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Regulation of the Inner Membrane Mitochondrial Permeability Transition by the Outer Membrane Translocator Protein (Peripheral Benzodiazepine Receptor)*

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Justina Šileikytė[‡1], Valeria Petronilli[‡], Alessandra Zulian[‡], Federica Dabbeni-Sala[§], Giuseppe Tognon[¶], Peter Nikolov[‖], Paolo Bernardi[‡2], and Fernanda Ricchelli[¶3]

From the [‡]Consiglio Nazionale delle Ricerche Institute of Neuroscience and Department of Biomedical Sciences, the [§]Department of Pharmacology and Anesthesiology, and the [¶]Consiglio Nazionale delle Ricerche Institute of Biomedical Technologies and Department of Biology, University of Padova, I-35121 Padova, Italy and the [‖]Institute of Organic Chemistry, Bulgarian Academy of Sciences, 1000 Sofia, Bulgaria

We studied the properties of the permeability transition pore (PTP) in rat liver mitochondria and in mitoplasts retaining inner membrane ultrastructure and energy-linked functions. Like mitochondria, mitoplasts readily underwent a permeability transition following Ca^{2+} uptake in a process that maintained sensitivity to cyclosporin A. On the other hand, major differences between mitochondria and mitoplasts emerged in PTP regulation by ligands of the outer membrane translocator protein of 18 kDa, TSPO, formerly known as the peripheral benzodiazepine receptor. Indeed, (i) in mitoplasts, the PTP could not be activated by photo-oxidation after treatment with dicarboxylic porphyrins endowed with protoporphyrin IX configuration, which bind TSPO in intact mitochondria; and (ii) mitoplasts became resistant to the PTP-inducing effects of *N*,*N*-dihexyl-2-(4-fluorophenyl)indole-3-acetamide and of other selective ligands of TSPO. Thus, the permeability transition is an inner membrane event that is regulated by the outer membrane through specific interactions with TSPO.

The mitochondrial permeability transition (PT)[4] is a sudden increase in the permeability of the inner mitochondrial membrane to solutes with molecular masses of up to 1500 Da. This process is due to opening of a voltage- and Ca^{2+}-dependent, cyclosporin A (CsA)-sensitive, high conductance channel called the permeability transition pore (PTP). Its involvement in pathological states and in the loss of cell viability is widely recognized, but its molecular identity remains elusive (1). The long-standing idea that the PTP may form at inner-outer membrane contact sites and that it may be constituted by the adenine nucleotide translocator (ANT) in the inner mitochondrial membrane (IMM) and the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane (OMM) has not been confirmed by genetic ablation of these proteins (2–4), yet the PT can be regulated by proteins that interact with the OMM such as hexokinase (5, 6) and by ligands of the OMM translocator protein of 18 kDa, TSPO (formerly known as the peripheral benzodiazepine receptor) (7–17). As of today, however, it is not clear whether the OMM is necessary for the PT to occur and what regulatory properties, if any, it may contribute to the PTP.

Among the variety of effectors that regulate the PTP open-closed transitions, oxidizing agents have received considerable attention, and changes in the redox state of pyridine nucleotides, glutathione, and sulfhydryl groups have been shown to play a prominent regulatory role (18–23). We have used chemical modifiers, photosensitizing agents, and light to explore PTP regulation by redox events. As photodamage is restricted to sites in close proximity to the photosensitizer, irradiation offered a unique opportunity to explore the role of specific protein residues in PT regulation (24–26). One remarkable finding was that hematoporphyrin IX (HP), which produces mainly singlet oxygen ($^1O_2$) upon irradiation, results in PT inhibition or activation depending on the light dose (24–26). For low light doses, HP inhibits the PT through photomodification of matrix-exposed His residues (24), followed by a drop in reactivity of critical matrix Cys residues (26). For higher doses, HP instead causes PTP reactivation through modification of distinct surface Cys residues (26). HP belongs to the class of dicarboxylic porphyrins endowed with protoporphyrin IX (PP) configuration. These porphyrins bind mitochondria with nanomolar affinity through TSPO (17, 27–31), and intriguingly, PP is a potent inducer of PTP opening (32). Given the long-standing proposals that the PTP is regulated by the OMM and that it may include TSPO itself (7–9, 11, 32–34), we studied the properties of the PTP in rat liver mitochondria and in digitonin-treated mitoplasts that retain

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[2] To whom correspondence may be addressed: Dept. of Biomedical Sciences, University of Padova, Viale Giuseppe Colombo 3, I-35121 Padova, Italy. Fax: 39-49-827-6361; E-mail: bernardi@bio.unipd.it.

[3] To whom correspondence may be addressed: Consiglio Nazionale delle Ricerche Inst. of Biomedical Technologies and Dept. of Biology, University of Padova, Via Ugo Bassi 58B, I-35121 Padova, Italy. Fax: 39-49-827-6348; E-mail: rchielli@bio.unipd.it.

[4] The abbreviations used are: PT, permeability transition; CsA, cyclosporin A; PTP, permeability transition pore; ANT, adenine nucleotide translocator; IMM, inner mitochondrial membrane; VDAC, voltage-dependent anion channel; OMM, outer mitochondrial membrane; HP, hematoporphyrin IX; PP, protoporphyrin IX; DP, deuteroporphyrin IX; CP, coproporphyrin III; PhAsO, phenylarsine oxide; $Cu(OP)_2$, copper-*o*-phenanthroline; MAO, monoamine oxidase; W, watts.

IMM ultrastructure and energy-linked functions. Our results indicate that mitoplasts undergo a Ca^{2+}-induced and CsA-sensitive PT and that the OMM plays a regulatory role exerted in part at least through TSPO.

