Photodynamic action of porphyrin on Ca^{2+} influx in endoplasmic reticulum: a comparison with mitochondria

Fernanda RICCHELLI^{*1}, Paola BARBATO^{*}, Monia MILANI^{*}, Silvano GOBBO^{*}, Christian SALET[†] and Giuliana MORENO[†]

^{*}Consiglio Nazionale delle Ricerche, Centro Metalloproteine, Dipartimento di Biologia, Università di Padova, Viale G. Colombo 3, 35131 Padova, Italy, and [†]Laboratoires de Biophysique et de Photobiologie, INSERM U 201 et CNRS URA 481, Muséum National d'Histoire Naturelle, 43 Rue Cuvier, 75231 Paris Cedex 05, France

We have studied the distribution properties of haematoporphyrin (HP) and protoporphyrin (PP) in mitochondria and endoplasmic reticulum after isolation from rat liver. The photosensitizing efficiency of porphyrin on the Ca^{2+} influx function of microsomes has been compared with that obtained on Ca^{2+} uptake in mitochondria. HP and PP are accumulated in microsomes to a greater extent than in mitochondria, both porphyrins binding to membrane protein sites. The Ca^{2+} influx functions of mitochondria and microsomes, before and after irradiation in the presence of HP or PP, were studied by following the changes in the free Ca^{2+} concentration in the medium as revealed by the variations in fluorescence intensity of the Ca^{2+} indicator Calcium

Green-1. For the same amount of incorporated porphyrin, the Ca^{2+} influx function of microsomes is degraded by irradiation more rapidly than that of mitochondria. The protective effect of dithiothreitol suggests that thiol groups in the Ca^{2+} -transporting enzyme are the preferential targets of the photodynamic effect. These results suggest that intracellular Ca^{2+} movements are altered primarily by the endoplasmic reticulum rather than by mitochondrial damage, in good agreement with other observations made in porphyrin-loaded irradiated cells.

Key words: Calcium Green-1, haematoporphyrin, microsomes, protoporphyrin, photosensitizing.

INTRODUCTION

It is well known that free Ca^{2+} ions modulate a wide variety of cellular functions. For this purpose, the cytosolic free Ca^{2+} concentration is buffered to approx. 100 nM, mainly by the combined action of membrane-bound Ca^{2+} transport systems of the plasma membrane, mitochondria and endoplasmic reticulum. Even slight alterations in the intracellular homeostasis of Ca^{2+} can induce irreversible damage and possibly cell death. Much attention has therefore been devoted to the disturbance of Ca^{2+} fluxes induced by a variety of chemicals or pro-oxidant agents [1–3]. Loss of Ca^{2+} homeostasis as an early event associated with oxidative stress induced by reactive oxygen metabolites and oxy radicals has been widely studied [3,4].

There has also been increasing interest in the damage to Ca^{2+} transport function induced by singlet oxygen ($^1\text{O}_2$), a very reactive oxidant species produced by irradiation in the presence of porphyrins or analogue molecules [5–14]. These sensitizers are currently used in the photodynamic therapy of solid tumours [15,16]. Photodynamic therapy causes repairable injury as well as irreversible damage leading to cell death (apoptosis and necrosis). Understanding the causes of the photodynamic damage is important in controlling and improving the photodynamic therapy process.

Published results show that, after cell irradiation in the presence of porphyrins, an increase in free cytosolic Ca^{2+} is observed. This increase can be transient or irreversible, depending on the duration of incubation of the cells with the sensitizer [10–12]. In the former the observed phenomenon seems to be correlated with cell repair processes of the photodamage induced by $^1\text{O}_2$. In the latter it could be a step leading to cell death. It is supposed that the transient increase in cytosolic Ca^{2+} is due to extracellular ion

influx, whereas the irreversible accumulation of Ca^{2+} is due to delivery from subcellular organelles. In the latter the intracellular Ca^{2+} movements are first impeded at the level of the endoplasmic reticulum with negligible alteration to mitochondrial Ca^{2+} uptake [12]. Mitochondrial disturbance is first revealed by an impairment of the ADP-stimulated respiration and a decrease in the intracellular ATP level. The higher sensitivity to photodamage observed for endoplasmic reticulum in the cells might be explained by a preferential inactivation of the Ca^{2+} -ATPase, coupled with a shortage of intracellular ATP. To test this hypothesis, studies have been undertaken on mitochondria and endoplasmic reticulum isolated from rat liver. The effects of irradiation on specific mitochondrial functions, including Ca^{2+} transport, have been studied in detail [5,6], whereas the alterations induced in the Ca^{2+} transport systems of endoplasmic reticulum are still unknown.

