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EFFECTS OF PHOTODYNAMIC ACTION ON ENERGY COUPLING OF Ca^{2+} UPTAKE IN LIVER MITOCHONDRIA

Christian Salet, Giuliana Moreno and Françoise Vinzens

Laboratoire de Biophysique, INSERM U.201, ERA du C.N.R.S. 951
Muséum National d'Histoire Naturelle, 61, Rue Buffon, 75005 PARIS (France)

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Summary. In mitochondria isolated from rat liver, incubated in the presence of 6×10^{-3} mM hematoporphyrin and irradiated with UV light at 365 nm, respiration, oxidative phosphorylation and Ca^{2+} uptake were measured in order to determine the respective photosensitivity of these functions. Irradiation with increasing doses produces uncoupling of oxidative phosphorylation followed by inhibition of Ca^{2+} uptake and finally arrest of respiration. Ca^{2+} uptake stimulated by the addition of ATP was also studied in mitochondria uncoupled by irradiation which were still able to concentrate Ca^{2+} aerobically. Anaerobic Ca^{2+} uptake driven by ATP hydrolysis was found to be similar in control and in irradiated mitochondria, suggesting a different photosensitivity for the ATPase as compared to the ATP-synthase activity.

Intracellular Ca^{2+} movements play a role of vital importance in cell function (1). As energized mitochondria lower the Ca^{2+} concentration of a suspending medium to a steady state value, it has been first proposed by Drahota *et al.* (2) that mitochondria effect a regulation of the concentration of free Ca^{2+} in the cytoplasm. We have previously indicated that the photodynamic action useful in photochemiotherapy (3) can modify Ca^{2+} transport in isolated mitochondria (4). In the present study we deal with the effects of hematoporphyrin (Hp) photosensitization on the mitochondrial Ca^{2+} uptake supported by either respiration or ATP hydrolysis. For increasing doses of radiation we have measured respiration, oxidative phosphorylation and Ca^{2+} uptake in order to determine the effects on the Ca^{2+} coupling as well as the photosensitivity of the Ca^{2+} carrier itself.

MATERIALS AND METHODS

Mitochondria were isolated from livers of Wistar rats as previously described (5). The final pellet was suspended in 0.25 M sucrose in order to achieve a concentration of 100 mg/ml of protein, measured according to the method of Lowry *et al.* (6). Irradiation of mitochondria was carried out in a thermostated (30°C) water-jacketed glass vessel containing 4 ml of respiratory medium (24 mM glycylglycine, 10 mM $MgCl_2$, 60 mM KCl, 7 mM KH_2PO_4 and 87 mM sucrose) which was stirred magnetically. $CaCl_2$ was added in order to get

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10^{-2} mM Ca^{2+} concentration in the respiratory medium at the beginning of each experiment. Other reagents such as Hp were eventually added as indicated below. Hematoporphyrin dihydrochloride (Mann Research Laboratories), with a purity better than 92 % as shown by chromatographic analysis on silicagel, was used without further purification. All the other chemicals were the finest available grade. Solutions were made in water doubly distilled in quartz. About 1 mg of mitochondria proteins per ml of respiratory medium containing 14 mM succinate + 5 µM rotenone or 14 mM glutamate as substrate was used for each experiment.

Oxygen consumption and Ca^{2+} movement were respectively followed using a Clark type electrode (Yellow Springs Instrument) and a Ca^{2+}-selective electrode (Radiometer F 2110 calcium selectrode with a calomel reference electrode radiometer K 401, connected to a Radiometer type PHM 64 pH-meter), inserted into the reaction chamber through three independent openings. The Clark electrode and the pH-meter were connected to a dual-channel recorder. The Ca^{2+}-selective electrode was calibrated according to the method described by Nicholls (7) using different concentrations of $CaCl_2$ in buffer containing 75 mM KCl, 5 mM potassium nitrilotriacetate and 50 mM Hepes.

Irradiation source was a Philips HPW 125 W lamp emitting at the wavelength of 365 nm. The power density at the level of the vessel was 5 mW/cm^2 as measured by a Black-Ray ultraviolet meter (J 221).

RESULTS

In all the experiments, the respiratory medium was supplemented with 6×10^{-3} mM Hp giving an optical density of 0.45/cm at 365 nm. Control experiments were carried out without irradiation. After placing mitochondria into the reaction chamber with glutamate as substrate, the level of Ca^{2+} decreases to reach a plateau at a concentration of 1 µM (Fig. 1) which suggests the absence of dark effects in the presence of Hp. The Ca^{2+} movement under irradiation is also represented in Fig. 1. Instead of reaching a steady state level, the Ca^{2+} movement reverses after 2 minutes of irradiation, *i.e.* a dose of 6×10^3 J/m^2, to return to the Ca^{2+} original concentration in a few minutes.

