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Role of the permeability transition pore complex in lethal inter-organelle crosstalk

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1. ABSTRACT

The endoplasmic reticulum (ER) function is critical for multiple cellular activities. Hence, impairment of the physiological function of the ER, such as accumulation of unfolded proteins or disturbance of lumenal calcium homeostasis, leads to an evolutionarily conserved adaptive response known as the ER stress response. Activation of this self-protective pathway gives the cell a chance to restore normal ER function. In the case of prolonged or severe stress conditions, or if the ER dysfunctions cannot be compensated, apoptosis is ultimately activated to eliminate stressed cells. Although the molecular mechanisms involved in ER stress-mediated apoptosis are poorly understood, it is known that ER and mitochondria can cooperate to induce cell death. In this review, we discuss the commitment and development of the lethal crosstalk between ER and mitochondria and focus on the role of the mitochondrial permeability transition pore complex in these processes.

2. INTRODUCTION

The endoplasmic reticulum (ER) is dedicated to synthesis, proper folding, modification and maturation of proteins, as well as lipid and membrane biogenesis. As a major intracellular calcium (Ca^{2+}) storage organelle, the ER plays a critical role towards maintenance of cellular Ca^{2+} homeostasis. Impairment of these physiological functions, including accumulation and aggregation of misfolded or unfolded proteins, alteration of protein N-glycosylation and disruption of the lumenal Ca^{2+} homeostasis, causes the so-called ER stress. A wide variety of human diseases have been associated to these cellular dysfunctions of the ER such as diabetes, obesity, viral infection, neurodegenerative disorders and cancer (1). To overcome the deleterious effect of ER stress, the organelle triggers three major specific responses: (i) the unfolded protein response (UPR), (ii) the ER-overload response (EOR) and (iii) the ER-associated degradation (ERAD). Activation of these self-protective pathways gives the cell a chance to restore ER

homeostasis by reducing general protein neosynthesis, increasing the folding and processing capacity of the ER and eliminating unfolded proteins from the ER (see (2) for review). In mammals, this coordinated and complex cellular response involves activation of multiple proteins including three proximal sensors: the PERK kinase (PKR-like ER protein), the transcription factor ATF6 (activating transcription factor 6) and the kinase/endoribonuclease IRE1 (Inositol-requiring enzyme 1). These transmembrane proteins are normally kept inactive through the interaction of their N-terminal lumenal domain with the ER-resident chaperone GRP78/BiP (Glucose-regulated protein 78 kDa/Binding immunoglobulin protein). Upon ER stress the dissociation of GRP78/BiP (allowing its binding to unfolded proteins) from the three sensors initiates the UPR (3, 4). After dissociation, ATF6 translocates to the Golgi apparatus, where it is cleaved into its active form by S1P/S2P proteases (Site-1 protease/Site-2 protease) (5), and then moves to the nucleus to activate transcription of the UPR-target genes (6). IRE1 and PERK are activated by dimerization and trans-autophosphorylation within their serine/threonine kinase domains. On the one hand, PERK activation leads to phosphorylation of eIF2 (eukaryotic initiation factor 2) which attenuates general protein translation (7). On the other hand, the endonuclease activity of IRE1 cleaves a small intron from XBP1 mRNA (X-box binding protein 1), previously induced by ATF6, generating an active splice variant that stimulates transcription of ER chaperones genes (8, 9). The UPR thus appears as a pro-survival response triggered to rescue ER homeostasis. Actually, in the case of prolonged or excessive stress condition, the ER dysfunction cannot be compensated and apoptosis is ultimately activated to eliminate damaged cells. However, the molecular mechanisms that control the switch from the pro-survival to the pro-apoptotic pathway are not yet fully understood. Nevertheless, some core proteins of the protective UPR pathway have been also implicated in the apoptotic response induced by ER stress. For instance, the inhibition of protein translation induced by PERK is not complete and expression of specific genes such as CHOP/GADD153 (C/EBP homologous protein/Growth arrest- and DNA damage-inducible gene 153) is paradoxically activated (10). This transcription factor has been demonstrated to promote apoptosis in several systems (11-13), presumably at least by inhibiting the expression of the anti-apoptotic protein Bcl-2 and activating the mitochondrial pathway of apoptosis (14, 15). Similarly, in HEK293T cells overexpression of IRE1 stimulates apoptosis (16), demonstrating that this enzyme could act as a pro-survival as well as a pro-apoptotic factor. Several lines of evidence suggest that IRE1 could contribute to ER stress-induced apoptosis by activating kinase cascades, in particular the TRAF2/JNK pathway (17, 18).

It is well established that caspases, a group of cysteine proteases that cleave proteins after aspartic acid residues, are the main effectors of apoptosis in eukaryotic cells. The link between ER stress and caspase activation has remained unclear until the characterization of caspase-12 as a mediator of ER stress-induced apoptosis in a murine model by Nakagawa and Yuan (19). In mice, the inactive procaspase-12, localized to the cytosolic face of the ER membrane, is thought to be proteolytically activated by Ca^{2+}-dependent calpain (20) or the IRE1 signaling pathway (21). Following its activation, which appears to be specifically involved in response to ER stress, caspase-12 can directly process initiator procaspase-9, independently of cytochrome c and Apaf-1 (apoptosis protease-activating factor 1), to activate the executive caspase-3 (22, 23). Nevertheless, the relevance of caspase-12 in ER stress-induced apoptosis has been questioned by different reports showing that in P19 mouse embryonal carcinoma (24) and B16/B16 murine melanoma (25) cells died to a similar extent, whether they express caspase-12 or not. Furthermore, the role of caspase-12 in human tissues remains controversial since deleterious mutation in its gene was reported to preclude the expression of a functional protease (26). Altogether these data suggest that the processing of caspase-12 occurring in ER stress-triggered apoptosis may be dispensable and that other mechanisms may be activated to ensure complete and efficient apoptotic cell death. In particular, a growing amount of evidences indicates that the ER, in addition to directly initiating its own apoptotic pathways, is intimately interconnected with mitochondria and can cooperate with this well-documented central orchestrator of the apoptotic program to induce apoptosis. This review explores the mechanisms through which ER and mitochondria might communicate to elicit apoptosis and focuses on the role of the mitochondrial permeability transition pore complex (PTPC) in these processes.