EXPERIMENTAL PROCEDURES

Reagents—HP, PP, deuteroporphyrin IX (DP), and coproporphyrin III (CP) were obtained from Frontier Scientific (Logan, UT), and stock solutions were prepared in dimethyl sulfoxide. FGIN1-27 (*N*,*N*-dihexyl-2-(4-fluorophenyl)indole-3-acetamide) and PK11195 (1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)isoquinoline-3-carboxamide) were generous gifts of Prof. Pietro Giusti (Department of Pharmacology and Anesthesiology, University of Padova). Digitonin, phenylarsine oxide (PhAsO), and Ro 5-4864 (4′-chlorodiazepam; 7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one) were from Sigma. Copper-*o*-phenanthroline ($Cu(OP)_2$) was prepared just before use by mixing $CuSO_4$ with *o*-phenanthroline at a molar ratio of 1:2 in bidistilled water. All chemicals were of the highest purity commercially available.

Preparation of Mitochondria—Liver mitochondria from Wistar rats were prepared by standard differential centrifugation. The final pellet was suspended in isolation buffer (250 mM sucrose, 0.5 mM EGTA/Tris, and 10 mM Tris-HCl, pH 7.4) to give a protein concentration of 80–100 mg/ml as measured by the biuret method. The quality of mitochondrial preparations was established as described previously (26).

Preparation of Mitoplasts—The mitochondrial suspension was added to solutions of varying digitonin concentrations in isolation buffer at a final protein concentration of 20 mg/ml. The resulting mixtures were then cooled in an ice-water bath and gently stirred for 20 min. The suspensions were centrifuged at 10,000 × *g* for 5 min, and the resulting pellets were washed twice with isolation buffer and finally resuspended in the same medium. The extent of OMM extraction was assessed based on the activity of monoamine oxidase (MAO) using benzylamine as a substrate (35). Extraction of MAO activity was ~90% after treatment of mitochondria with 0.09–0.12 mg of digitonin/mg of mitochondrial protein (see Fig. 2).

Western Blotting—Proteins were solubilized in Laemmli gel sample buffer, separated by 15% SDS-PAGE, and transferred electrophoretically to nitrocellulose membranes using a Mini Trans-Blot system (Bio-Rad). Western blotting was performed in phosphate-buffered saline containing 3% nonfat dry milk with polyclonal antibodies prepared in rabbits against purified bovine heart F_1-ATPase, anti-GRIM19 monoclonal antibody (Santa Cruz Biotechnology), anti-apoptosis-inducing factor polyclonal antibody (Exalpha Biologicals, Inc.), anti-Bcl-x_L polyclonal antibody (Cell Signaling Technology), and anti-VDAC1 monoclonal antibody (a generous gift of Dr. F. Thinnes). Immunoreactive bands were detected by enhanced chemiluminescence (Millipore).

Permeability Transition and Calcium Retention Capacity—PT was induced at 25 °C in standard medium (250 mM sucrose, 10 mM Tris/MOPS, 5 mM succinate, 1 mM P_i/Tris, 10 μM EGTA/Tris, 0.5 μg/ml oligomycin, and 2 μM rotenone, pH 7.4). Osmotic swelling was followed as the decrease in 90° light scattering at 540 nm with a PerkinElmer Life Sciences LS50 spectrofluorometer (36). The calcium retention capacity of mitochondria and mitoplasts was measured in the same medium supplemented with 0.5 μM calcium green-5N (excitation at 480 nm and emission at 530 nm).

Mitochondrial and Mitoplast Membrane Potential—Changes in membrane potential difference ($\Delta\psi_m$) in mitochondria and mitoplasts were followed based on the accumulation of pyronin G (3 μM) as monitored by the changes in emission fluorescence intensity at $\lambda = 580$ nm (excitation, $\lambda = 520$ nm) (37).

Porphyrin Uptake and Photosensitization of Mitochondria and Mitoplasts—Mitochondria and mitoplasts were suspended in standard medium, and porphyrins were added under gentle stirring at room temperature. After 2 min, the suspensions were centrifuged at 4000 × *g* for 2 min, and porphyrin concentrations were determined fluorometrically by calibration plots after extraction of supernatants and pellets with 2% SDS. Porphyrin-mediated photosensitization of mitochondria and mitoplasts was achieved as described previously (26). Briefly, preparations were incubated for 1–2 min in the dark with the desired concentration of porphyrin and then irradiated at 365 nm in a thermostatted glass reaction vessel with a Philips HPW 125-watt lamp. The fluence rate at the level of the preparations (40 watts (W)/m^2) was measured with a calibrated quantum photo radiometer (Delta OHM HD 9021). All irradiations were performed at 25 °C under magnetic stirring. Proper controls were carried out to verify that neither incubation with the photosensitizer in the dark nor illumination in the absence of porphyrin produced any appreciable changes in the parameters under study. The structures of the porphyrins studied in this work are shown in Fig. 1.

To compare the photoactivity of these different porphyrins on the PTP, it was essential to establish first how much of each was taken up by mitochondria and mitoplasts. Determinations of porphyrin uptake revealed that HP, PP, and DP exhibited higher incorporation yields in mitochondria (~30–40% of the total added) than in mitoplasts (~20–30%) (see Table 1; see also Ref. 38 for HP and PP). The membrane affinity of the highly hydrophilic tetracarboxylic CP was much lower and equal for mitochondria and mitoplasts (incorporation yield of ~10–13%). For each porphyrin, the concentration was adjusted in the range of 0.2–1.5 nmol of porphyrin taken up per mg of protein to obtain comparable amounts of membrane-bound compound, which was measured spectrofluorometrically. The rates of permeabilization were then studied after irradiation times ranging from 10 to 240 s at 40 W/m^2 (total light doses between 0.04 and 0.96 J/cm^2). Under these conditions, (i) porphyrins were bound to mitochondria and mitoplasts as monomers, the only species that is appreciably photoactive (39); (ii) no measurable effects were induced in the dark (data not shown); and (iii) the molar absorption at the irradiation wavelength used (365 nm) was very similar for all porphyrins (difference within 10%), which reflects an equivalent number of absorbed photons (data not shown).

