability[15~22]. Thus, it seemed to be obvious to study the effect of oleficin also on the Mg^{2+} and Ca^{2+} content of mitochondria.

Effect of Oleficin on the Mg^{2+} and Ca^{2+} Content of Mitochondria

Oleficin decreases both the Mg^{2+} and Ca^{2+} content of mitochondria (Figs. 6, 7). The release induced was complete in the presence of EDTA. It can also be seen that for maximal depletion of Ca^{2+} about four times as much oleficin was needed than for Mg^{2+}. The depletion of Mg^{2+} was complete—in contrast to Ca^{2+}—only in the presence of EDTA.

Discussion

From the swelling experiments presented it can be concluded that oleficin increases the permeability of the mitochondrial inner membrane to K^+, Na^+, $Tris^+$, Tea^+, Mg^{2+}, proton and chloride, thus no selectivity is seen. However, it does not facilitate the influx of Ca^{2+} into ruthenium red treated mitochondria. At the same time the antibiotic efficiently deplets both Mg^{2+} and Ca^{2+} from the mitochondria.

The stimulation of respiration by oleficin can be explained on the basis of complex ion movements induced by the antibiotic. For the oleficin caused stimulation of the state 4 respiration at low K^+ concentration, the active reuptake of Ca^{2+} and its cycling induced by the antibiotic can be made responsible. This conclusion is drawn from the finding that both EGTA and ruthenium red significantly diminished the stimulatory effect of oleficin on state 4 respiration. The stimulation remaining in the presence of EGTA or ruthenium red can be the consequence of the induced transport either of K^+, proton or Mg^{2+}. The fact that EDTA decreases much more the oleficin stimulated respiration (at a higher concentration of the antibiotic) than EGTA indicates that under these conditions the reuptake of Mg^{2+} lost can also occur.

In favour of a Mg^{2+} uptake speaks also the finding that maximal stimulation of the "basal" ATPase activity by the antibiotic could be obtained only in the presence of added Mg^{2+}.

The progressive inhibition by oleficin of respiration stimulated either by ADP, DNP or the antibiotic itself in high KCl medium might also be a consequence of the non-selective permeability increase of the inner membrane. It seems likely that under these conditions K^+ and Cl^- are taken up simultaneously, thus the membrane potential can not be sufficiently discharged resulting in a inhibition of respiration.

The inhibition of the DNP-activated ATPase activity by oleficin can be explained on the basis of depletion of Mg^{2+} from mitochondria caused by the antibiotic. This results in a decrease of the concentration of Mg-ATP complex, the real substrate of ATPase. This interpretation is supported by the finding that the inhibition can be prevented by added Mg^{2+}, indicating also that in the presence of the antibiotic Mg^{2+} can be taken up through the inner membrane into the matrix space.

A detergent-like action to be responsible for the non-selective permeability increase caused by oleficin can be excluded by two facts. Firstly, oleficin is effective at low concentrations compared to detergents. Secondly, the ATPase activity of the mitochondria remains sensitive to carboxyatractylo-

side, a specific inhibitor of the ADP/ATP translocator, even in the presence of the highest concentration of oleficin used (not shown). This means that in the presence of oleficin the adenine nucleotides can only cross the inner membrane through the specific translocator, thus the membrane did not become leaky.

We suggest that the oleficin induced non-selective permeability increase of the mitochondrial inner membrane is a consequence of Mg^{2+} depletion caused by the antibiotic. The preventing effect of added Mg^{2+} on the oleficin induced swelling of mitochondria in isoosmotic solutions of monovalent cations supports above all this conclusion. There are several reports indicating that Mg^{2+} depletion results in a permeability increase of the mitochondrial inner membrane[15,17~19]. However, it cannot be ruled out that Ca^{2+} might also play a role in this non-selective permeability increase as suggested by others[20~22].

Finally the question arises, by what mechanism does oleficin increase the permeability of the mitochondrial inner membrane to Mg^{2+}? Though oleficin can bind and transport from an aqueous phase into an organic one Ca^{2+} and Mg^{2+}, but not K^+ and Na^+, it does not facilitate the uptake of Ca^{2+} into ruthenium red treated mitochondria, in contrast to the synthetic Ca-ionophore as found by CARONI *et al.*[14] Furthermore, regarding its ability to bind bivalent cations and to deplete them from mitochondria oleficin resembles very much to the ionophore antibiotic A23187, described by REED and LARDY[11]. However, oleficin in contrast to A23187 does not facilitate the transport of Ca^{2+} through the membrane of erythrocytes in either direction (B. SARKADI—personal communication —investigated by methods described[23]). It appears also that oleficin transports bivalent cations into an organic bulk phase less effectively than A23187 does. From these facts we think that the mechanism by which oleficin induces a Mg^{2+} transport through the mitochondrial inner membrane is different from the mechanism of action of ionophores in the classical sense. It is worth to mention in this respect that primycin, an antibiotic of quite different structure, increases the permeability of the mitochondrial inner membrane to Mg^{2+} in a similar manner[24].

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