3. THE MITOCHONDRIAL PATHWAY OF APOPTOSIS

Apoptosis is a tightly regulated, genetically predetermined program by which metazoans eliminate unwanted, infected, mutated, dysfunctional and damaged cells. The two major and well-characterized signaling cascades leading to apoptosis are the extrinsic pathway (also known as the “death receptor pathway”) and the intrinsic pathway (also called the “mitochondrial pathway”). The extrinsic pathway is activated through binding of exogenous ligands to death receptors at the cell surface. The death receptor family includes the CD95/Fas receptor, the Tumor Necrosis Factor-α (TNF-α) receptor and Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) receptor (reviewed in (27)). Binding of ligands causes oligomerization of the receptors, which triggers the assembly of the death-inducing signaling complex (DISC). This complex induces the activation of initiator caspase-8 and -10, followed by the processing of downstream executive caspases and the subsequent dismantling of the cell.

The intrinsic pathway involved mitochondria as central integrators and coordinators of the apoptotic process and is usually activated by cell-internal stimuli such as DNA damage, growth factor deprivation, oxidative stress, chemotherapeutic drugs or signals emanating from other organelles (e.g. nucleus, lysosomes and ER). Although very different, all these stimuli favor mitochondrial membrane permeabilization (MMP), which results in the release of

apoptogenic factors from the intermembrane space (IMS) to the cytosol (for complete review see (28)). These IMS proteins include caspase activators such as cytochrome c (a mobile electron carrier of the respiratory chain) (29, 30), Omi/HtrA2 (Omi stress-regulated endoprotease/High temperature requirement protein A2) (31-33), Smac/DIABLO (second mitochondria-derived activator of caspases/direct inhibitor of apoptosis-binding protein with low pi) (34, 35), as well as caspase-independent death effectors such as EndoG (Endonuclease G) (36) and AIF (Apoptosis-Inducing Factor) (37, 38). Released cytochrome c associates with the adaptor Apaf-1, dATP and the initiator procaspase-9 to form a multiprotein complex called apoptosome, which in turn promotes caspase-9 auto-processing and subsequent activation of downstream effector caspases (39, 40). In addition, cytochrome c can also translocate to the ER, where it binds to the IP3R (Inositol 1,4,5-trisPhosphate (IP3) Receptor) Ca^{2+}-channel, resulting in increased Ca^{2+} release and amplification of the apoptotic process (41). Omi/HtrA2 and Smac/DIABLO can indirectly activate caspases by binding to IAPs (Inhibitor of apoptosis) thus releasing their suppressive effect on caspase activity (31-35). When redistributed to the cytosol, AIF and EndoG translocate to the nucleus to induce a caspase-independent chromatin condensation and DNA fragmentation (36, 37). Although they were initially considered to be independent, the receptor and mitochondrial pathways can be interconnected in some model of apoptosis (42). Indeed, the death receptor-mediated activation of caspase-8 can lead to the cleavage of the BH3-only protein Bid into its truncated form t-Bid, which then translocates to mitochondria to promote MMP (43, 44).

4. PTPC AND THE MITOCHONDRIAL MEMBRANE PERMEABILIZATION

MMP is considered as the "point-of-no-return" in many models of apoptosis involving the mitochondrial pathway (28). Nevertheless, the precise mechanisms by which mitochondrial mediators are released during MMP are still a matter of controversy. Different models, not mutually exclusive, have been proposed in which permeabilization of mitochondrial membranes may engage only the outer membrane (OM) or both OM and inner membranes (IM). To explain mitochondrial outer membrane permeabilization (MOMP), three major mechanisms have been put forward: (i) activation of pro-apoptotic proteins of the Bcl-2 family such as Bax, Bid or Bak is accompanied by mitochondrial intramembranous homo- or hetero-oligomerization, allowing megachannel formation with subsequent release of IMS proteins (45, 46); (ii) activated pro-apoptotic members of Bcl-2 family may also translocate to the OM and interact with components of PTPC, favoring release of apoptogenic factors (47-51); (iii) destabilization of the mitochondrial lipid bilayer would elicit formation of apoptotic pores, large enough to allow redistribution of IMS protein to the cytosol (52, 53).

Alternatively, the model involving permeabilization of both the OM and IM is based on the formation of a polyprotein complex, called PTPC (Figure 1), at the contact sites between outer and inner mitochondrial membranes (54-56). Although the composition of this giant channel has not been clearly established, it is believed that the scaffold structure of PTPC is based on the dynamic interactions between the voltage-dependent anion channel (VDAC, an unspecific pore protein also known as mitochondrial porin) localized in the OM, the adenine nucleotide translocase (ANT, a specific ADP/ATP transporter) in the IM and cyclophilin D (CypD, a peptidyl-prolyl isomerase) in the matrix. Additional proteins were also found associated with PTPC including hexokinase (HK), peripheral benzodiazepine receptor (PBR), creatine kinase (CK) and members of the Bcl-2 family. These proteins are generally not considered as part or the pore itself but are rather supposed to regulate PTPC (57). Under physiological conditions, a dynamic opening/closing of PTPC controls the homeostasis of mitochondria and regulates the matrix volume, the Ca^{2+} flux, the redox equilibrium and the matrix pH. During apoptosis, PTPC opens as a lethal non-specific pore, thus increasing the permeability of the IM for small molecules of MM < 1500 Da, a phenomenon known as the mitochondrial permeability transition (MPT) (58, 59). Consequently to the extended opening of PTPC, mitochondria undergo a massive entry of water that favors matrix swelling, remodeling of cristae and - owing to the difference of surface between IM and OM - local ruptures of the OM. These events lead to the dissipation of the mitochondrial membrane potential ($\Delta\Psi_m$) and to the release of soluble pro-apoptotic proteins from the IMS. Many studies carried out on purified mitochondria or *in cellulo* have demonstrated an induction of PTPC-dependent MMP in response to a myriad of stimuli including calcium, pro-oxidant drugs (e.g tert-butylhydroxyperoxide, diamide, phenylarsine oxide), chemotherapeutics, GD3 gangliosides, activators of either ANT (e.g atractyloside) or PBR (e.g. PK11195), Bax-BH3-domain-derived peptides and viral proteins (e.g. Vpr) (reviewed in (54, 60)). In addition, the connection between PTPC and apoptosis has been reinforced by demonstration that inhibitors of PTPC can also negatively regulate cell death. Indeed, the VDAC channel blockers NPPB (5-nitro-2-(phenylpropylamino)-benzoate), DIDS (dihydro-4,4′ diisothiocyanostilbene-2,2′-disulphonic acid), phloretin and NADH (61, 62), inhibitors of ANT such as BA (bongkrekic acid), ADP and ATP (50, 63) and the two ligands of CypD cyclosporin A (CsA) and sangliferhin A (64, 65) were shown to inhibit or delay MMP and subsequent apoptotic process. Noteworthy, sustained PTPC opening was also involved in necrosis (a cell death triggers in response to severe insult), in particular after ischemia/reperfusion injury of the heart, presumably due to a dramatic fall in ATP level (66, 67). However, more recently, genetic knock-out studies were conducted on mice invalidated for either VDAC or ANT or CypD casting some doubts on the precise composition of PTPC. In opposition to many data implicating VDAC in MMP and apoptosis (reviewed in (68, 69)), a recent report of Baines *et al.* (70) demonstrates that deletion of the three VDAC isoforms did not modify the MMP and the cellular apoptosis in response to Ca^{2+}, oxidative stress and the pro-apoptotic proteins Bax and Bid, suggesting that VDAC would be dispensable for MPT and cell death. The