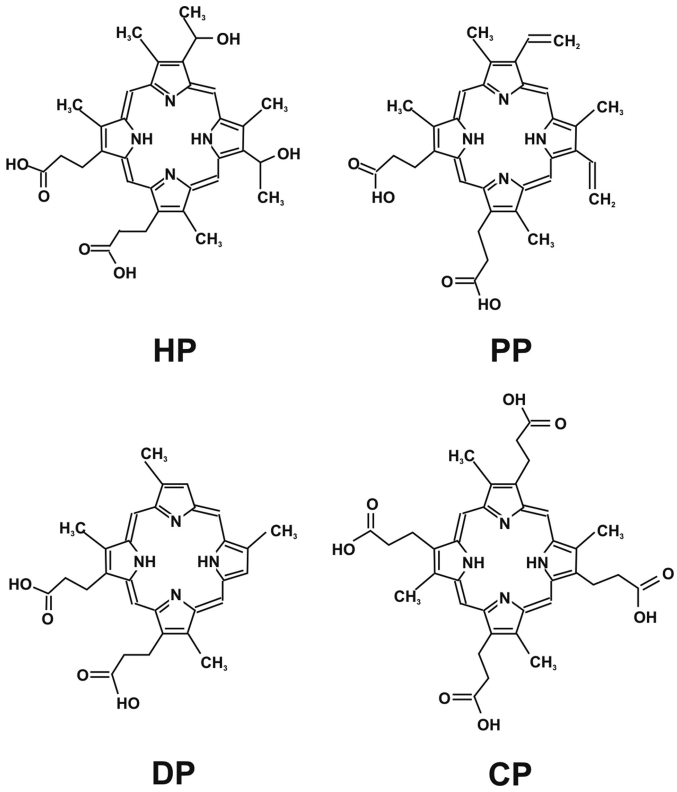

FIGURE 1. **Structure of porphyrins.** The chemical structures of HP, PP, DP, and CP are shown.

Transmission Electron Microscopy—Mitochondria and mitoplasts were fixed for 30 min at 4 °C using glutaraldehyde at a final concentration of 1.5% (v/v) in 0.1 M cacodylate buffer, pH 7.2, and post-fixed with 1% OsO_4. Thin sections (60 – 80 nm) were stained with uranyl acetate in alcohol (50%) and lead citrate. Observations were made with an FEI Tecnai F12 transmission electron microscope.

RESULTS

We carried out a titration of mitochondria with increasing concentrations of digitonin, and we assessed the release of MAO and maintenance of the $\Delta\psi_m$ by the resulting organelle preparation. About 90% of MAO activity was solubilized between 0.09 and 0.12 mg of digitonin/mg of mitochondrial protein, and a lower $\Delta\psi_m$ was detected only with the highest digitonin concentration (Fig. 2*A*).

Ultrastructural observation by transmission electron microscopy showed that treatment with 0.04 mg/mg digitonin caused partial removal of the OMM (Fig. 2*B*, *panel b*, compare with intact mitochondria in *panel a*), whereas with 0.09 mg/mg digitonin, the OMM was effectively removed. The electron-dense matrix was preserved and delimited by a highly convoluted, slightly unwound IMM (Fig. 2*B*, *panel c*). At this concentration of digitonin, characteristic features of mitoplasts were also foldings or finger-like protrusions of the IMM and the absence of cristae and intracristal spaces projecting into the matrix typically found in mitochondria. A few mitoplasts were round and swollen, and this population became predominant after treatment with 0.12 mg/mg digitonin, which resulted in the formation of spherical vesicles (Fig. 2*B*, *panel d*).

We also studied the profile of marker proteins at increasing digitonin concentrations. We found that 0.09 mg/mg digitonin extracted all of OMM Bcl-x_L and nearly all VDAC, with a marginal effect on the intermembrane apoptosis-inducing factor, whereas it left an intact amount of IMM GRIM19 and the β-subunit of the ATP synthase complex, which resides largely in the matrix space (Fig. 2*C*). We also tested an anti-TSPO antibody (a generous gift of Dr. Vassilios Papadopoulos), which unfortunately did not react with the rat protein. However, similar digitonin titrations performed in mouse liver mitochondria revealed an identical digestion pattern as in rats and led to the complete disappearance of TSPO at 0.09 mg/mg digitonin (data not shown). On the basis of the ultrastructural data and protein profiles, we therefore chose 0.09 mg of digitonin/mg of mitochondrial protein for subsequent experiments.

We tested the ability of mitoplasts to undergo the PT with the sensitive calcium retention capacity test, which measures the threshold Ca^{2+} load required to open the pore. Like mitochondria (Fig. 2*D*, *trace a*), mitoplasts readily took up a train of Ca^{2+} pulses (Fig. 2*D*, *trace a′*) in a process that was fully sensitive to ruthenium red, an inhibitor of the Ca^{2+} uniporter (data not shown). In mitoplasts, the threshold Ca^{2+} required for opening of the PTP, which is marked by a precipitous release of the previously accumulated Ca^{2+}, was about one-third of that necessary to open the pore in intact mitochondria. In both preparations, the Ca^{2+} threshold was more than doubled in the presence of 1 μM CsA (data not shown). Mitoplast Ca^{2+} uptake could not be explained by residual intact mitochondria after digitonin treatment. Indeed, a titration with concentrations of mitochondria ranging between 0.1 and 1 mg/ml revealed that 0.4 mg/ml mitochondria was necessary to match the calcium retention capacity of 1 mg/ml mitoplasts (data not shown). This is well above the residual MAO activity, which would account for a maximum of 0.10 mg/ml intact mitochondria. Given that intact mitochondria were not seen in EM images of preparations treated with 0.09 mg/mg digitonin, we concluded that Ca^{2+} uptake was entirely due to mitoplasts (see also below).