MATERIALS AND METHODS

Chemicals

Haematoporphyrin IX (HP) and protoporphyrin IX (PP) (respectively 85 % and 95 % pure by HPLC analysis) were purchased from Porphyrin Products (Logan, UT, U.S.A.). Stock solutions (1 mM) of both porphyrins were prepared in ethanol. Rotenone, oligomycin, antimycin A, thapsigargin, ADP and ATP were obtained from Sigma (St. Louis, MO, U.S.A.). The fluorescent Ca^{2+} indicator Calcium Green-1 and the Ca^{2+} calibration buffer kits were purchased from Molecular Probes (Eugene, OR, U.S.A.). All other reagents were the purest commercially available products.

Abbreviations used: HP, haematoporphyrin IX; $^1\text{O}_2$, singlet oxygen; PP, protoporphyrin IX; Φ_F , fluorescence quantum yield.

¹ To whom correspondence should be addressed (e-mail rchielli@civ.bio.unipd.it).

Spectroscopic studies

Absorption measurements were performed with a Hewlett Packard 8452A spectrophotometer. For mitochondrial and microsomal suspensions the contribution from light scattering was corrected by using subcellular organelles without porphyrin as a reference. The porphyrin concentrations were calculated at the maxima of the Soret bands (400 or 406 nm) by using the following molar absorption coefficients (ϵ): 140000 M⁻¹·cm⁻¹ for HP, and 120000 M⁻¹·cm⁻¹ for PP in ethanol [17]. The absorption spectrum of the Ca²⁺ indicator Calcium Green-1 was monitored in the range 350–600 nm (absorption maximum at 506 nm; ϵ 90000 M⁻¹·cm⁻¹).

Steady-state fluorescence measurements were performed with a Perkin-Elmer LS 50B spectrofluorimeter. Fluorescence emission spectra of HP (emission λ_{max} 626 nm) and PP (emission λ_{max} 636 nm) were monitored in the range 550–700 nm (excitation λ in the range 400–530 nm). The emission of Calcium Green-1 (maximum of the peak at 530 nm) was excited at 480 nm.

Preparation of mitochondria and microsomes

Mitochondria and microsomes were isolated from the liver of Wistar rats. Mitochondria were prepared by differential centrifugation from a 12% (w/v) homogenate in 0.25 M sucrose/0.1 mM EGTA/10 mM Tris/HCl (pH 7.4) buffer. After sedimentation of the nuclear fraction at 600 *g* for 10 min at 4 °C, mitochondria were sedimented from the supernatant by centrifugation at 6800 *g* for 20 min. The fluffy layer was carefully discarded and the pellet, resuspended in the initial buffer, was centrifuged at 12100 *g* for 5 min. The final mitochondrial pellet was diluted in the sucrose-containing buffer to give a concentration of 80–100 mg/ml mitochondrial proteins, measured by the method of Waddell and Hill [18].

Liver microsomes were prepared by centrifugation of the post-mitochondrial supernatant fraction at 100000 *g* for 50 min. The pellet was washed three times with the initial buffer. For the last washing, buffer without EGTA was used. The final suspension contained a protein concentration of approx. 40–50 mg/ml.

Studies of HP and PP uptake by mitochondria and microsomes

Isolated mitochondria (0.5 mg/ml) or microsomes (1 mg/ml) were suspended in 2 ml of a medium containing 125 mM KCl, 4 mM MgCl₂, 2 mM KH₂PO₄ and 25 mM Hepes, pH 7.2 (test medium [19]), and incubated for 2 min with HP and PP at variable concentrations in the range 2–14 nmol of porphyrin added per mg of protein. The mitochondrial samples were centrifuged at 11300 *g* for 2 min; the dye concentration was determined spectrophotometrically both on the supernatant and the pellet after extraction with 2% (w/v) SDS [17]. For microsomes, after incubation the various porphyrin-containing suspensions (1 mg/ml) were mixed with 2 ml of 20% (w/v) trichloroacetic acid. After 10 min in an ice bath, the samples were centrifuged for 10 min at 11600 *g*. The dye concentration (in nmol/mg of microsomal protein) was determined both in the supernatant, from the absorption spectrum of the dicationic form of the porphyrin ($\epsilon_{406}^{\text{HP}}$ 316000 M⁻¹·cm⁻¹; $\epsilon_{412}^{\text{PP}}$ 139000 M⁻¹·cm⁻¹) and in the pellet after extraction for 24 h with ethanol.