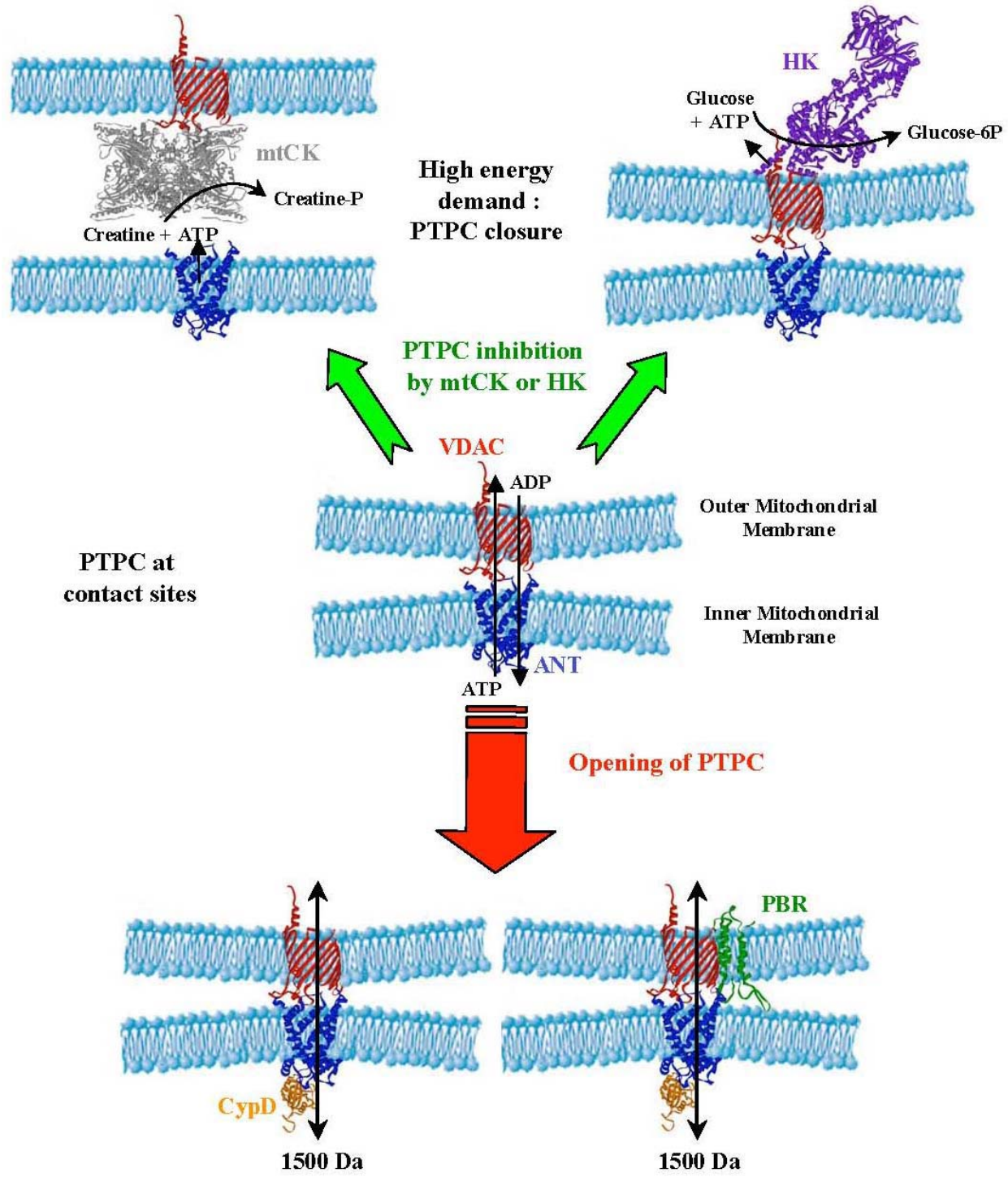

Figure 1.The putative PTPC model. At mitochondrial contact sites (middle part of the figure), PTPC consists of ANT/VDAC complexes that can be maintained in a close state by Hexokinase (HK) or mitochondrial Creatine Kinase (mtCK) depending on the tissue. In response to death stimuli, matricial Cyclophilin D (CypD) can associate with ANT/VDAC to generate a high conductance channel of low selectivity that causes the so-called process, MPT (Mitochondrial Permeability Transition). In some tissues, PBR can also associate to VDAC. PDB accessions numbers, ANT: 10KC; mtCK: 1CRK; HK: 1BG3; CypD:2BIT. VDAC model was provided by V. De Pinto (179).

requirement for ANT was questioned by Kokoszka *et al.* (71), who demonstrated that mitochondria purified from liver of mice lacking both ANT1 and ANT2 isoforms still undergo MPT, although concentrations of Ca^{2+} required were higher. In addition, they observed no difference in the sensitivity of hepatocytes deficient for both ANT1 and ANT2, when compared to controls, in response to Fas ligand or TNF-α. Thus, the authors concluded that ANTs are non-essential components of PTPC, a statement that is still controversial. Indeed, since ANTs belongs to a large family of structurally related mitochondrial carriers, it has been proposed that ANT deficiency would be complemented by another ANT-like protein of the IM (72), a proposal that is reinforced by the recent discovery of a novel isoform of ANT (ANT4) in mice (73). Moreover, Pereira *et al.* (74) showed that in yeast lacking the three ANT orthologues AAC1/2/3 (ADP/ATP carrier), MMP, cytochrome c release and apoptosis are impaired, leading to the conclusion that AAC are required for MMP in *Saccharomyces cerevisiae*. The role of CypD in MMP was investigated in CypD knock-out mice (*Ppif* $^{-/-}$) by four independent groups (75-78). In mitochondria purified from *Ppif* $^{-/-}$ mice, PTPC opening was still observed in response to Ca^{2+} but required higher concentrations than wild-type (76, 77), whereas no difference were observed in response to others stimuli such as ubiquinone 0, depolarization, and thiol oxidants (76). Interestingly, the absence of effect of CsA on the PTPC in *Ppif* $^{-/-}$ mitochondria corroborates the well-admitted notion that CypD is the target of CsA in mitochondria (76). According to all authors, the response to various apoptotic stimuli appeared similar in cells isolated from deficient and wild-type mice, while cells lacking CypD were resistant to PTPC opening and cell death triggers by necrotic stimuli such as H_2O_2 and Ca^{2+} overload, suggesting that CypD-dependent MPT is central is necrosis but not in apoptosis. Nevertheless, on the one hand, *Ppif* $^{-/-}$ fibroblasts were also demonstrated to be resistant to H_2O_2-induced PTPC opening and cell death with apoptotic features (as measured by TUNEL and annexin V assays), and on the other hand, transgenic mice overexpressing cyclophilin D in the heart show mitochondrial swelling and spontaneous MMP-dependent apoptosis of cardiomyocytes (75), casting a doubt about the exact role of CypD in cell death. Finally, the different above-mentioned reports raised numerous questions on the exact nature of PTPC, an issue that is still under intensive investigation.