A set of experiments was carried out to study PT regulation in mitoplasts. Specifically, we checked whether the pore could still be regulated by two distinct classes of matrix- and surface-exposed sulfhydryl groups previously identified in intact mitochondria (20, 22, 26). These thiols can be discriminated based on their reactivity with the membrane-permeant dithiol cross-linker PhAsO and the membrane-impermeant thiol oxidant $Cu(OP)_2$, respectively (20, 22, 26). Both mitochondria (Fig. 3*A*) (22) and mitoplasts (Fig. 3*A′*) underwent permeabilization and swelling upon addition of PhAsO (*traces a*), in a process that was prevented by CsA (*traces b*) or by 20 μM *N*-ethylmaleimide (*traces c*), indicating that the PhAsO-reactive matrix thiols (19) maintained their PTP regulatory activity after removal of the OMM. PTP-dependent permeabilization of both mitochondria (Fig. 3*B*) and mitoplasts (Fig. 3*B′*) was also seen after the addition of $Cu(OP)_2$ (*traces a*) in a process that maintained its sensitivity to CsA (*traces b*) but was not

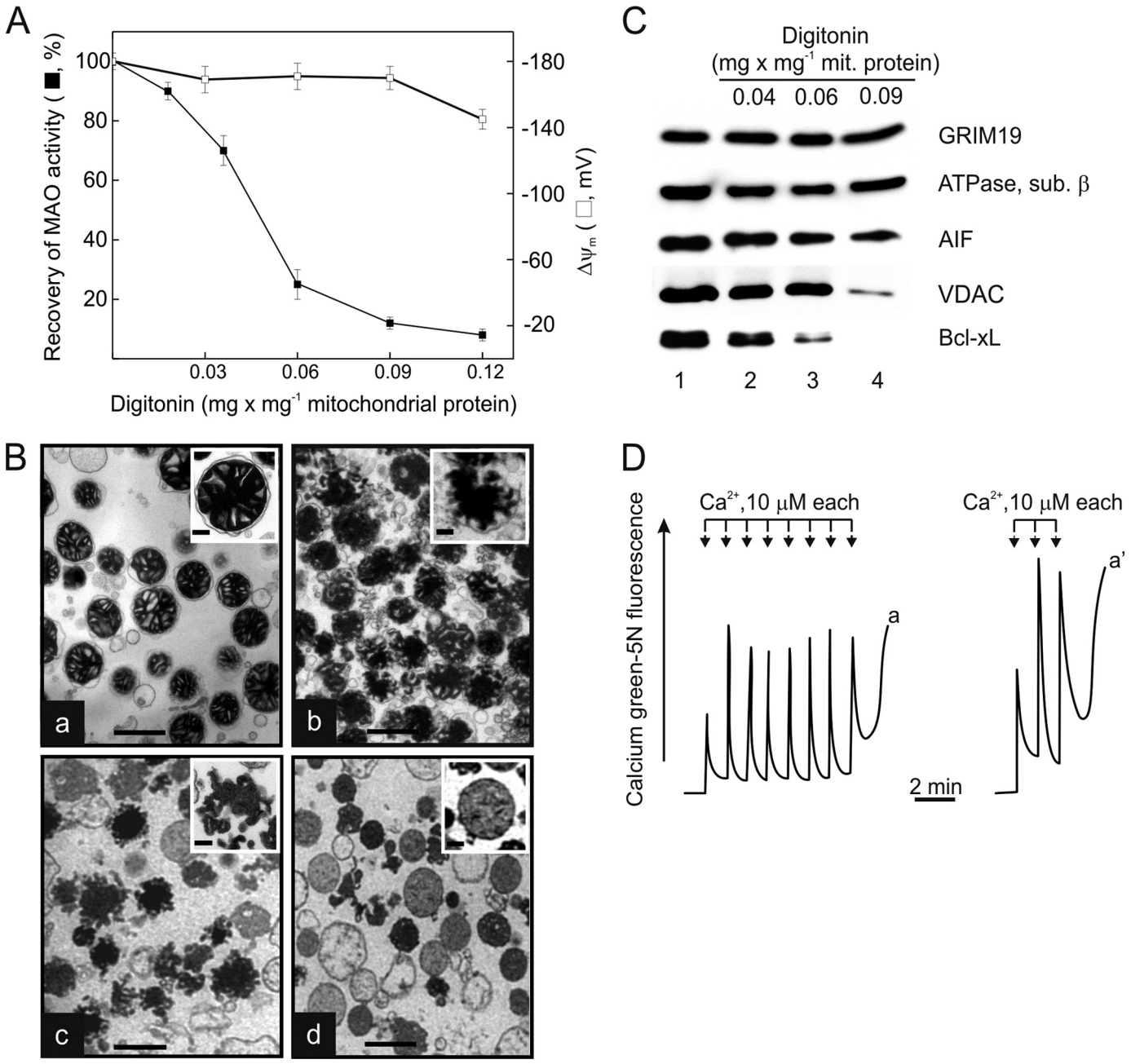

FIGURE 2. **Properties of mitochondria and digitonin-treated mitoplasts.** *A*, mitochondria (20 mg/ml) were incubated for 20 min with the indicated amounts of digitonin. The suspensions were then diluted and pelleted by centrifugation as described under "Experimental Procedures." For each preparation, MAO activity (■) and $\Delta\psi_m$ (□) were determined. The activity was calculated as percent of the starting untreated mitochondria. *Error bars* represent means ±S.D. of four experiments. *B*, transmission electron microscopy of mitochondrial preparations without treatment with digitonin (*panel a*) and after treatment with 0.04 (*panel b*), 0.09 (*panel c*), and 0.12 mg (*panel d*) of digitonin/mg of protein. *Scale bars* = 1 μm in the *main panels* and 0.2 μm in the *insets*. *C*, Western blot analysis of untreated mitochondria (*lane 1*) and of preparations obtained after treatment of mitochondria with the indicated amounts of digitonin (*lanes 2–4*). *AIF*, apoptosis-inducing factor; *mit.*, mitochondrial; *sub.