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PITFALLS IN THE USE OF ELECTRON MICROSCOPY TO STUDY THE MITOCHONDRIAL MEMBRANE PERMEABILITY TRANSITION IN APOPTOTIC CELLS AND PELLETS: WHERE DO WE STAND IN RELATION TO THE INCIDENCE OF MITOCHONDRIAL SWELLING IN APOPTOSIS?

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ABSTRACT

The importance of apoptosis as a form of programmed cell death was recognized in the 1980s, whereas the central role of mitochondria in controlling this process was identified in the mid-1990s. An important event in apoptosis is the collapse of the mitochondrial transmembrane potential ($\Delta\Psi m$), with the ensuing loss of the selective permeability of the inner membrane resulting in swelling of the hyperosmolar mitochondrial matrix. This event is known as the mitochondrial permeability transition (MPT). After swelling of the intermembrane space, the outer membrane ruptures, exposing the permeable inner membrane. An increasingly swollen matrix covered by the inner membrane eventually herniates into the cytoplasm through the breach formed in the outer membrane (OM). The increase in surface area of the inner mitochondrial membrane (IMM) involves the unfolding of membrane stored in the cristae. This membrane movement is osmotically driven since the cytoplasm has a lower osmolality. The proteins partly embedded in the inner membrane are thus exposed to the cytoplasm. In nine out of ten electron microscopy studies of isolated mitochondria expressing the permeability transition, the existing ruptures of the OMM were overlooked. The MPT can also be recognized in individual mitochondria by using fluorescent probes that are not retained in these organelles once the $\Delta\Psi m$ is lost. In cases in which there is no rupture of the OMM, cytochrome c must be released from mitochondria with impermeable inner membranes. Examination of several hundred of the more than 61,000 published papers on programmed cell death revealed that the key signaling events of apoptosis, such as the onset of the MPT, mitochondrial swelling and cytochrome c release to the cytoplasm, are influenced by factors such as the cell type and presence of apoptogenic agents. These two factors need to be examined in any assertion that non-swollen mitochondria are more frequent than swollen mitochondria in apoptotic cells after opening of the mitochondrial transition pore. This view differs from data obtained so far for human and laboratory animal cells in our laboratory, and also from literature reports for these same cells.

Key words: Apoptosis, cytochrome c release, electron microscopy, membrane permeability transition, mitochondrial cristae, outer mitochondrial membrane

General considerations

This minireview deals with a theme closely related to one of the two main mechanisms that mitochondria use to release intermembrane and intracristal proteins to the cytoplasm, namely, rupture of the outer mitochondrial membrane mediated by the state of the mitochondrial permeability transition (MPT) [73]. Transmission electron microscopy (TEM) has shown that rupture of the outer membrane is very common in apoptotic cells [73]. Such rupture provides a simple, efficient means of exposing intermembrane proteins such as cytochrome c, apoptosis inducing factor (AIF) and proteins of the inner membrane involved in electron transport and oxidative phosphorylation to the cytoplasm. The alternative pathway for releasing apoptogenic intermembrane proteins from mitochondria involves the permeation or translocation of these proteins through a structurally intact outer membrane. One of the most

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widely recognized means of protein permeation or translocation through the outer membrane involves mitochondrial outer membrane permeabilization (MOMP), with rupture of the outer mitochondrial membrane being considered as a particular case of MOMP [24,47,89]. In the following discussion, these two mechanisms for releasing mitochondrial proteins to the cytoplasm will be considered as etymologically and physically distinct processes.

The intermembrane proteins released during apoptosis include the inhibitors of apoptosis proteins (IAPs) Smac/DIABLO and Omi/HtrA2 and, depending on the cell type, procaspases 2, 3 and 9 [12,69,79-81,92]. The latter activate the machinery that will mediate the intracellular events during apoptosis. Kinetic studies have suggested that different intermembrane proteins are transferred to the cytoplasm by specific channels located in the outer and/or inner membrane(s) (for further discussion, see Belizário *et al.* in this issue).

The role of mitochondria in apoptosis: a late recognition

In the early 1970s and 1980s, one of the issues about necrosis that was commonly discussed in Pathology courses was how to experimentally and conceptually establish the point of no return for cells destined to die. The concept of cell death was expanded in the classic paper by Kerr, Wyllie and Curie in 1972 [40]. In addition to necrosis, cells may die silently, but always with the same fine structural features. Indeed, the universal phenotype of apoptotic cells indicated that the activation of a common set of molecules was involved in cell death. Judging by the large number of reports on apoptosis published yearly, with ~61,000 papers published mainly in the last two decades [33], the original report by Kerr *et al.* [40] is unprecedented in the biomedical literature. The lack of attention given to structural alterations in the mitochondria of apoptotic cells [40,41] resulted in these organelles being considered as secondary players in this phenomenon until the mid 1990s.

The finding that apoptotic nuclear changes required the presence of mitochondria [61] was an important advance in our understanding of the role of these organelles in apoptosis. This observation was strengthened by the pioneering contributions of French research groups who reported that a decrease in the mitochondrial potential was an early step in apoptosis and that the MPT was a critical event in this type of cell death [55,64,90,91]. These findings and the discovery of the role of intermembrane cytochrome c in inducing apoptosis [51], together with the demonstration that cytochrome c interacted with cytoplasmic proteins [25], highlighted the central role of mitochondria in promoting the onset of apoptosis.