Irradiation of microsomes and mitochondria in the presence of porphyrin

Mitochondrial (0.5 mg/ml) or microsomal (1 mg/ml) fractions suspended in the test medium were incubated for 2 min in the dark with the desired quantities of porphyrin. Succinate (5 mM),

as energizing substrate, and rotenone (4 μ M) were added to the test medium in the mitochondrial preparations. Irradiation was performed on 1.4 ml of magnetically stirred suspensions with a 125 W Philips HPW lamp (almost monochromatic light emission at 365 nm) at a fluence rate of 33 W/m² in a thermostatically controlled glass reaction vessel (30 °C).

Fluorimetric determination of Ca²⁺ influx in microsomes and mitochondria

The photodynamic damage to the Ca²⁺ fluxes in the two organelles was followed by determining the changes in the free Ca²⁺ concentration in the medium by means of the fluorescent Ca²⁺ indicator Calcium Green-1, which is cell impermeant. On binding to Ca²⁺ this indicator exhibits an increase in fluorescence emission intensity with a negligible shift in wavelength. Ca²⁺ calibration buffer kits, provided by Molecular Probes, were used to obtain calibration curves and the values of the dissociation constant (K_d) of the Ca²⁺ indicator at the chosen temperature, ionic strength and pH. The kits consisted of Ca²⁺/EGTA solutions of precisely known free Ca²⁺ concentrations in the range 0–39.8 μ M. At the highest Ca²⁺ concentration the indicator is fully saturated.

Determinations of free Ca²⁺ were performed in 2 ml of mitochondrial (0.5 mg/ml) or microsomal (1 mg/ml) suspensions in the test medium to which 0.5 μ M Calcium Green-1 was added, in the absence (control) and in the presence of porphyrin before irradiation (dark control) and after irradiation, at 30 °C. For microsomes, ATP (3 mM) was added as the energy source for the accumulation of Ca²⁺. The respiratory inhibitor antimycin A and the mitochondrial ATPase inhibitor oligomycin were also present in the medium to prevent Ca²⁺ uptake by mitochondria that might have been present as contaminants in the microsomal fraction.

The variations in fluorescence of the Ca²⁺ indicator were correlated with the free Ca²⁺ concentrations in the medium, as described in [20].

RESULTS

Distribution properties of HP and PP in mitochondria and microsomes

HP and PP reach their maximal accumulation into microsomes in less than 60 s (results not shown). However, PP needs a further 3 h for complete monomerization (results not shown). It is well known that porphyrin monomeric species are required for efficient photosensitization [21].

The effect of increasing porphyrin concentrations (in the range 2–14 nmol/mg) on the HP and PP uptake by mitochondria and microsomes was studied after incubation for 2 min. As shown in Figure 1, PP was taken up by both organelles in larger quantities than HP. Moreover, microsomes accumulated both porphyrins to a greater extent than did mitochondria.

Experiments were conducted to discriminate between protein sites and lipid domains as the final dye microenvironment in microsomes. Analogously to previous reports on the distribution properties of HP and PP in mitochondria [22], the nature of dye localization in microsomes was studied by following the changes in the dye fluorescence quantum yield (Φ_F) in the temperature range 10–35 °C. The values of Φ_F for HP and PP were calculated relative to that of rhodamine B in ethanol ($\Phi_F = 0.98$ at 20 °C). The results obtained were arranged to give Arrhenius plots (Figure 2) in accordance with the method of Kirby and Steiner [23]:

$$\ln(\Phi_F^{-1} - 1) = \ln f_i/k_F - (E_a/RT)$$

Figure 1 Dependence of porphyrin uptake by microsomes and mitochondria on the dye concentration

Isolated microsomes (1 mg/ml) ($\square$) and mitochondria (0.5 mg/ml) ($\bullet$) were suspended in a medium containing 125 mM KCl, 4 mM MgCl_2 , 2 mM KH_2PO_4 and 25 mM Hepes, pH 7.2, and incubated with different concentrations (in the range 2–14 nmol/mg) of HP (upper panel) or PP (lower panel). The incubation time was 2 min. The dye uptake was determined as described in the Materials and methods section.