*, subunit. *D*, the incubation medium contained 250 mM sucrose, 10 mM Tris/MOPS, 5 mM succinate, 1 mM P_i, 10 μM EGTA/Tris, 0.5 μM calcium green-5N, 0.5 μg/ml oligomycin, and 2 μM rotenone, pH 7.4, at 25 °C. The calcium retention capacity was measured in mitochondria (1 mg/ml; *trace a*) or mitoplasts prepared with 0.09 mg of digitonin/mg of protein (1 mg/ml; *trace a'*) by the sequential addition of a train of 10 μM Ca^{2+} pulses at 1-min intervals (*arrows*).

inhibited by 20 μM *N*-ethylmaleimide (*traces c*), as also described in detail previously for intact mitochondria (22, 26). These results prove that the $Cu(OP)_2$-reactive external thiols are located in the outer face of the IMM rather than in the OMM.

In intact mitochondria, the PT can be either inhibited or activated by photo-oxidative stress mediated by HP depending on whether low or high doses of light are applied, respectively (26). We tested whether these peculiar photosensitizing properties of HP are maintained in mitoplasts. Mitochondria or mitoplasts (Fig. 4, *closed* and *open squares*, respectively) were incubated with 2 μM HP and irradiated for times ranging between 30 and 240 s at 40 W/m^2, corresponding to light doses between 0.12 and 0.96 J/cm^2. PTP opening was then triggered by Ca^{2+}. These experiments showed that irradiation for up to 100 s inactivated the PTP in both mitochondria (26)

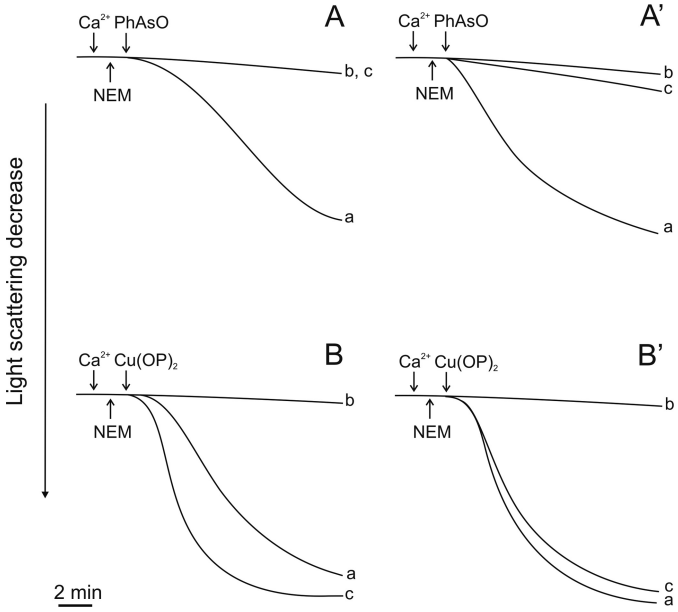

FIGURE 3. **Effect of PhAsO and Cu(OP)$_2$ on light scattering in mitochondria and mitoplasts.** The experimental conditions were as described for Fig. 2*C*, except that calcium green-5N was omitted. Light scattering was measured at 540 nm in mitochondria (1 mg/ml; *A* and *B*) or mitoplasts (1 mg/ml; *A′* and *B′*). *A* and *A′*, 5 μM Ca^{2+} and 5 μM PhAsO were added where indicated (*traces a*); 1 μM CsA was added before (*traces b*); and 20 μM *N*-ethylmaleimide (*NEM*) was added where indicated (*traces c*). *B* and *B′*, 5 μM Ca^{2+} and 3 μM Cu(OP)$_2$ were added where indicated (*traces a*); 1 μM CsA was added before the organelles (*traces b*); and 20 μM *N*-ethylmaleimide was added where indicated (*traces c*).