Inappropriate mention of mitochondrial outer membrane rupture

The lack of any mention of mitochondrial alterations in apoptotic cells by Kerr *et al.* [40], and statements such as "The cell atrophies, but mitochondria and other organelles remain physically intact [13]," encouraged some authors to consider the absence of mitochondrial alterations as a hallmark of apoptotic cells [34]. A further complicating factor is the lack of structural data in several reports that supposedly investigated rupture of the outer mitochondrial membrane [19,22,23,27,69].

Early elucidative references on the transmission electron microscopy of mitochondria

The studies of Allen *et al.* [2], Galili *et al.* [20] and Morris *et al.* [60] were among the first to provide electron micrographs of swollen mitochondria in apoptotic lymphoid cells. Galili *et al.* [20] made the important observation that apoptotic cells show cytoplasmic decompartmentalization followed by chromatin condensation. We have also frequently observed this phenomenon in various cell types, but it is often not mentioned in reports on the fine structure of apoptotic cells. Cytoplasmic decompartmentalization is indirect evidence of early alterations in the cytoskeleton of apoptotic cells.

Rupture of the outer mitochondrial membrane was a viable conjectural possibility before it was acutally demonstrated by TEM [4]. In the early stages of apoptosis, mitochondria become progressively more swollen and the capacity of the outer membrane for distension is more limited than that of the inner membrane, because of its much larger surface [65,66]. This idea is discussed in detail by Marzo *et al.* [57], who predicted a structural sequence that matched with our results showing that the initial rupture of the outer membrane is small, and that a junction-like region is formed between both membranes (A. Sesso, unpublished observations). These authors anticipated that the "Bax-ANT complex could form a gap junction-like conduit at the inner-outer mem-

brane contact site or regulate the permeability of the outer and inner mitochondrial membranes in a coordinate fashion, or both". A disturbance "in volume homeostasis of the mitochondrial matrix could provoke a local mechanical disruption of the outer membrane" [57].

The mitochondrial permeability transition (mpt)

A considerable amount of biochemical and physiological information was known about mitochondria before the recognition of their importance in apoptosis. Haworth and Hunter [31,32,35,36] showed that the sudden increase in the permeability of the inner membrane of isolated mitochondria to solutes of up to 1500 kDa was a consequence of the opening of a pore or megachannel located between both mitochondrial membranes. This change in permeability was referred to as a mitochondrial permeability transition (MPT) and the pore was known as a permeability transition pore (PTP). Isolated mitochondria in the PT state are swollen. In these early electron microscopic observations, as well as in some quite recent reports (see below), the inner mitochondrial membrane was reported to have disappeared while the outer membrane was maintained [3,35]. Pore opening increases the permeability of the mitochondrial inner membrane, leading to the equilibration of ions between the matrix and the intermembrane space, with uncoupling of the respiratory chain and loss of the H^+ gradient across the inner membrane. The maintenance of this gradient is essential for the synthesis of ATP. Another associated alteration is the expansion of the hyperosmolar matrix caused by the increased influx of fluid. This concept of pore opening is now broadly accepted [7,94], and there is evidence that the opening of a single pore is sufficient to cause collapse of the transmembrane potential during the onset of MPT [94].

Mitochondria with a ruptured outer membrane (mrom) are frequent in apoptotic cells

The paper by Vander Heiden *et al.* [83] is frequently quoted as showing rupture of the outer mitochondrial membrane. However, in their Figs 3 they show, in early apoptotic cells, mitochondria with the shape of maximal amplitude swelling with ruptured outer membrane. The ensuing release to the cytoplasm of cytochrome c is shown in their Fig. 1A. An analysis of the procedures and results shown, does not permit to discard that the data they presented represent the first demonstration that MPT precedes the release of mitochondrial intermembranous proteins to the cytoplasm. These authors presented a micrograph (Fig. 3C of their manuscript) of a limited section of a mitochondrion with a questionable [68] and never reproduced discontinuity of the outer membrane. Curiously, like other reports discussed below, the authors apparently overlooked the fact that their Fig. 3B exhibited extremely swollen mitochondria with a ruptured outer membrane. The morphology of mitochondria with a ruptured outer membrane (MROM) in apoptotic cells was described by Angermüller *et al.*