Figure 2 Arrhenius plots of the Φ_F of porphyrins in microsomes

The values of Φ_F of HP ($\blacksquare$) and PP ($\square$) at various temperatures were calculated from the integrated emission spectra on excitation at 510 nm and referred to that (0.13) at 20 °C. Each experimental point is the average from three experiments performed at variable concentrations of porphyrin in the range 2–10 nmol/mg and dissolved in the test medium. From the slopes of the plots, E_a values of 0.04 $\text{kJ}\cdot\text{mol}^{-1}$ for HP and 0.05 $\text{kJ}\cdot\text{mol}^{-1}$ for PP were calculated.

where k_p is the first-order rate constant for the direct emission of fluorescent radiation by the excited state, E_a and f_i are the activation energy and the frequency factor for the various radiationless deactivation processes of the excited state, R is the gas constant (in $\text{kJ}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$) and T is the absolute temperature. Very low values, 0.04 kJ/mol for HP and 0.05 kJ/mol for PP, were obtained from the slopes of the Arrhenius plots (Figure 2).

Irradiation effects on the Ca^{2+} influx in microsomes

The experiment shown in Figure 3 describes the changes in the fluorescence intensity of the Ca^{2+} indicator Calcium Green-1 at 530 nm (excitation at 480 nm) in a suspension of microsomes (1 mg of protein/ml) in the test medium supplemented with mitochondrial inhibitors and the corresponding variations in free Ca^{2+} calculated as described in the Materials and methods section. To convert the fluorescence intensities to Ca^{2+} concentrations a K_d of 0.8 μM was used, as calculated from the calibration curves (results not shown).

Because the maximal capacity of microsomes to accumulate Ca^{2+} is very low, less than 10 ng-ions/mg of protein [19], the experiments were carefully planned to minimize the external Ca^{2+} contaminants. The addition of ATP to the medium, which has an initial free Ca^{2+} concentration of approx. 0.4 μM , activated the Ca^{2+} -ATPase pump. Consequently, a rapid decrease in the free Ca^{2+} concentration was produced up to a steady-state level of approx. 0.2 μM , at which microsomes generally buffered the free Ca^{2+} in the medium (Figure 3, trace a). The involvement of the Ca^{2+} -ATPase pump in the observed Ca^{2+} accumulation was proved by the effect of thapsigargin, a specific inhibitor of Ca^{2+} -ATPases from the endoplasmic reticulum of animals [24]: the ATP-driven accumulation of Ca^{2+} into liver microsomes was completely blocked by pretreatment with 2 μM thapsigargin (Figure 3, trace b). Moreover, the addition of 4 μM thapsigargin to Ca^{2+} -loaded microsomal vesicles led to an immediate discharge of the sequestered Ca^{2+} (Figure 3, trace c). The absence of Ca^{2+} uptake before the addition of ATP clearly indicates that mitochondria, possibly present as contaminants in the microsomal fraction, were fully inactivated by antimycin and oligomycin. In the absence of porphyrin and irradiation (control), the velocity (v) of the Ca^{2+} uptake process, calculated from the initial slope of trace a in Figure 3, was approx. 0.12 nmol of Ca^{2+} /min per mg.

The experiments of Ca^{2+} uptake were repeated for porphyrin-loaded microsomes before (dark control) and after irradiation. Figure 4 shows results with microsomes after interaction with 6 nmol of HP/mg (i.e. 1.92 nmol of HP taken up/mg; see Figure 1) and 45 s irradiation at a fluence rate of 33 W/m^2 . As can be easily calculated from the initial slope of trace a in Figure 4, in the absence of irradiation porphyrin alone caused a relevant effect on v , which decreased from the initial value of the control (approx. 0.12 nmol of Ca^{2+} /min per mg) to approx. 0.07 nmol of Ca^{2+} /min per mg (porphyrin dark effect). Irradiation further affected both the velocity and the maximal capacity of Ca^{2+} accumulation (Figure 4, trace b). Protection against irradiation effects was observed in the presence of dithiothreitol (Figure 4, trace c).