5. ROLE OF PTPC IN STRUCTURAL AND FUNCTIONAL INTERACTIONS BETWEEN ER AND MITOCHONDRIA

In contrast to prokaryotes, a specific feature of eukaryotes is the existence of organelles surrounded by a membrane that define the sub-cellular compartmentation and ensure the permeability barrier necessary to intracellular homeostasis. Consequently, to enhance the reliability of signal recognition and to limit undesired side effects on other compartments, signal transmission between different organelles is often facilitated by close appositions. Thus, although ER and mitochondria are distinct specialized compartments, many reports have provided convincing evidence for the existence of local interactions between these two organelles (see (79) for recent review). In fact, ER and mitochondria form a highly dynamic interconnected network within which they cooperate to synthesize mitochondrial cytochrome c oxidase (80), a 45-kDa glycoprotein of the IM (81) and phospholipids (82, 83) and to control intracellular Ca^{2+} signaling and cell death (84-88).

5.1. PTPC and ER-mitochondria physical interactions

Physical associations between ER and mitochondria were observed in different experiments of electron microscopy and cosedimentation (89-91). However, they were initially suspected of being, at least in part, artifacts of chemical fixation process. To rule out this latter hypothesis, Rizzuto and co-workers have designed a set-up, in living HeLa cells, in which ER and mitochondria were labeled with two differently colored GFP mutants, and conclusively demonstrated the existence of close appositions of the two organelles, that were called "microdomains" (92). They also estimated that 5 to 20% of the surface of the mitochondrial network is associated with ER cisternae in HeLa, while tomographic examination of thick sections of rat liver indicates that 50% of the mitochondria are directly interconnected to ER membranes (93). More recently, it has been beautifully shown by electron tomography that the physical links between ER and mitochondria in mammalian liver have length of ~10 nm at the smooth ER and ~25 nm at the rough ER and are of protein nature, as they were disrupted by limited proteolysis (94). The proteins involved in the dynamic interactions of the two organelles are still largely unidentified, however, a number of potential candidates have been recently proposed. For example, the autocrine motility factor receptor, a marker of a smooth subdomain of the ER, exhibits direct interactions with mitochondria on electron micrographs and thus was suggested to link the two organelles (95). Another interesting candidate could be PACS-2 (Phosphofurin Acidic Cluster Sorting protein 2), a multifunctional sorting protein, that was localized on ER and mitochondria by confocal microscopy (96). Depletion of PACS-2 by RNA interference (i.e. siRNA) induces extensive mitochondrial fragmentation and uncoupling of the fragmented mitochondria from the ER, leading the authors to conclude that PACS-2 is required for intimate association of ER with mitochondria. These mitochondrial fission and dissociation from the ER result from the cleavage of the ER cargo receptor BAP31 (B Cell receptor Associated Protein) into its p20 form. The demonstration by another group that overexpression of this p20 product stimulates the recruitment of the fission protein DRP-1 (dynamin-related protein 1) to mitochondria (97), links PACS2 to the mitochondrial morphogenesis machinery and suggests that fission-fusion proteins could also modulate ER-mitochondria interactions. Interestingly, DRP-1, which is a dynamin GTPase implicated in mitochondrial fission (98, 99), was localized in both ER and mitochondria (100, 101). Furthermore, inhibition of DRP-1 by overexpression of dominant-negative mutants or microinjection of neutralizing DRP-1 antibodies dramatically alters distribution and morphology of both ER and mitochondria, leading the authors to suggest that DRP-1 could regulate

the dynamic interactions between the two organelles (100). Along the same line, the integral protein of the OM hFis1 (human fission protein 1), another member of the mitochondrial fission machinery, was demonstrated to interact with DRP-1 (102) and to elicit an apoptotic process that requires the ER gateway (103), indicating that hFis1 might also participate in regulation of ER-mitochondria associations. A missing link in the connection between ER-mitochondria and the protein of the fission machinery could be PTPC. Indeed, reports from different groups show that overexpression of hFis1 or DRP-1 sensitizes cells to Ca^{2+}-induced MPT (104) and regulates PTPC opening (103). It is therefore plausible to assume that interactions between proteins of the fission machinery and members of PTPC could favor and/or stabilize ER and mitochondria associations during ER-mediated apoptotic process, an interesting issue that needs further investigations.