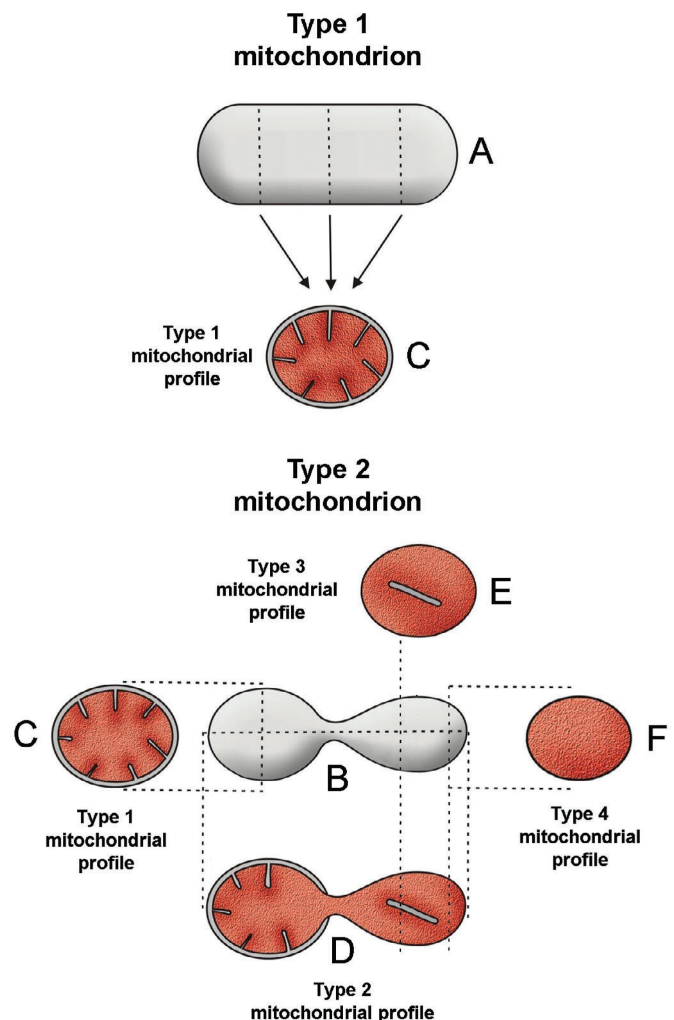

Figure 1. Scheme showing that a mitochondrion with a ruptured outer membrane (type 2 mitochondrion) may yield four types of sections, depending on the angle of incidence. In the absence of a ruptured outer membrane (type 1 mitochondria), all of the mitochondrial sections show both membranes.

[4], Kwong *et al.* [48] and Feldemann *et al.* [15]. The latter authors also observed that the mitochondria of the apoptotic hepatocytes they analyzed were in a state of permeability transition. The same profiles were observed in TEM images taken in parallel with the electron microscopic tomographic reconstruction of mitochondria, in apoptotic cells *in situ* [59] or after isolation and exposure to various treatments [18,70]. In these six papers [4,15,18,48,59,70], the mitochondria had a swollen matrix covered by an expanding inner membrane that herniated into the cytoplasm.

Depending on the angle of sectioning, a mitochondrion with ruptured outer membrane may show one of four profiles (Figs. 1 and 2A). Profile type 2 has a ruptured outer membrane (Fig. 1D and the profiles numbered 2 in Fig. 2A and following figures). One of the extremities of these hour-glass-like profiles is covered by both mitochondrial membranes, while the opposite pole is covered only by the inner membrane. In profile types 3 and 4, the inner membrane bordered a swollen matrix with or without remnants of cristae (Fig. 1E and F, respectively. Profile 3 in Fig. 2A was derived from a plane passing orthogonally through CG whereas profile 4 was derived from a plane that passed through EF). Profile 1 may be derived from a normal mitochondrion or from a type 2 mitochondrion (Fig. 1C, while the profile in Fig. 2 may or not be derived from a plane that passed through AE).

Curiously, the report by Vander Heiden *et al.* [83] has been amply cited as showing MROM whereas the six papers mentioned above have generally not been cited when rupture of the outer membrane is considered as one of the alternatives to explain the release of apoptotic mitochondrial proteins into the cytoplasm. A preliminary report of our findings of mitochondria with a ruptured outer membrane in various types of cells in apoptosis was presented in a meeting of the Brazilian Society for Electron Microscopy in 1998 [72]. The general morphology of mitochondria with a ruptured outer membrane that we had thoroughly examined [73] was identical to that described by the six reports mentioned above.

Type 2 or 3 mitochondrial profiles have been observed in five types of apoptotic cells in rat tissues and in cultured cells such as HeLa, HL-60, K-562, LLC-WRC-256, PC-12, WEHI-3 and WEHI-164 [73]. When WEHI-3 and K-562 cells were exposed to various drugs, in some cases there was no type 2 mitochondrial profile in apoptotic cells. However, type 2 mitochondrial profiles were observed in WEHI-3 cells treated with novobiocin, the teneposide VM26 and vimblastine but not with okadaic acid. K-562 cells exposed to brefeldine A, VM 26 and thapsigargin also showed type 2 profiles, while those treated with vimblastine, oligomycin and nigericin did not. Mitochondria with a ruptured outer membrane were not seen with drugs that caused marked structural changes, such as a reduction in mitochondrial size and the formation of a hyperdense matrix.

Mitochondria with a ruptured outer membrane (MROM) are generally not identified in TEM images of pelleted mitochondria in the permeability transition state

The configuration of MROM *in situ* [4,15,48,73] can only be explained by recognizing that the inner mitochondrial membrane has lost its selective permeability and allows the influx of fluid into a progressively more swollen mitochondrial matrix. In only one of ten papers published up to 2006 in which TEM was used to describe the morphology of isolated mitochondria undergoing a permeability transition was rupture of the outer mitochondrial membrane clearly stated and shown [9]. In another paper, this rupture, although not mentioned in the text, can be seen by using a magnifying lens to examine the micrographs directly in the journal page [67]. These ruptures were not clear in the website images.

In several papers [3,75,76,82,85], isolated mitochondria undergoing PT have been reported to lose their inner membrane, with the outer membrane remaining intact. These papers initially cite Almofti *et al.* [3] as a source of similar observations (but see below). Should this loss of membrane be the case, then mitochondria would have only one membrane delimiting a space with or without remnants of cristae, as occurs with mitochondrial profiles 3 and 4. The lack of an inner membrane would agree with the loss of the permeability of this organelle. In none of these reports is rupture of the outer membrane mentioned.