The porphyrin dark effect on the initial rate of Ca^{2+} accumulation by microsomes was studied in detail for different amounts of added dye, and a comparison between HP and PP was performed. The concentration ranges of added dye were 1.7–7.2 nmol/mg (for HP) and 0.5–2.7 nmol/mg (for PP), which correspond to a range of microsome-bound dye of 0.4–2.3 nmol/mg for both porphyrins (see Figure 1). As shown in Figure 5, for the same amount of incorporated porphyrin, there was a larger inhibition of v by PP.

Figure 3 Microsome-mediated regulation of the free Ca^{2+} concentration in the medium

Microsomes (1 mg/ml) were suspended in the test medium to which rotenone (4 μM), antimycin A (0.2 μM) and oligomycin (12.6 μM) were added, and the Ca^{2+} pump was activated by the addition of 3 mM ATP (trace a). In trace b, 2 μM thapsigargin (Tg) was present in the medium before the addition of ATP. In trace c, 4 μM thapsigargin was added after the accumulation of Ca^{2+} by microsomes. The concentrations of free Ca^{2+} in the medium were calculated from the changes in the fluorescence intensity at 530 nm (excitation at 480 nm) of Calcium Green-1 (0.5 μM) by using a value of K_d of 0.8 μM as the dissociation constant of the Ca^{2+} /Ca $^{2+}$ -indicator complex, as described in the Materials and methods section.

Figure 4 Microsome-mediated regulation of the free Ca^{2+} concentration in the medium after irradiation in the presence of HP

The experimental conditions were as described in the legend to Figure 3 (trace a) except that 6 nmol/mg HP was present in the test medium. Trace a, sample not irradiated (dark control); trace b, sample irradiated for 45 s before the addition of ATP; trace c, sample supplemented with 1 mM dithiothreitol and irradiated for 45 s before the addition of ATP. Irradiation was performed at 30 °C with a 125 W Philips HPW lamp at a fluence rate of 33 W/m².

Figure 6 shows the dependence of the Ca^{2+} uptake velocity on the duration of irradiation for three chosen concentrations of microsome-bound HP (0.64, 1.28 and 1.92 nmol/mg). The experimental values of v were normalized to that of the control (in the absence of porphyrin).

The photosensitizing efficiency of PP, measured after 3 h of incubation with the endoplasmic reticulum to obtain complete dye monomerization, was apparently higher than that of HP (results not shown). This can be explained simply on the basis of the higher dark effect (see Figure 5). In fact, HP and PP are

Figure 5 Changes in the Ca^{2+} influx function of microsomes as a function of HP and PP concentration in the absence of irradiation (dark effect)

The Ca^{2+} influx function is shown as the velocity (v) of the Ca^{2+} accumulation by microsomes, measured from the initial slope of the accumulation curves. All v values were normalized to that of the control (in the absence of porphyrin). The experimental conditions were as reported in the legend to Figure 3.

distributed in similar microenvironments in microsomes (the present paper) and produce $^1\text{O}_2$ with equal yields [17].

Comparison between the irradiation effects on the Ca^{2+} influx in microsomes and mitochondria

Figure 7 shows the regulation of the free Ca^{2+} concentration in the medium, which is mediated by mitochondrial transport systems, followed by the changes in fluorescence of Calcium Green-1. The addition of mitochondria to the test medium

Figure 6 Changes in the Ca^{2+} influx function of HP-loaded microsomes as a function of the irradiation duration

The Ca^{2+} influx function is shown as the velocity (v) of the Ca^{2+} accumulation by microsomes, measured as reported in the legend to Figure 5, for 0.64 (●), 1.28 (▲) and 1.92 (■) nmol of HP taken up/mg of protein. Irradiation was performed at 30 °C with a 125 W Philips HPW lamp at a fluence rate of 33 W/m².