The role of PTPC members in coupling ER and mitochondria has been more intensively studied in experiments designed to elucidate the molecular mechanisms enabling Ca^{2+} transfer between the two organelles. It has been postulated that to generate an efficient local Ca^{2+} signal at ER-mitochondrial junctions, both the ER Ca^{2+} release sites and the mitochondrial Ca^{2+} uptake sites should be present at the interface area. In fact, different reports have established that in close contacts between the ER and mitochondria, microdomains of high [Ca^{2+}] may be generated upon opening of ER specific receptors, such as RYR (ryanodine receptor) or IP3R, to enable efficient Ca^{2+} exchange ((92, 105) and (106) for recent review). Furthermore, within microdomains, a quasi-synaptic organization permits a direct "channeling" of Ca^{2+} ions from the ER into mitochondria (107). Whilst the nature of the putative channel complex is not fully determined, the existence of interactions between RYR/IP3R and components of PTPC were described. Indeed, VDAC, already known as a large channel which transports anions, cations, and various metabolites including substrates and nucleotides across the OM (108-110), was also demonstrated to possess Ca^{2+} binding sites and to be highly permeable to Ca^{2+} when purified and reconstituted into lipid bilayers or liposomes (111). These results suggest that VDAC could control Ca^{2+} transport into the mitochondria and thus could have an active role in regulation of the mitochondrial Ca^{2+} homoeostasis. This issue was addressed *in cellulo* by Rizzuto's group, who observed that in HeLa cells and skeletal myotubes, overexpression of VDAC, which augments the presence of VDAC in microdomains, increases the efficiency of Ca^{2+} accumulation into the mitochondrial matrix in response to agonists-stimulated Ca^{2+} release from either ER or SR (sarcoplasmic reticulum) (112). Accordingly, site of close contacts between ER and mitochondria were shown to be enriched in both IP3R/RYR (113-116) and VDAC (117, 118) proteins. In addition to VDAC, RYR and IP3R were found associated with other members of PTPC. Indeed, we have observed, by immunoprecipitation and subsequent proteomic analysis, that RYR and IP3R dynamically interact with the PTPC core protein ANT, this interaction being modulated during chemotherapy-induced apoptosis (119). Since ANT is a transmembrane protein of the inner mitochondrial membrane, one would argue that its interaction with the ER receptors should require another protein from the OM, such as VDAC, an issue that is still unresolved. Nevertheless, it was observed that after treatment of mitochondria with digitonin, some of the resulting mitoplasts (without OM) remained closely associated to ER (120), suggesting that direct interactions between ER and IM proteins, such as IP3R/ANT complexes, could participate to the linkage of the two organelles. Furthermore, the hypothesis of a direct Ca^{2+} channeling from IP3-gated channels to VDAC was recently reinforced by the demonstration that IP3R and VDAC physically interact through the molecular chaperone grp75 (glucose-related protein 75) (121). Thus, it would be interesting to determine whether IP3R-grp75-VDAC trimolecular complex facilitates only the transfer of Ca^{2+} from ER to mitochondria or plays also a role in coupling the two organelles. However, observations by electron microscopy that in DT40 IP3R knock-out cells associations between ER and mitochondria are similar to wild-type cells suggest that linkage of the two organelles still exist independently of the presence of IP3R (94).

Although the elucidation of the molecular mechanisms that underlie the connections between ER and mitochondria needs further investigations, these different findings point to PTPC as a potential "anchor" for ER proteins and suggest that this megachannel could play a fundamental role in ER-mitochondria apoptotic communication, an issue that will be analyzed in more details in the following paragraphs.

5.2. PTPC and lethal crosstalk between ER and mitochondria

The organization of ER and mitochondria in a dynamic interconnected network facilitates the exchange of metabolites and molecules that regulate numbers of physiological processes of these organelles. In this review, we limit ourselves to discuss some of the exciting results that describe the role PTPC in the lethal ER-mitochondria crosstalk. Thus, one of the most critical questions is: how ER and mitochondria communicate to induce cell death? Based on the observations that ER is the major intracellular Ca^{2+} store and that this ion is a well-known inducer of apoptosis and necrosis in many cell systems (reviewed in (122)), its role in the lethal crosstalk between ER and mitochondria has been the matter of intense investigation during last decades.

In the 1950s-1960s, it was first demonstrated that mitochondria can take up Ca^{2+}, leading to the emerging concept that mitochondria could contribute to Ca^{2+} homeostasis ((123-125) and for a historical review see (126)). More recently, the development of fluorescent tools that specifically target mitochondria, such as Ca^{2+}-sensitive photoprotein aequorin (127), fluorescent Ca^{2+} indicator Rhod-2 (128, 129) and GFP-based fluorescent probes pericams and cameleons (130, 131), allowed to demonstrate the capacity of mitochondria to promptly and transiently accumulate Ca^{2+} within their matrix (and then release it) in response to stimulation of ER/SR receptors (127, 132-137). This mitochondrial uptake of Ca^{2+} released

from ER is thought to be facilitated by the existence of microdomains of close appositions of the two organelles, as discussed in the previous paragraph (106). A major breakthrough in the understanding of the mechanisms underlying the lethal interplay between ER and mitochondria was the discovery that massive and sustained accumulation of Ca^{2+} in mitochondria can lead to MPT. In fact, the concept of MPT has emerged from a pioneer work of Hunter *et al.* (138) which reported that in purified mitochondria, Ca^{2+} induces a configurational transition from the aggregated to the orthodox state, associated with a non specific increase in the permeability of the inner membrane. Thereafter, different groups have demonstrated that Ca^{2+}-dependent MPT results from opening of a voltage-dependent, CsA- and ADP-inhibitable, high-conductance mitochondrial membrane channel (see (139) for complete review), which was identified as PTPC by Szabo and Zoratti (140, 141). In summary, these different findings indicate, on one hand, that Ca^{2+} release by IP3R or RYR may be taken up by mitochondria, where it can generate a PTPC-dependent Ca^{2+}-induced Ca^{2+}-release transient (mCICR) that propagates Ca^{2+} signals from one mitochondrion to another (142), and on the other hand that sustained accumulation of Ca^{2+} in mitochondria leads to PTPC-dependent MPT. Therefore, Ca^{2+} seems to be a primary language of the lethal ER-mitochondria crosstalk and PTPC was suggested to be one of its main targets.

As mentioned in the introduction, in case of severe and long lasting ER stress, ER homeostasis cannot be restored and death process is triggered. ER stress can be pharmacologically induced by ER-targeted toxins that impair ER function such as tunicamycin (TN, inhibitor of N-glycolsylation) and brefeldin A (BFA, inhibitor of the transport of proteins from ER to Golgi apparatus), or perturb lumenal Ca^{2+} homeostasis, such as thapsigargin (TG, inhibitor of SERCA pumps) or A23187 (Ca^{2+} ionophore). ER stressors were demonstrated to induce mitochondrial alterations, conclusively indicating that ER stress may activate the mitochondrial pathway of apoptosis (143-150). For instance, in human cancer cells, A23187, TN, TG and BFA were shown to triggers the signs of MMP, including mitochondrial relocalisation of Bax, dissipation of $\Delta\Psi_m$ and release of apoptogenic factors (cytochorme c, AIF) (144, 150). Moreover, different laboratories have observed a protection conferred by the PTPC inhibitor CsA against thapsigargin- or A23187-induced MMP and apoptosis (149, 151-154). It was therefore tempting to assume that ER stress-mediated MMP and subsequent apoptosis could result from opening of PTPC.