The report by Almofti *et al.* [3] illustrates what has happened to the interpretation of electron micrographs of this phenomenon. These authors wrote that in mitochondria swollen by Ca^{2+} "the structure of the inner membrane disappeared as reported previously [references 19, 28 and 29 which they cited correspond to references 36, 6, and 39 in the present paper].

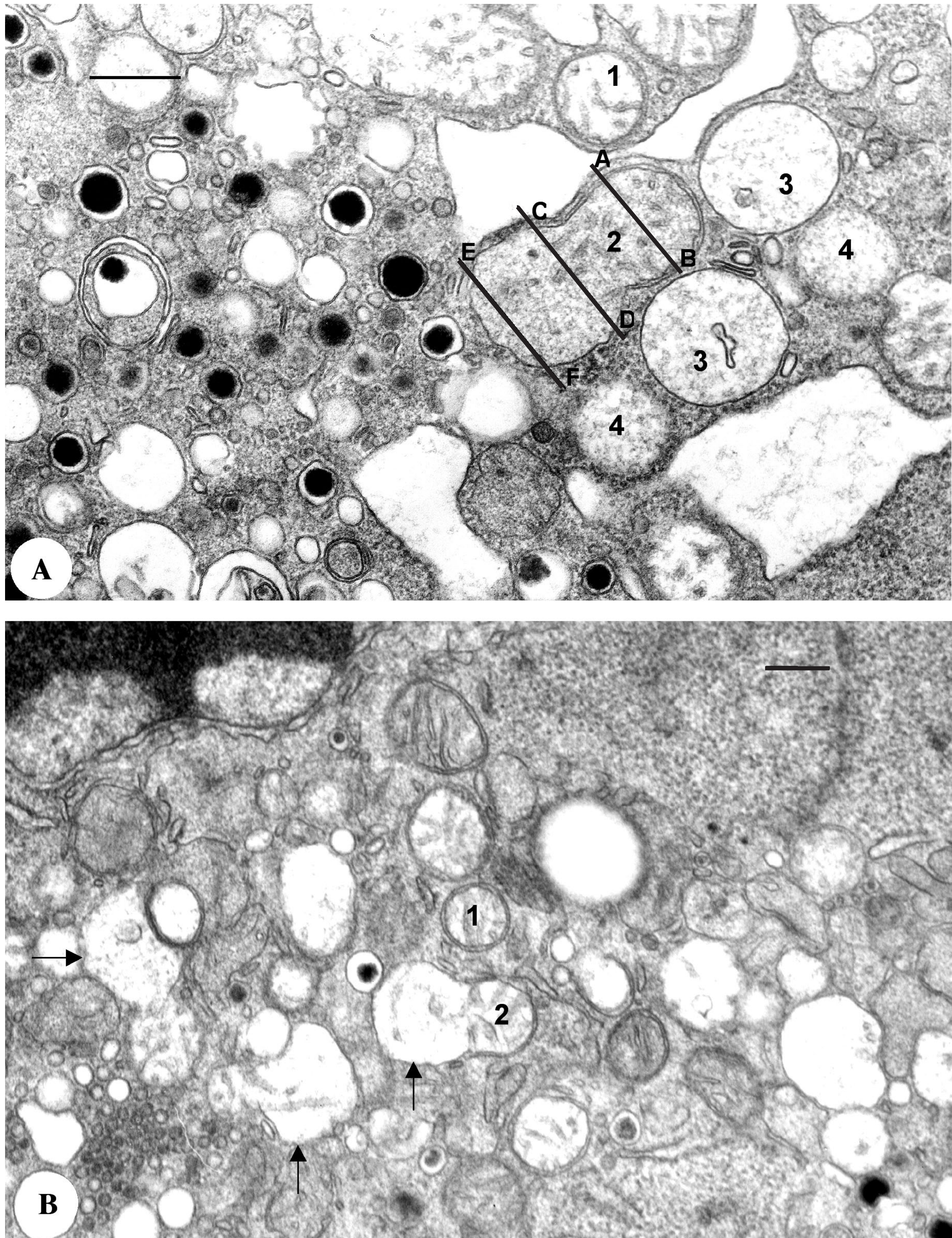

Figure 2. PC-12 cells deprived of serum for 8 and 16 h. Panel **A** illustrates how a type 2 mitochondrion may give rise to four types (1-4) of profiles. Panel **B** shows three type 2 mitochondrial profiles with a very large area of the matrix covered by the inner membrane (**arrows**). Most of the exposed inner membrane is derived from the matrix. The type 3 profiles (numbered 3) contain remnants of cristae. Bar = 0.5 µm

However, mitochondria treated with Ag^{+} had novel configurations, in which there was less increase in mitochondrial volume, and the structure of the inner mitochondrial membrane did not completely disappear (Fig. 6C)". What did not completely disappear was the outer mitochondrial membrane which was shown only partially, while the inner membrane was intact throughout its course. When the degree of swollenness was lower, as referred to in Fig. 6C, the authors detected profiles containing both mitochondrial membranes. This assertion indicates that the maximal amplitude of swelling was one of the factors involved in disruption, and that this swelling was eventually hindered by the compression of both membranes, resulting in the type 2 mitochondrial profile.