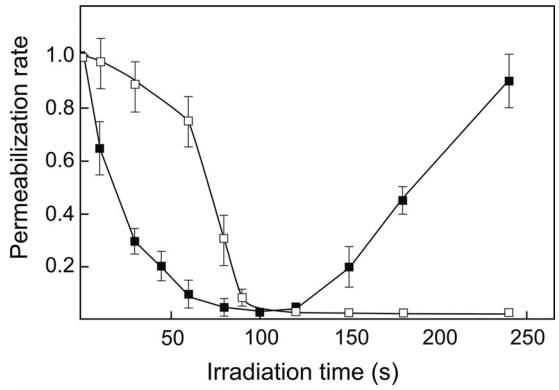

FIGURE 4. **Effect of the irradiation time in mitochondria and mitoplasts loaded with HP.** Mitochondria (■) and mitoplasts (□) at 0.5 mg/ml were loaded with 2 μM HP for 1 min at 25 °C in standard medium before irradiation at 40 W/m^2 for the indicated times; permeabilization was then studied as the light scattering change at 540 nm following the addition of 60 μM (mitochondria) or 30 μM (mitoplasts) Ca^{2+}. The permeabilization rates were normalized to that of the control (non-irradiated organelles). *Error bars* represent the mean ± S.D. of four experiments.

and mitoplasts, although the latter were significantly more resistant (Fig. 4). In both preparations, inactivation occurred through photomodification of His residues, as indicated by the counteracting effect of diethyl pyrocarbonate, which prevents the addition of $^1O_2$ to the His imidazole ring and its irreversible degradation (data not shown, but see Ref. 26). On the other hand, mitoplasts were strikingly resistant to the activation of the PTP that in mitochondria follows irradiation for times longer than 100 s (Fig. 4). It must be stressed that the mitoplast preparations (i) maintained the ability to take up Ca^{2+} in the full range of light doses investigated, indicating that they retained energy coupling and a membrane potential, and (ii) readily underwent large amplitude swelling upon addition of alamethicin, indicating that they maintained their structural integrity (data not shown). We deduce that in intact mitochondria HP interferes with PTP-regulating OMM sites that are lost in mitoplasts.

TABLE 1

Porphyrin incorporation in mitochondria and mitoplasts

Mitochondria and mitoplasts (1 mg/ml protein) were incubated for 2 min in standard medium in the presence of HP, PP, DP, and CP ranging from 1 to 6 nmol of porphyrin/mg of protein. After 2 min, the suspensions were centrifuged at 4000 × *g* for 2 min, and porphyrin concentrations were determined fluorometrically by calibration plots after extraction of supernatants and pellets with 2% SDS. All data are the average of at least three independent experiments and are expressed as percent of the total amount of porphyrin added.

	Mitochondria	Mitoplasts
HP	30 ± 4	20 ± 2
PP	37 ± 5	25 ± 4
DP	40 ± 5	30 ± 5
CP	13 ± 3	10 ± 3

HP binds TSPO with nanomolar affinity (27). To test whether this OMM protein mediates HP-dependent PTP photoactivation, we extended our investigation to DP and PP, dicarboxylic porphyrins that display even higher affinity than HP in binding TSPO (27). We also tested CP (Table 1; see "Experimental Procedures" for details), a tetracarboxylic porphyrin whose binding to TSPO is very weak (27).

Like HP, photoactivated PP and DP caused a concentration-dependent PT inhibition in both mitochondria and mitoplasts (Fig. 5, *A* and *A′*, respectively). PP and DP were more effective than HP, whereas CP was totally inactive. As the efficiency of $^1O_2$ production is very similar for all porphyrins in the monomeric state (~0.7) (39), the resulting effects (or lack thereof) must depend on the different localization of specific porphyrins in the mitochondrial and mitoplast membranes. It should be noted that the PTP-active porphyrins were more effective in mitochondria than in mitoplasts (Fig. 5, compare *A* and *A′*), suggesting the TSPO facilitates their diffusion to the "internal" PTP regulatory sites.

We next investigated the effects of these porphyrins on PTP after prolonged irradiation. Mitochondria and mitoplasts were allowed to accumulate a very small Ca^{2+} load (10 nmol/mg of protein) that was not sufficient for spontaneous PTP opening yet was permissive for the subsequent effect of irradiation. In mitochondria, PTP opening was readily detected with PP, DP, and HP (Fig. 6, *closed symbols*); of note, the relative potency of porphyrins in eliciting photosensitization of the PTP closely correlated with their relative affinities for TSPO binding (27). Strikingly, in these protocols, all porphyrins were instead totally inactive in mitoplasts (Fig. 6, *open symbols*).

Several TSPO ligands have been reported to cause PTP opening (7–9, 11, 13, 16). We tested the effects of PP, PK11195, Ro 5-4864, and FGIN1-27 (40) on Ca^{2+} release in Ca^{2+}-loaded mitochondria treated with ruthenium red, a sensitive measure of pore opening (41). In these protocols, the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone caused Ca^{2+} release in both mitochondria and mitoplasts (Fig. 7, *A* and *A′*, respectively, *traces b*) through a fully CsA-sensitive process (*traces a*). Strikingly, the addition of 40