Fig. 2 shows the effects of various durations of irradiation on respiration and oxidative phosphorylation. In state IV (*i.e.* without ADP), respiration decreases with the dose of radiation in the presence of succinate + rotenone, while it is stimulated before decreasing in the presence of glutamate. In both cases, oxidative phosphorylation as measured by the respiratory control ratio (RCR), *i.e.* the rate of consumption of oxygen in state III (addition of ADP) divided by the rate in state IV, is inhibited after 1 min of irradiation. Shown also in Fig. 2 the duration of irradiation which reverses the Ca^{2+} movement.

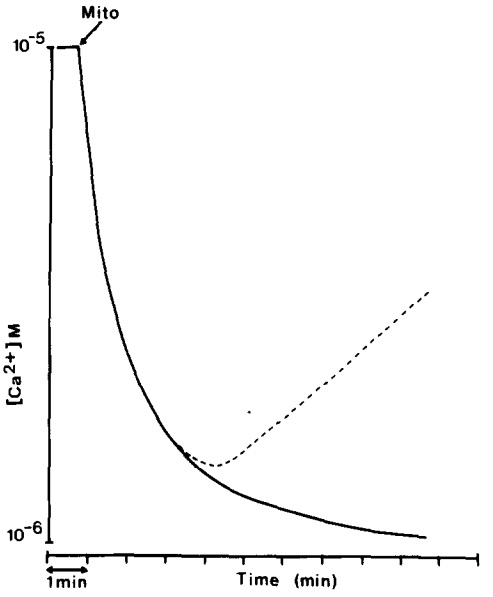

Figure 1 : Ca^{2+} uptake in control (——) and irradiated (---) mitochondria in the presence of 6 x 10^{-3} mM hematoporphyrin. The reaction medium contained 24 mM glycylglycine, 10 mM $MgCl_2$, 60 mM KCl, 7 mM KH_2PO_4, 87 mM sucrose and initially 10^{-2} mM Ca^{2+}. Rat liver mitochondria (1 mg/ml) were added to 4 ml of medium maintained at 30° C and stirred magnetically. 14 mM glutamate was used as substrate.

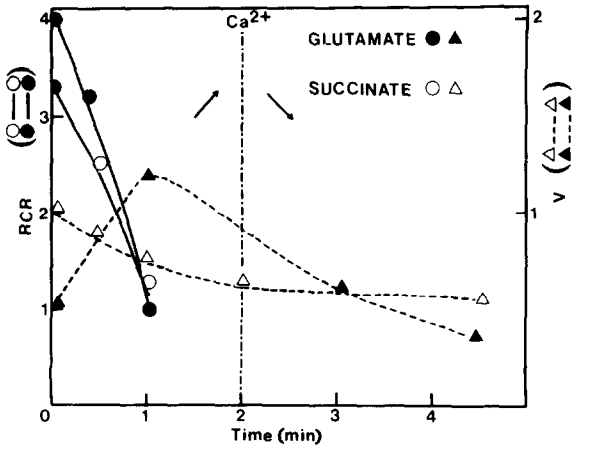

Figure 2 : Ratio V of O_2 consumption (---), *i.e.* the rate of O_2 consumption after irradiation over the rate of consumption before irradiation of mitochondria in state IV (without ADP) versus the duration of irradiation in minutes. Respiratory control ratio (RCR) (——), *i.e.* the rate of consumption of O_2 in state III (in the presence of ADP) divided by the rate of consumption in state IV is also presented. Experimental conditions as in Fig. 1 with 14 mM succinate + 5 µM rotenone (open symbols) or 14 mM glutamate (closed symbols) as substrates. The duration of irradiation which reverses the Ca^{2+} movement is also indicated (·-·).

In another set of experiments, respiration has been stimulated with addition of $CaCl_2$ instead of ADP, as previously shown by Chance (8) or Rossi and Lehninger (9). After 1 minute of irradiation this stimulation also disappears (data not shown), the mitochondria being energized by succinate or glutamate.

Other experiments have been performed to see whether or not uncoupled mitochondria, which are yet able to concentrate Ca^{2+} aerobically, could concentrate Ca^{2+} by ATP hydrolysis in the absence of oxygen. In mitochondia loaded with 40 nM Ca^{2+}/mg of protein, release of Ca^{2+} is observed when oxygen is exhausted in the respiratory chamber (Fig.3). At this stage the addition of ATP induces a similar Ca^{2+} uptake in unirradiated and in 90 s irradiated mitochondria.