Examination of the three papers cited by Almofti *et al.* [3] revealed no mention of the disappearance of the inner mitochondrial membrane. Figure 6B in Almofti *et al.* [3] illustrates similar findings to those in Fig. 4B in [75], Fig. 2B in [76], Fig. 5B in [85] and also Fig. 3 in [82], all of which show extremely swollen mitochondria possessing only one membrane, i.e., mitochondrial profiles types 3 and 4, and misinterpreted as being the outer mitochondrial membrane (sic). Profile types 2, 3 and 4 were probably shown in a report by Igbavboa and Pfeiffer [38] on mitochondrial fractions expressing PT.

In addition to the extreme swollenness, another fact that likely complicated the observations in isolated mitochondria was the much poorer structural preservation of these organelles compared to mitochondria with a ruptured outer membrane embedded in the cell cytoplasm *in situ* [4,15,48,73]. The general absence of type 2 mitochondrial profiles in these studies of apoptotic cells is also intriguing. In contrast, type 2 mitochondrial profiles can be seen in Figures 2 (cultured murine NS-1 apoptotic cell) and 11 and 12 (apoptotic hepatocyte bodies in macrophage and hepatocyte cytoplasm) of [42], and in Fig. 2A,B shown here.

The importance of TEM in studies of mitochondrial alterations has declined since the 1970s and 1980s, partly because of the length of time (5-7 years) required to train a good electron microscopist and partly because of the high cost of equipping and maintaining an EM laboratory [1,21,26,58]. The importance of EM is clearly illustrated by the fact that rupture of the outer membrane was observed in the three papers from laboratories with facilities for tomographic electron microscopic reconstructions of mitochondria.

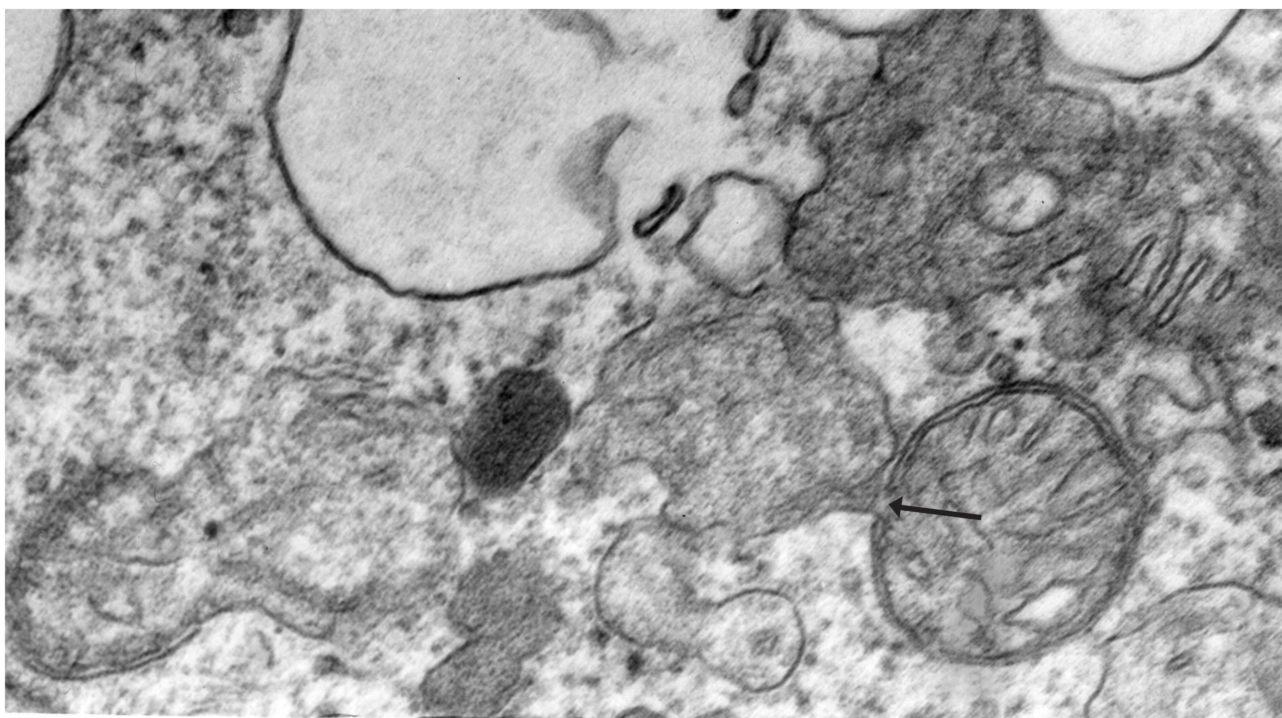

Figure 3. BHK cells exposed to 6 μM camptothecin for 16 h. The type 2 mitochondrial profile on the right shows a minute opening of the outer membrane (**arrow**). In both profiles, the external matrices covered by the inner membrane appear to have been expelled through the apertures of the outer membranes.