To explore the functional links between ER-released Ca^{2+}, PTPC and mitochondrial alterations during ER stress, we have monitored $[Ca^{2+}]_m$ and the opening of PTPC, by using the permeant Ca^{2+}-sensitive probe Rhod2-AM and the calcein-cobalt assay (155-158), respectively. This latter method relies on the loading of cells with the fluorescent probe calcein (MW=620Da) and its quencher cobalt (Co^{2+}). When loaded into cells calcein distributed within all cell compartments, whereas Co^{2+} is exclude from mitochondrial matrix due to the impermeability of the IM. Consequently, under physiological conditions mitochondria fluoresce, while upon PTPC opening, the calcein is no longer entrapped in the matrix and its fluorescence is quenched by the Co^{2+}, a phenomenon that can be recorded by flow cytometry (Figure 2). We observed that ER stress agents, which generate a sustained accumulation of Ca^{2+} into mitochondria, led to PTPC opening, MMP and subsequent nuclear apoptosis, whereas physiological stimuli such as the IP3-mobilizing agent histamine, which triggered only a transient increase in $[Ca^{2+}]_m$, did not cause mitochondrial alterations nor apoptosis (150). Thus, PTPC appears to play a key role in the Ca^{2+}-dependent apoptotic communication between ER and mitochondria during ER stress. By taking advantage of cell lines overexpressing Bcl-2 or vMIA (viral mitochondrial inhibitor of apoptosis, encoded by the Cytomegalovirus UL37 gene) or knock down for Bax or Bak, we demonstrated that (i) Bcl-2 and vMIA prevent ER stress-mediated PTPC opening and (ii) Bax invalidation decreased MPT, whereas Bak invalidation did not modify the response to ER stressors (150). Accordingly, a protection against ER stress-mediated MMP was observed in other cell systems after overproduction of Bcl-x_L or vMIA (144). The protective effect of vMIA may rely on its ability to interact with the PTPC member ANT (but not VDAC) (159) or to sequester Bax in an inactive form at the mitochondria (160, 161). Members of Bcl-2 family are well-known regulators of MMP, which directly interact with mitochondrial membranes to control the release of IMS proteins (162). In the apoptotic signaling pathway triggers by ER-dependent Ca^{2+} mobilization, Bcl-2 family proteins are thought to regulate cell death not by acting on mitochondria but through their localization at the ER. Indeed, Bcl-2_{ER} seems to protect cell against Ca^{2+}-mediated MMP by reducing the amount of ER releasable Ca^{2+} (163, 164) and/or by functionally interacting with IP3R (165, 166). Conversely, Bax_{ER} could sensitize cells to ER stress by increasing the pool of Ca^{2+} releasable from the ER (167, 168), probably by regulating the phosphorylation state of IP3R (169). A pharmacological approach allowed us to better define the molecular mechanisms of Ca^{2+} transfer and the role of PTPC in the lethal dialogue between ER and mitochondria in whole cells. In addition, to study these processes free from all of the complex side reactions that occur in cells, we have developed an original cell-free system in which we can confronted gradient-purified mitochondria and ER vesicles purified by ultracentrifugation (150). These pure organelles can be isolated from mouse liver or brain or from human cancer cell lines. In both whole cells and cell-free system treated with ER stress agents (e.g. TG, TN, A23187), we demonstrated that BAPTA-AM (a Ca^{2+} chelator) and 2-APB (2-AminoethoxydiPhenyl Borate (170)), an inhibitor of IP3R, led to partial inhibition of ER stress-mediated Ca^{2+} entry in mitochondria and nuclear apoptosis, in accord with the well-known role of IP3R in Ca^{2+}-dependent apoptosis (reviewed in (171)). Besides, 2-APB also delays PTPC opening, mitochondrial swelling and dissipation of $\Delta\Psi_m$, indicating the important role of IP3R in PTPC-dependent mitochondrial apoptosis triggers by Ca^{2+} accumulation into mitochondria. To enter the mitochondrial matrix Ca^{2+} moves from the cytosol to the IMS across the OM and then