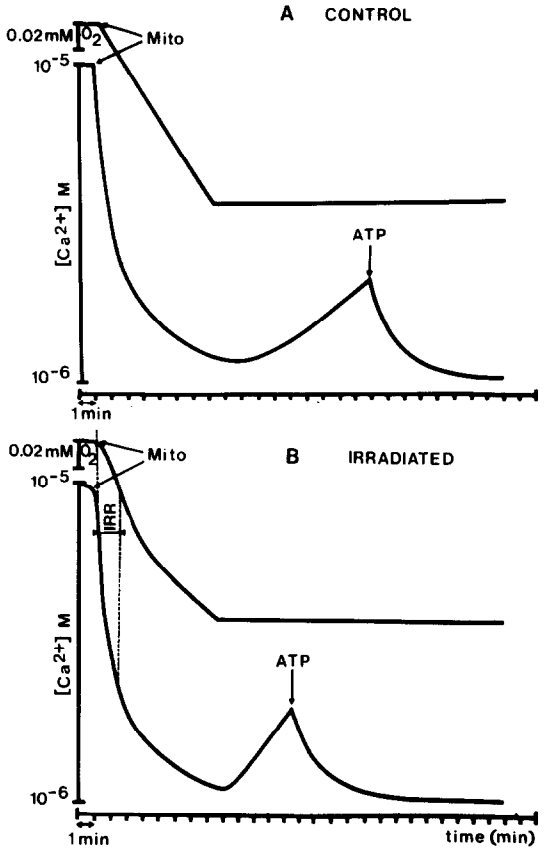

Figure 3 : Ca^{2+} and O_2 simultaneous movements in control (A) and in 90 s irradiated (B) mitochondria. Experimental conditions as in Fig. 1 with succinate as substrate. Release of Ca^{2+} is observed when O_2 is exhausted in the vessel and addition of 0.4 mM ATP (arrow) produces a new Ca^{2+} uptake in both cases.

DISCUSSION

Photodamages induced by porphyrins result chiefly from reactions with singlet oxygen generated by energy transfer from the porphyrin first triplet excited state (10). At the cellular level, the main target of these reactions which can lead to cell death is not well defined. But it seems that the photodamage location is where the photosensitizer can concentrate. For instance protoporphyrin is more toxic to lipophilic structures than a less hydrophobic derivative such as uroporphyrin (11). In this context, the recent finding of Berns *et al.* (12) who observed by microspectrofluorimetry that HPD, a derivative used in the treatment of cancer, concentrates in mitochondria of single cells, makes particularly relevant a detailed study of the photodynamic modifications produced at the level of these organelles. In the presence of oxygen several photosensitizers, have been shown to impair oxidative phosphorylation and respiration when isolated mitochondria are irradiated (13). Effects on Ca^{2+} transport in similar conditions are unknown. In the present study, the effects on mitochondrial functions with special emphasis on Ca^{2+} uptake are described after Hp photosensitization. Ca^{2+} uptake through a channel or a carrier of the inner mitochondrial membrane is directly dependent upon the proton electrochemical potential (for a general review see (14)). Our findings illustrate that uncoupled mitochondria are still able to concentrate Ca^{2+} by a respiration driven process ; in a first step, irradiation acts similar to oligomycin, the well-known inhibitor of phosphorylation. But as the duration of irradiation increases, the Ca^{2+} uptake is inhibited in turn while the respiration continues. This fact suggests that the photosensitivity of the Ca^{2+} carrier lies between those of the proton translocating ATPase and the electron transfer chain.

Moreover our experiments on Ca^{2+} uptake driven by ATP pyrophosphorolysis suggest a different susceptibility for the ATPase and the ATP-synthase activity in the $F_1 - F_0$ complex. It has been reported by Hackney (15) that the β subunit of the soluble component F_1 is rapidly modified by photodynamic action in the presence of bilirubin as a photosensi-

tizer. The apparent discrepancy between the modification of this hydrolytic site as shown by electrophoresis and the present results, where the ATPase activity is lost after the ATP-synthase activity, suggests the existence of other photodamage(s) particularly at the level of the membrane factor which modulates the catalytic and physical properties of F_1. But it is also likely that Hp and bilirubin have different affinities for the various parts of the inner mitochondrial membrane. In that case, formation of singlet oxygen in various sites could produce different damages.

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