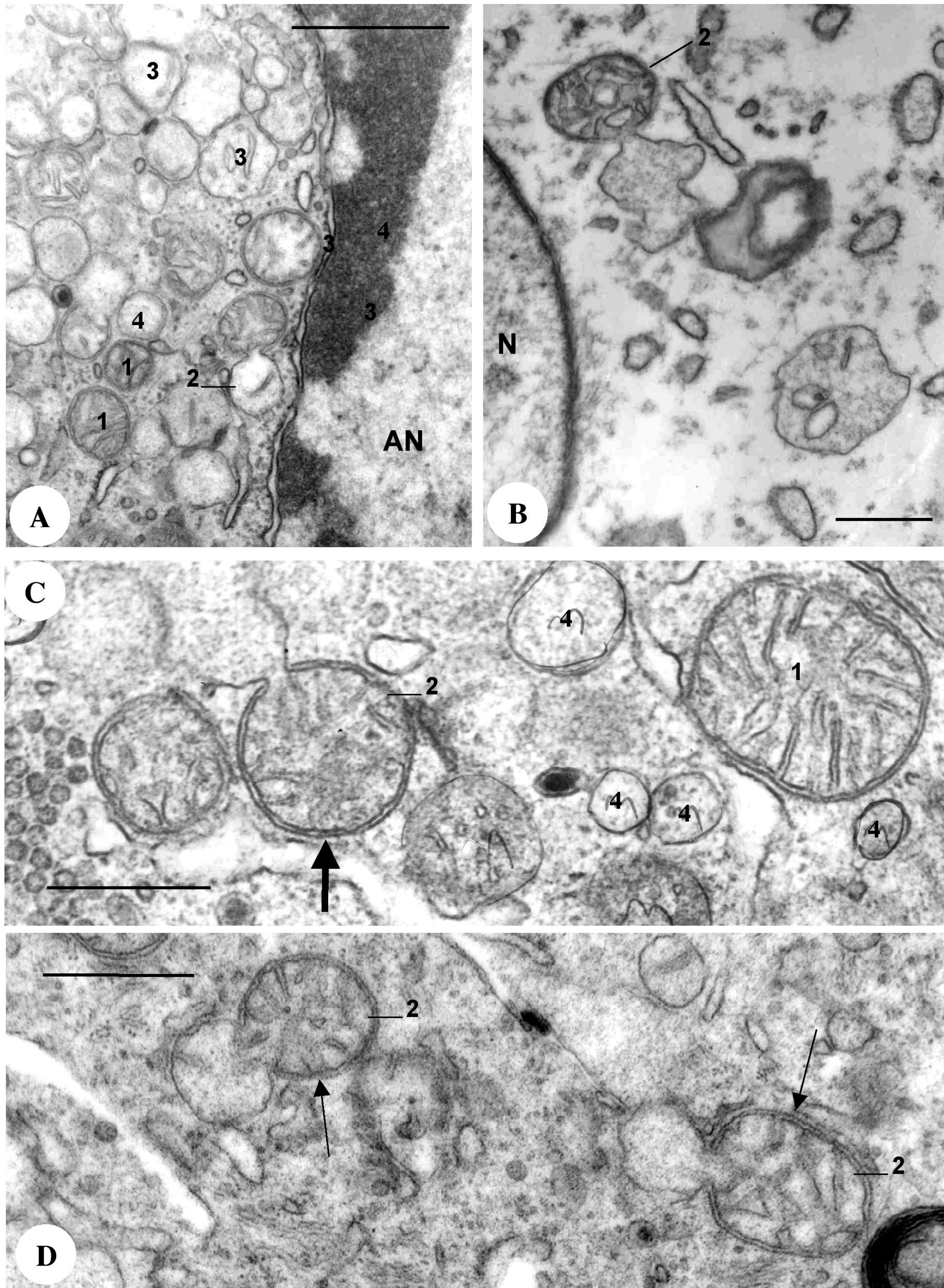

Figure 4. PC-12 cells deprived of serum for 4 (**A**), 16 (**B**) and 16 h (**C**), respectively. Panel **B** is from a non-apoptotic fibroblast exposed to 0.5 μM staurosporine for 16 h. The apertures of the outer membrane are very small in panels **A** and **B** (both indicated by **2**) but become progressively wider in panels **C** and **D**. In panel **A**, the profile types are numbered from **1** to **4**. Profile type 4 is also indicated in panel **C**. Bar = 0.5 μm