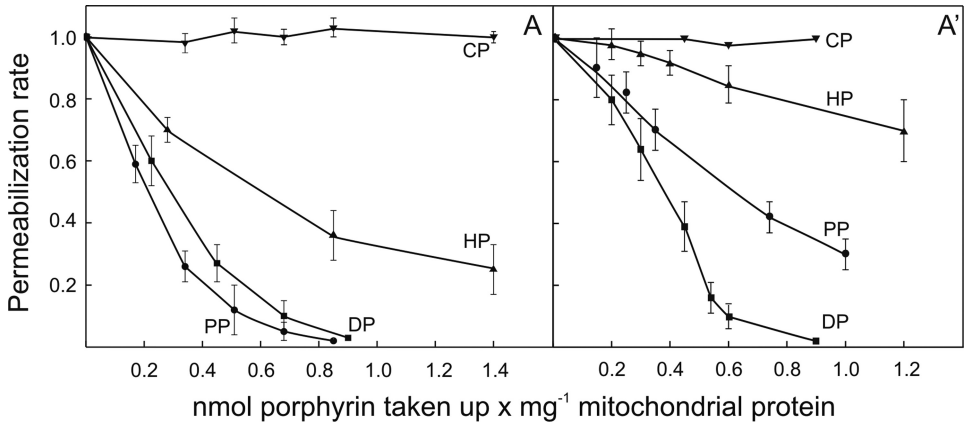

FIGURE 5. **Effect of DP, HP, PP, and CP on light-dependent inactivation of the permeability transition.** Mitochondria (*A*) and mitoplasts (*A'*) at 0.5 mg/ml were incubated for 1 min with concentrations of each porphyrin giving a loading of 0.2–1.5 nmol of porphyrin/mg of protein at 25 °C (for details, see Table 1 and "Experimental Procedures"). Preparations were irradiated for 45 s at a fluence rate of 40 W/m^2 and then supplemented with 60 μM (*A*) or 30 μM (*A'*) Ca^{2+} to trigger the PT, which was followed as the changes in 90° light scattering at 540 nm. The permeabilization rate was normalized to that of the control (in the absence of porphyrins). *Error bars* represent the mean ± S.D. of three experiments.

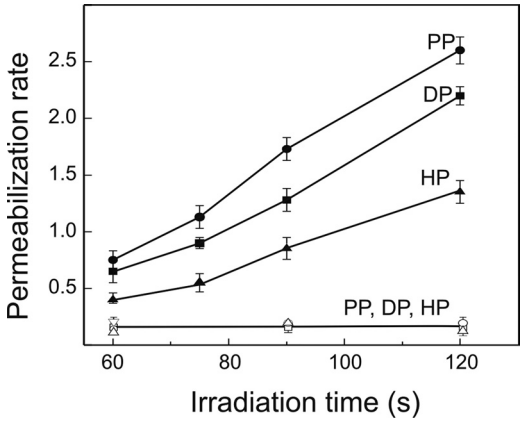

FIGURE 6. **Effect of DP, HP, and PP on light-dependent activation of the permeability transition.** Mitochondria (*closed symbols*) or mitoplasts (*open symbols*) at 0.5 mg/ml were incubated for 1 min at 25 °C with concentrations of each porphyrin (PP, *circles*; DP, *squares*; HP, *triangles*) giving a loading of 1.2 nmol of porphyrin/mg of protein (for details, see Table 1 and "Experimental Procedures"). Preparations were then supplemented with 5 μM Ca^{2+} (a concentration not sufficient to induce PTP opening *per se*) and irradiated for the indicated times at a fluence rate of 40 W/m^2. The PT was followed as the change in 90° light scattering at 540 nm. *Error bars* represent the mean ± S.D. of three experiments.

μM FGIN1-27 caused CsA-sensitive PTP opening in mitochondria but not in mitoplasts (Fig. 7, *A* and *A'*, respectively, *traces c*). A full titration was carried out with FGIN1-27, Ro 5-4864, and PK11195, which confirmed the striking resistance of digitonin-treated mitoplasts to the PTP-inducing effects of TSPO ligands (Fig. 8). Similar results were obtained for concentrations of PP above ~6 μM (data not shown).

DISCUSSION

An important result of this work is the demonstration that a CsA-sensitive PT can occur in digitonin-treated mitoplasts, *i.e.* in the absence of an intact OMM. The PT of mitoplasts maintains most of the basic features of the PT observed in mitochondria (Ca^{2+} dependence, desensitization by CsA, activation by oxidants, inactivation by HP at low irradiation times), thus indicating that the PT is fundamentally an IMM event that can occur in the absence of an intact OMM. These findings heavily bear on our understanding of the nature and location of the PTP and call into question the widespread conviction that the pore forms at contact sites between the IMM and OMM and that the pore itself spans both membranes (42).

The basis for this model of the PTP is the presence of ANT and VDAC (together with many other proteins) in detergent membrane extracts that possess hexokinase activity and display transport properties that resemble those of the PTP after reconstitution in liposomes (43). Similar preparations (which were, however, not even enriched in ANT and VDAC) had been previously shown to possess electrophysiological properties similar to those of the PTP, although atractylate caused channel closure (44) rather than the opening that would be expected based on studies in intact mitochondria (45). We believe that this model should be abandoned because (i) a Ca^{2+}-dependent and CsA-sensitive PT can occur in mice in which both isoforms of ANT have been genetically ablated (2); (ii) the PTP of VDAC1-null mice is indistinguishable from that of wild-type animals (3); (iii) a PT can occur in mitochondria of cells in which the genes for all three VDAC isoforms have been deleted or silenced (4); (iv) the 32-kDa protein binding to the PTP modulator cyclophilin D under conditions that favor pore opening is not ANT (46); and (v) the PT can occur in the absence of an intact OMM (this study). Although trace amounts of VDAC could be detected in our mitoplasts (Fig. 2*C*), we would like to stress that the absence of an intact OMM was documented by electron microscopy (Fig. 2*B*) and, most importantly, by the lack of effects of TSPO ligands (see below).

These results should not be taken to imply that the OMM is not relevant for PTP modulation. On the contrary, another important result of this work is the demonstration that the OMM plays a prominent regulatory role and that this is in part at least exerted through TSPO. Our data are consistent with a dual role of TSPO (i) as a *regulatory protein* for PTP modulation when it binds its selective ligands such as FGIN1-27, PK11195, Ro 5-4864, and PP and (ii) as a *transport protein* for PTP-active compounds that are then transferred to their PTP regulatory site(s) in the IMM or in the matrix.