Figure 7 Mitochondria-mediated regulation of the free Ca^{2+} concentration in the medium

Mitochondria (0.5 of mg protein/ml) were added to the test medium supplemented with 5 mM succinate as energizing substrate plus 4 μM rotenone. Changes in the free Ca^{2+} concentration in the medium were followed as changes in the fluorescence intensity of the Ca^{2+} indicator Calcium Green-1, as described in the legend to Figure 3. Where indicated (arrows), CaCl_2 (20 μM) or EGTA (0.1 mM) were added.

containing a high initial Ca^{2+} concentration (more than 40 μM , at which concentration the Ca^{2+} indicator is fully saturated) produced a very sharp decrease in the free ion until a steady-state level of approx. 0.5 μM was reached. The addition of pulses of Ca^{2+} (20 μM) gave rise to rapid increases in the free Ca^{2+} concentration in the medium, followed by a return to the original steady-state concentration; this is typical of the buffering capacity of mitochondria. A similar but opposite response was provoked by the addition of the Ca^{2+} chelator EGTA at 0.1 mM. In this case, there was net release of Ca^{2+} from the mitochondria into the medium. This test was used to check the integrity of the Ca^{2+} transport system of our mitochondrial preparations. The Ca^{2+} uptake process was measured as the initial rate (v) of

Figure 8 Comparison between the irradiation effects on the Ca^{2+} influx in HP-loaded microsomes and mitochondria

The Ca^{2+} influx function is expressed as value of v , the initial velocity of the Ca^{2+} uptake process, in the different experimental conditions, normalized to that of the control (without porphyrin). The values of v , after 45 s of irradiation of mitochondria (●) or microsomes (○) loaded with different amounts of HP, were calculated as described in the legend to Figure 5 for microsomes, and from the initial slope of the uptake curve after a pulse of 20 μM CaCl_2 for mitochondria. The fluence rate was 33 W/m².

the Ca^{2+} influx when mitochondria were supplemented with 20 μM CaCl_2 (control). This rate was affected only marginally by the addition of different HP concentrations (in the range 0.4–2.5 nmol/mg of incorporated dye) in the dark (dark control). After irradiation under the same experimental conditions as those described for microsomes, a decrease in the Ca^{2+} influx rate and in the capacity of buffering extramitochondrial Ca^{2+} could be observed only for long durations of irradiation and/or high porphyrin concentrations (results not shown). The values of v calculated after 45 s of irradiation of mitochondria loaded with different amounts of HP, normalized to that of the control, are shown in Figure 8 together with the corresponding values for microsomes. For the same amount of incorporated porphyrin, the Ca^{2+} influx function of microsomes was more rapidly degraded than that of mitochondria.

DISCUSSION

Most of the porphyrins that have been tested for their photo-oxidizing effects on cells, including HP and PP, preferentially associate with cell membranes. Cytotoxicity *in vitro* resulting from porphyrin photosensitization has been well documented and a number of subcellular sites have been implicated as sites of damage, including plasma membrane, nucleus, mitochondria and lysosomes (reviewed in [15,25,26]). Many observations from published results suggest that, in the cytoplasm, mitochondria are the major target of photodynamic attack. Inside the membrane, the primary site of action of the sensitizers is highly restricted to the regions very close to their localization site, owing to the high probability of the quenching of $^1\text{O}_2$ during its lifetime (the diffusion path of $^1\text{O}_2$ is less than 100 nm in biological membranes [27]). Therefore studies of the distribution properties of photosensitizers in the cells and in the subcellular organelles can provide valuable information about the identity of the primary targets in photosensitization processes. The nature of the target affects the final response of the cell to the photodamage.

Anionic porphyrins are less selectively distributed in the cell membrane compared with cationic molecules such as Victoria Blue BO [28], which induces photodamage that is strictly localized to mitochondria. With porphyrins, other membrane systems might be altered: we have previously reported that in cells in culture after a long incubation with Photofrin, the endoplasmic reticulum, as well as mitochondria, is a major target of photodynamic effect [12]. In particular we observed a higher sensitivity to porphyrin photosensitization of the Ca^{2+} transport function of endoplasmic reticulum than that of mitochondria. In the present study we were interested in comparing the distribution properties of the main components of Photofrin, HP and PP, and their photosensitizing efficiencies in isolated microsomes and mitochondria to explain the findings observed in cells in culture.