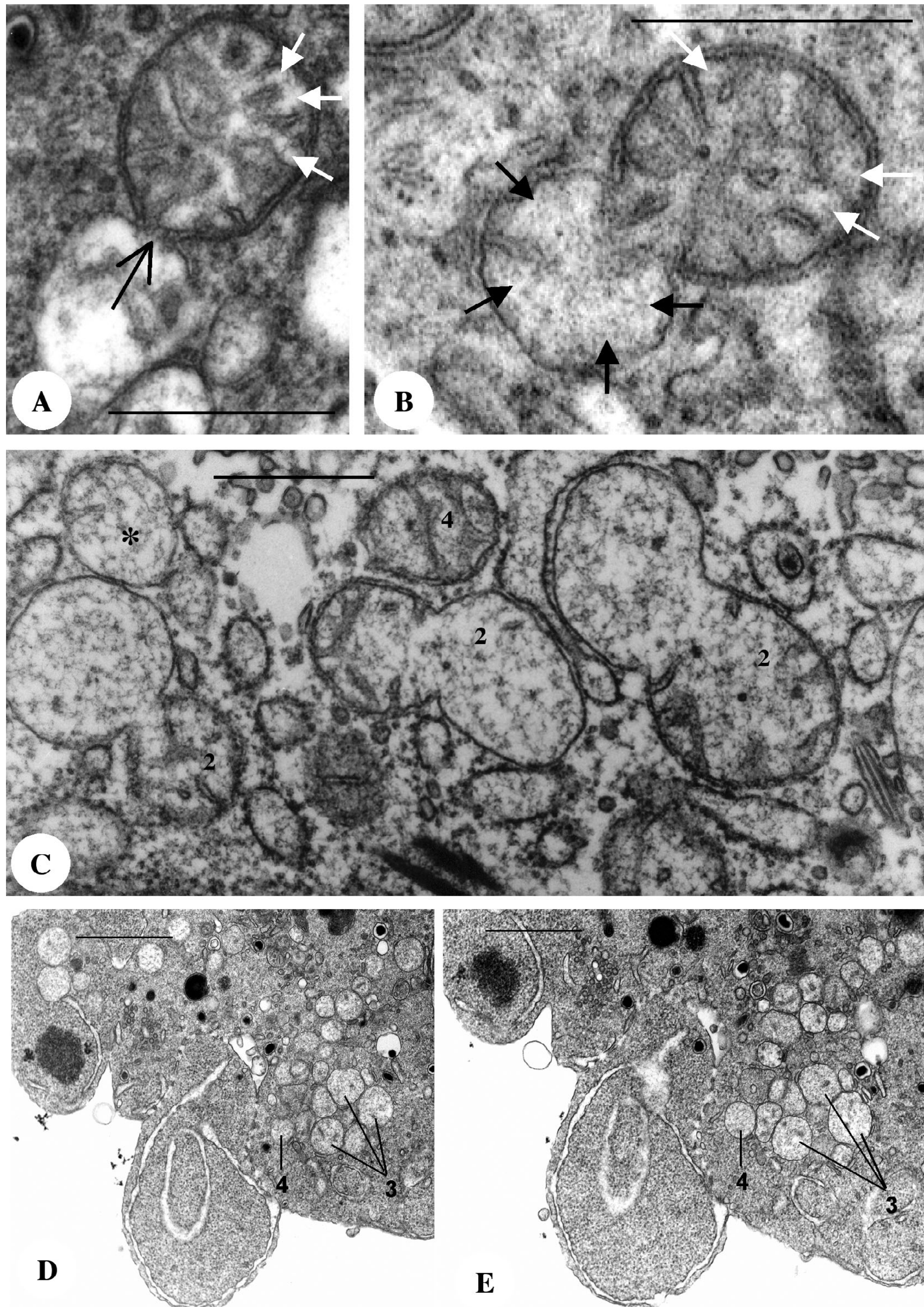

Figure 5. Panel A, PC-12 cells deprived of serum for 4 h. Panel **B** is an enlarged view of mitochondria indicated by an arrow on the left of panel 4**D**. In both panels, the matrix covered by both membranes has dense and clear regions, the latter indicated by **white arrows**. Panel **C** shows BHK cells exposed to 6 μM camptothecin for 16 h. Panels **D** and **E** are from semiserial sections through PC-12 cells deprived of serum for 8 h in which apoptotic bodies are being dismantled. The incidence of profile types 3 and 4 in these sections was extremely high, raising the possibility of mitochondrial-derived sections with only one membrane covering the matrices. These profiles were reminiscent of the profile for isolated mitochondria (Fig. 8) treated with KCl and sucrose to give an 3 mOsm. Bar = 0.5 μm