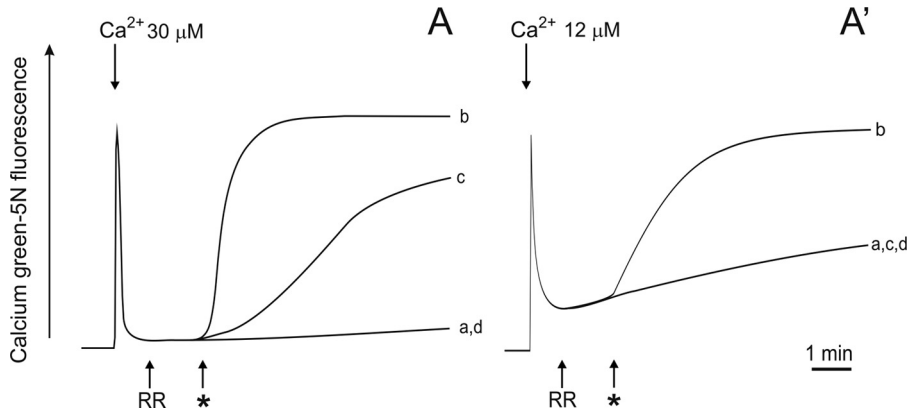

FIGURE 7. **Effect of uncoupler and FGIN1-27 on PTP-dependent Ca^{2+} release in mitochondria and mitoplasts.** Mitochondria (1 mg/ml; *A*) or mitoplasts (1 mg/ml; *A'*) were incubated as described for Fig. 2*D*. In *traces a* and *d*, the medium was supplemented with 1 μM CsA. Where indicated, Ca^{2+} was added, followed by 0.1 μM ruthenium red (*RR*) and, at the *asterisk*, by 0.5 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (*traces a* and *b*) or 40 μM FGIN1-27 (*traces c* and *d*).

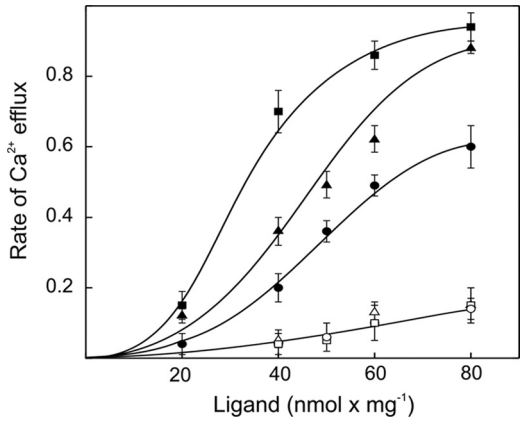

FIGURE 8. **Effect of TSPO ligands on PTP-dependent Ca^{2+} release in mitochondria and mitoplasts.** Mitochondria or mitoplasts (1 mg/ml) were incubated as described for Fig. 2*D*, and experiments were performed as shown in Fig. 7 by adding the stated amounts of FGIN1-27 (*squares*), Ro 5-4864 (*triangles*), or PK11195 (*circles*) after ruthenium red to mitochondria (*closed symbols*) or mitoplasts (*open symbols*). The rate of Ca^{2+} efflux was normalized to that obtained after the addition of 0.5 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

The first role is exemplified by the lack of pore reactivation by light in mitoplasts treated with porphyrins that bind TSPO in intact mitochondria (Figs. 4 and 6). This remarkable finding demonstrates that high light doses in mitochondria treated with HP, DP, and PP do not cause unspecific damage to the IMM because damage should be more prominent, rather than absent, in preparations that are not protected by the OMM. Instead, our data show that porphyrin- and light-dependent PTP reactivation is a specific event mediated by the OMM and suggest that the PTP regulatory sites are contributed by TSPO. In keeping with this hypothesis, in intact mitochondria, PTP activation could be induced in the presence of porphyrins characterized by a PP-like configuration that bind TSPO with nanomolar affinity (27, 28), whereas the PP-unrelated CP was ineffective (data not shown). Furthermore, the photoactivity of dicarboxylic porphyrins (PP > DP ≫ HP) followed exactly their order of affinity for TSPO, as calculated from the values of their inhibition constants for [^{3}H]PK11195 binding (27). Selectivity is the basis for the physiological role of TSPO, which allows the transport of natural dicarboxylic porphyrins (such as PP), heme precursors, heme itself, and cholesterol to mitochondria (28, 47–50). Strong evidence in favor of a regulatory role of TSPO in inner membrane permeability is provided by the observation that FGIN1-27, PK11195, Ro 5-4864, and relatively high concentrations of PP (~6 μM or higher) cause PTP opening in mitochondria (7–16) but not in mitoplasts (Figs. 7 and 8). We believe that the liganded state of TSPO modulates the PTP through protein-protein interactions that may be similar to those described for dephosphorylated Bad and Bcl-x_L (51).

The second role of TSPO is revealed by the effects of PP, DP, and HP, which at low concentrations do not affect the pore yet are able to induce PTP inhibition through photooxidation of matrix His residues in both mitochondria and mitoplasts (Fig. 5). Higher porphyrin concentrations are required in mitoplasts, indicating that transport through TSPO facilitates diffusion of porphyrins to their relevant matrix or IMM sites of action. In other words, these data demonstrate that compounds whose PTP-dependent effects are facilitated via transport through TSPO do not necessarily exert their effects through TSPO itself.

TSPO is a member of an evolutionarily conserved family of ubiquitous proteins able to bind small molecule drugs, cholesterol, and porphyrins (17). TSPO has been conserved from bacteria to mammals, and its inactivation induces an early embryonic-lethal phenotype in mice (52). In mammals, TSPO is highly expressed in the adrenal cortex (53), particularly in the zona glomerulosa (54), which is consistent with its role in steroid hormone synthesis. Its ubiquitous distribution suggests, however, more general functions, including heme biosynthesis, apoptosis, cell proliferation, and PTP modulation (49). A thorough study is under way to define which PTP ligands of pathophysiological relevance act through TSPO.

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