The results obtained in this paper show a higher affinity of HP and PP for microsomes compared with mitochondria, the increases in uptake being 30 % and 70 % respectively (Figure 1). The comparable values of activation energies (0.04–0.05 kJ/mol) calculated from the Arrhenius plots suggest a similar accommodation of both porphyrins into the microsomal membrane. Previous studies on model systems indicate that these values are typical of protein sites, whereas porphyrins in lipid domains exhibit E_a values in the range 4–10 kJ/mol [29]. When the dye is supplied to microsomes after dispersion in lipid vesicles, the porphyrin localization does not change (results not shown). The occurrence of a dark effect and the dependence of the inhibition of Ca^{2+} uptake on the porphyrin dose, which shows a saturation effect (Figure 5), would be indicative of binding of the dye to Ca^{2+} -ATPase sites, which are critical for the pumping of Ca^{2+} . The binding of porphyrin to other microsomal proteins cannot, however, be excluded. In fact, a rapid degradation of cytochrome *P*-450 and acyl-coenzyme A:cholesterol-O-acyltransferase has been shown to occur after cell photosensitization in the presence of porphyrins [30–32].

The porphyrin distribution properties in microsomes are different from those previously observed in mitochondria. Indeed, various localization patterns were obtained in mitochondria depending on the hydrophobicity of the dye, the duration of incubation and the nature of the dye carrier (namely liposomes). In particular, protein sites or lipid domains of the mitochondrial membranes could be discriminated [17,22].

The Ca^{2+} uptake ability in microsomes is rapidly degraded after irradiation in the presence of even small amounts of porphyrin (see Figure 6). The dark effect has an important role in the observed degradation of Ca^{2+} influx function. The targets of photodynamic action in the Ca^{2+} -ATPase are probably thiol groups, as demonstrated by the protective effect of dithiothreitol (Figure 4). Other $^1\text{O}_2$ -producing photoactivators such as fluorescein, eosin, erythrosin and phthalocyanines were also shown to inactivate the Ca^{2+} -transporting protein of sarcoplasmic reticulum as a result of the oxidation of sensitive thiol groups [33,34]. With rose bengal, however, histidine residues were the preferential targets [35].

We checked the intrinsic sensitivity to photodamage of the Ca^{2+} uptake systems of microsomes and mitochondria by measuring the changes in Ca^{2+} influx induced by the same amount of incorporated HP. As shown in Figure 8, the mitochondrial Ca^{2+} influx system is still almost intact at HP concentrations that give a 60 % loss of Ca^{2+} accumulation capability in microsomes. It must be pointed out that the Ca^{2+} uptake process in endoplasmic reticulum is modulated exclusively by the Ca^{2+} -ATPase pump, whereas in mitochondria it is an electrogenic process driven by the electrochemical gradient across the inner mitochondrial membrane, a passive uniporter facilitating Ca^{2+} diffusion [36]. Inhibition of Ca^{2+} uptake is obtained after the

collapse of the electric field built up by respiratory chain proton pumps or ATP hydrolysis. However, it has been reported [6,37] that photodamage of the electron transport chain is not a primary event after photodynamic action. Irradiation with increasing doses of light in the presence of HP produces first an inhibition of oxidative phosphorylation followed by an inhibition of Ca^{2+} uptake and finally an arrest of respiration. These and other studies [22] suggest that HP is distributed between various mitochondrial proteins and that the enzymes involved in ATP synthesis are the first targets of photodynamic action, probably as a consequence of an intrinsic higher photolability or a preferential porphyrin accumulation. As for PP, in mitochondrial membranes this dye is embedded in lipid domains and its photosensitizing efficiency is low compared with that of HP [17].

Thus a combination of a large accumulation of porphyrin and a higher photodegradation of the Ca^{2+} influx should contribute mainly to the preferential targeting of the Ca^{2+} transport system of the endoplasmic reticulum compared with mitochondria observed in Photofrin-loaded irradiated cells [12]. Our experiments on isolated organelles indicate that, in cells, the photo-inactivation of Ca^{2+} transport by endoplasmic reticulum is probably due to the high photolability of the Ca^{2+} pump rather than the loss of intracellular ATP induced by an alteration to the mitochondrial energetic system.

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