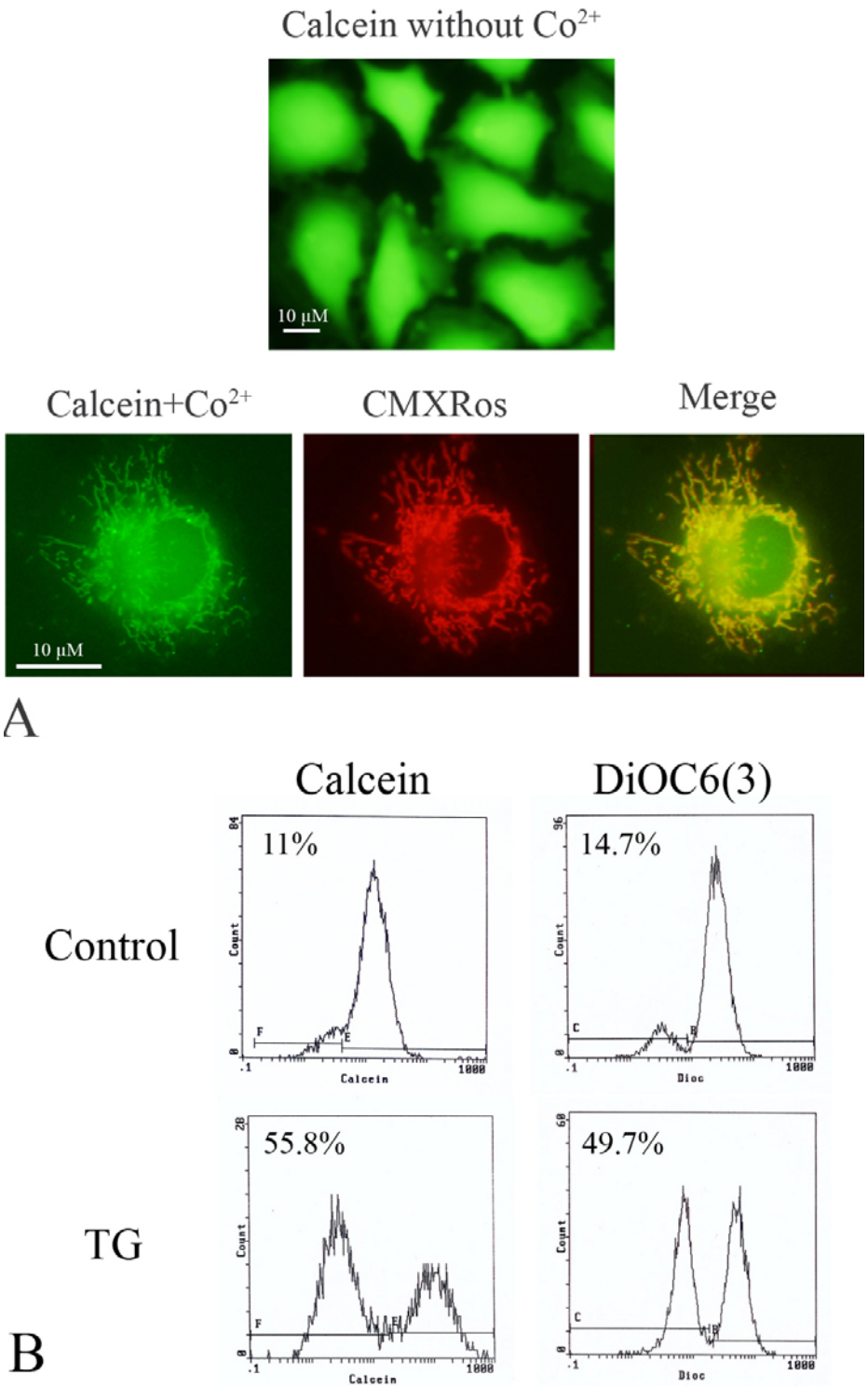

Figure 2. Measurement of PTPC opening by calcein/cobalt assay. HeLa cells were incubated for 15 min with 1 µM calcein without Co^{2+} (A, upper panel) leading to a cytosolic diffuse green fluorescence. When 100 µM of Co^{2+} is added, the cytosolic fluorescence is quenched and mitochondria appeared as a bright network. The green calcein fluorescence perfectly fits with the red fluorescence of the Mitotracker CMXRos as observe on merge micrograph (A, lower panel). (B) Cytometric analysis of cells stained with calcein/Co^{2+} or DiOC6(3) (mitochondrial potential probe) left untreated (control) or incubated with the ER toxin thapsigargin (TG). Percentages represent cells with low calcein fluorescence (calcein) or with low $\Delta\Psi_m$ (DiOC6(3)). White bars correspond to 10 µM.

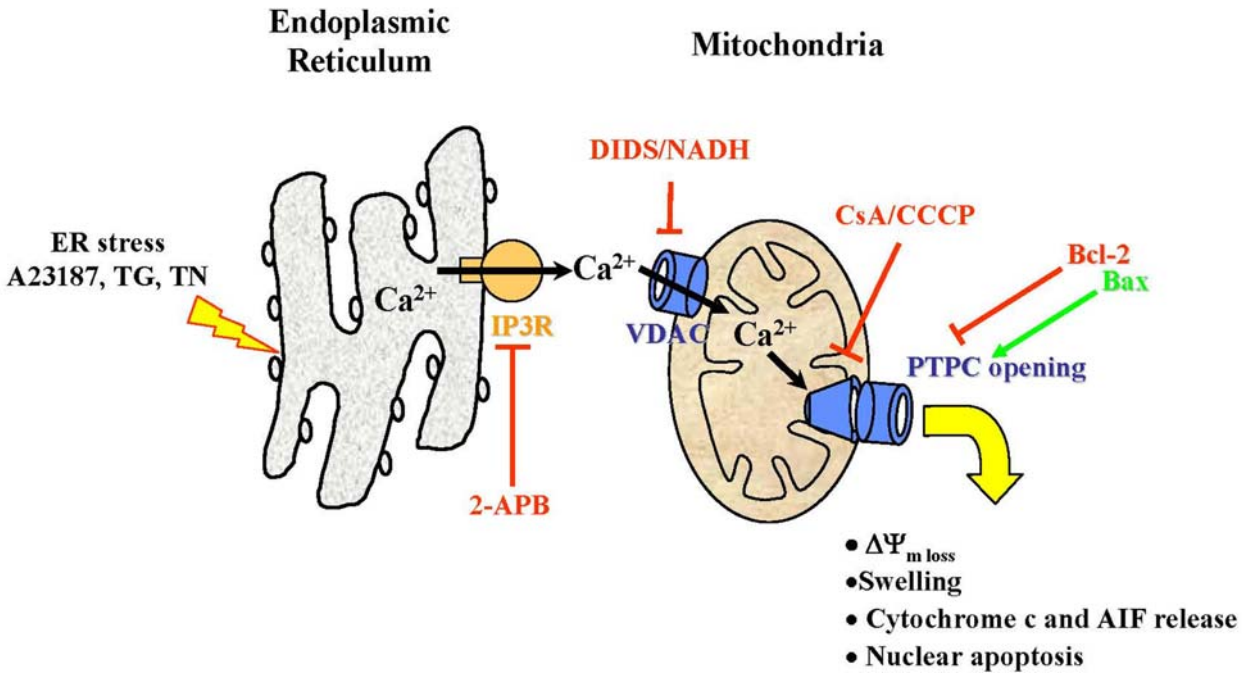

Figure 3. PTPC-dependent apoptotic signaling between ER and mitochondria in response to ER stress. Experimentally induced ER stress (A23187, TG, TN) leads to IP3R-mediated release of ER lumenal Ca^{2+}, mitochondrial accumulation through VDAC and Ca^{2+}-dependent opening of PTPC. The opening of PTPC then promotes PMM, release of apoptogenic factors and subsequent nuclear apoptosis. The proteins involved in the apoptotic pathway triggered by ER stress was determined by pharmacological studies using inhibitors of IP3R (2-APB, 2-AminoethoxydiPhenyl Borate), VDAC (DIDS, dihydro-4,4′ diisothiocyanostilbene-2,2′-disulphonic acid and NADH) and PTPC (CsA, cyclosporine A and the protonophore CCCP, carbonyl cyanide 3-chlorophenylhydrazon). The anti-apoptotic protein Bcl-2 was demonstrated to inhibit PTPC opening in response to ER stress, whereas this latter process was favored by the pro-apoptotic protein Bax.

from the IMS to the matrix across the IM. Evidence has been presented that Ca^{2+} delivery across IM occurs through the Ca^{2+} uniporter, a possibly gated channel, whom molecular identity and nature remain unknown (172, 173). We have seen in the previous section that Ca^{2+} can cross the OM through VDAC (111, 112, 174). In the presence of VDAC blockers, such as DIDS (4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid) and NADH (in the cell-free system), mitochondrial Ca^{2+} uptake, PTPC opening, mitochondrial alterations and nuclear apoptosis are significantly reduced. Our results therefore demonstrate that VDAC can efficiently participate in IP3R-released Ca^{2+} entry into mitochondria in response to ER stressors TG and TN, *in cellulo* as well as in cell-free system. By contrast, it appears that Ca^{2+} accumulation in mitochondrial matrix does not involved other core proteins of PTPC since inhibitors of ANT (BA, Bongkrekic acid) and cyclophilin D (CsA) were unable to modify mitochondrial Ca^{2+} uptake. On the other hand, BA and CsA, which prevent PTPC opening, also reduced mitochondrial swelling, loss of $\Delta\Psi_m$ and hypoploidy induced by ER toxins, indicating that PTPC is required for ER-stress induced mitochondrial alterations and apoptosis. Along the same lines, knockdown of cyclophilin D by siRNA significantly inhibits Ca^{2+}-dependent MMP and cytochorme c release provoked by TG treatment of human leukemic cells, corroborating the important role of PTPC members in ER stress-induced apoptosis (175). In summary, when functions (TN) or Ca^{2+} homeostasis (TG) of the ER is severely perturbed, a stress response is activated and a PTPC-dependent apoptotic dialogue is engaged between ER and mitochondria (Figure 3): Ca^{2+} released from the ER through IP3R is taken up by mitochondria via VDAC and/or other OM channels, accumulates then in the matrix where it stimulates the opening of PTPC, which in turn promotes mitochondrial swelling, rupture of the OM and liberation of apoptogenic factors.

In conclusion, the data discussed above settled Ca^{2+} and PTPC as key components of the lethal crosstalk between ER and mitochondria. Nevertheless, one can not rule out that signaling molecules other than Ca^{2+} ion could be released from ER to target PTPC and induce apoptosis. In agreement, we have observed that the ER stressor BFA provokes the opening of PTPC and subsequent mitochondrial apoptosis without inducing Ca^{2+} release from ER (Figure 4). The nature of the molecule(s) involved in BFA-mediated lethal communication between ER and mitochondria is still unidentified and requires further investigation. In addition, renewed attention has been recently given to the gangliosides, as intracellular messengers of cell death and potential mediators of apoptosis in response to ER stress (176). For example, among the different gangliosides, GD3, found at the ER-mitochondria contacts sites (177), was demonstrated to

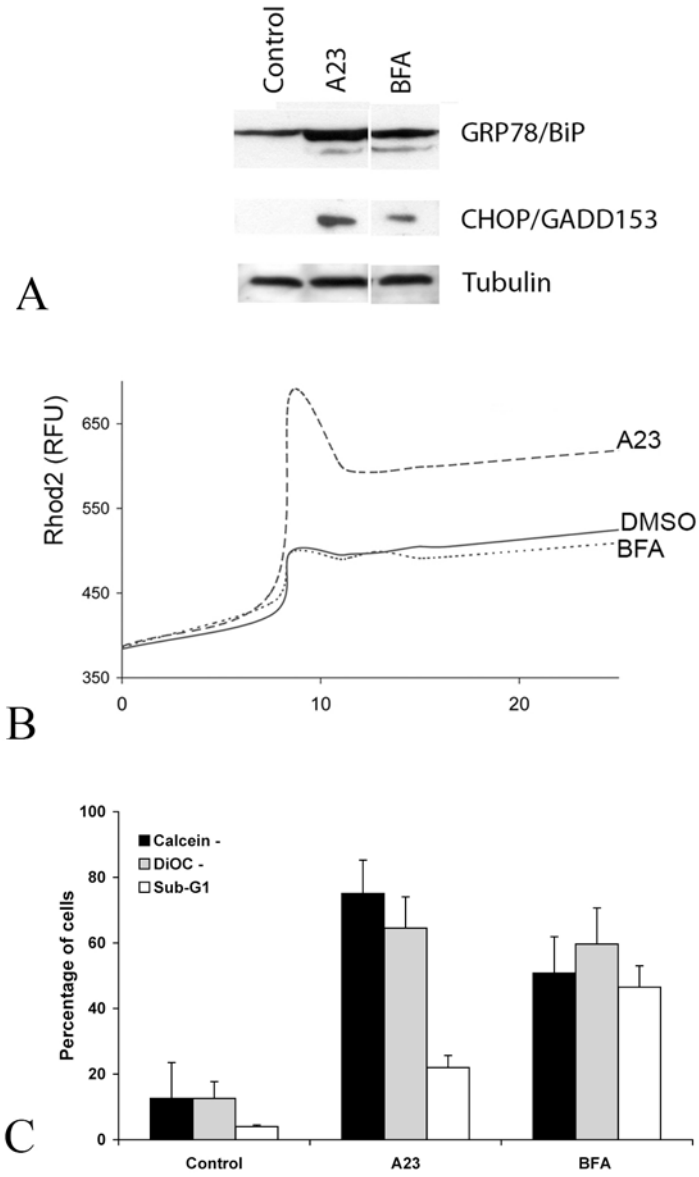

Figure 4. BFA-induced ER stress triggers the opening of PTPC without inducing Ca^{2+} release. (A) A23187 (A23) and brefeldin A (BFA) triggers overexpression of ER stress markers GRP78/BiP and CHOP/Gadd153. (B) Although BFA does not induced mitochondrial Ca^{2+} uptake compared to A23 (B, increase in Rhod-2 fluorescence), both ER stressors triggers PTPC opening (calcein -), loss of $\Delta\Psi_m$ (DiOC-) and nuclear apoptosis (Sub-G1).

promote opening of PTPC in a Ca^{2+}-independent manner (178) and thus appears as an interesting signaling molecule of the PTPC-mediated ER-mitochondria lethal interplay. However, future research will be fundamental in better understanding the molecular mechanisms that connect PTPC to the apoptotic crosstalk between ER and mitochondria.

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Abbreviations : AIF: Apoptosis-Inducing Factor; ANT: adenine nucleotide translocase; BFA: brefeldin A; Ca^{2+}: calcium; CypD: cyclophilin; $\Delta\Psi_m$: mitochondrial membrane potential; DRP-1: dynamin-related protein 1; ER: endoplasmic reticulum; IMS: intermembrane space; MMP: mitochondrial membrane permeabilization; MPT: mitochondrial permeability transition; IP3R : Inositol 1,4,5-trisPhosphate Receptor; OM: outer membrane; IM; inner membrane; MOMP: mitochondrial outer membrane permeabilization; PTPC: mitochondrial permeability transition pore complex; RY: ryanodine receptor; TN: tunicamycin; TG: thapsigargin; UPR: unfolded protein response; VDAC: voltage-dependent anion channel.

Key Words: PTPC, Endoplasmic Reticulum, Mitochondria, Apoptosis, Calcium, Review

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