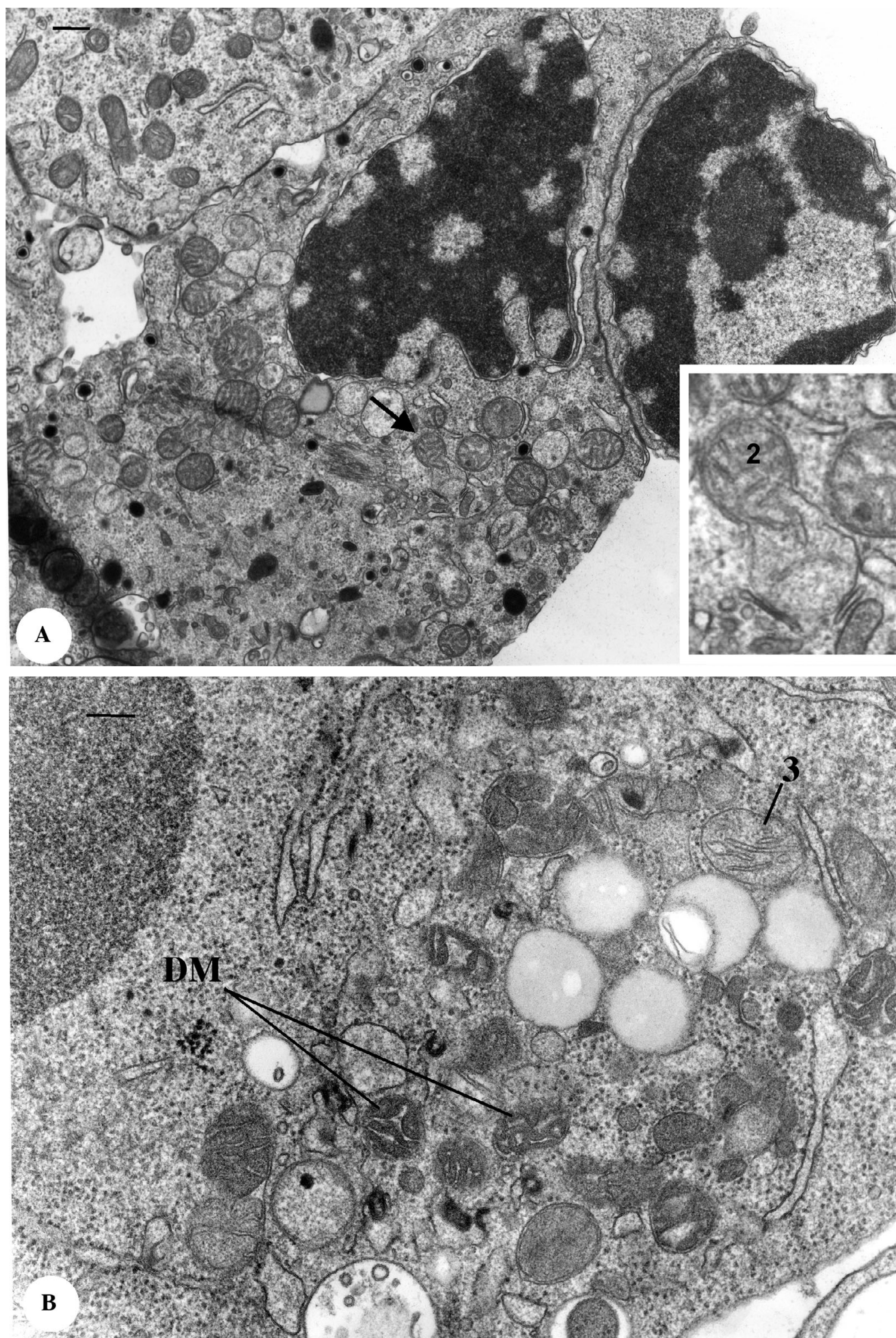

Figure 6. PC-12 cell deprived of serum for 16 h (**A**) and WEHI-3 cells exposed to 0.5 nM okadaic acid for 5 h (**B**). The cytoplasm in **A** contained numerous type 1 (common) mitochondrial profiles similar to those of non-apoptotic cells in the upper left. The **arrow** indicates a type 2-mitochondrial profile shown enlarged in the inset. In **B**, the **arrow** indicates a type 3 mitochondrial profile. This was the only indication of a ruptured outer mitochondrial membrane in this section and in the entire grid. Bar = 0.5 μm

Electron microscopy as a powerful complementary tool for analyzing the incidence of mrom in apoptotic cells *in situ*

In 8 out of 10 studies mentioned in the preceding section, there was no report of a ruptured outer mitochondrial membrane in isolated organelles expressing PT. Nevertheless, the authors declared having observed, or one can see in the published electron micrographs, mitochondrial sections with only a single membrane covering a clear, generally large area of matrix. In all of these studies, unimembranous, mitochondrial-derived profiles were described or clearly shown in the electron micrograhs. These unimembranous profiles can only have derived from MROM, as illustrated in Figs. 1 and 2A. The data shown here and the results of other studies [9,67] indicate that there is no other satisfactory explanation for these observations. Indeed, the 10 electron microscopic studies of isolated mitochondria mentioned above are more than sufficient to definitively prove that the morphology of the MPT is what is shown *in situ* in Figs. 2-7A.

Aspects of the morphology of the mpt commonly seen *in situ* and in pellets of mitochondria

The initially very small aperture of the outer membrane widens progressively after the onset of the MPT (type 2 profile identified as number 2 in Fig. 4A-D). A swollen matrix covered by the inner membrane is expelled outwardly through each of these ruptured regions of the outer membrane. Images of the initial rupture of the outer membrane suggest that once the mitochondrial matrix covered by the now permeable inner membrane is exposed to the cytoplasm, external fluid is sucked into the matrix compartment which has a higher osmolality. The region near the rupture of the outer membrane rapidly expands and additional matrix volume escapes (possibly via the same initial aperture) to the nearby cytoplasm, which has a lower osmolality. This morphology reveals the existence of the PT at the point of rupture of the outer membrane. These observations raise questions regarding the permeability of the inner membrane in reports of apoptosis in which no MROM is described and intermembranous proteins are released by channels. Our data suggest that in these cases the inner membrane must remain completely impermeable.

Although the PT occurs at the initial rupture of the outer membrane, it is unclear whether its expansion is instantaneous. Independently of the time required to compromise the entire surface of the inner membrane, it remains unclear whether external fluid gains entrance to the matrix compartment through the region covered by both membranes and whether the inner membrane is permeable. In Fig. 5A and B, the poles of the MROM covered by both membranes show dense and clear regions. The extremities of the white arrows are in the clear regions. These images are compatible with the possibility that these clear regions represent sections of matrix infiltrated with fluid that has permeated through the nearby inner mitochondrial membrane. Notice that the opening in the outer mitochondrial membrane in Fig. 5A is about the same size as the opening shown by the arrow on the right in Fig 3. Figure 5B is an enlarged view of the type 2 mitochondria profile indicated on the left of Fig. 4D. Here, the black arrows indicate the surface of fluid entrance into the region covered only by the inner membrane. This entrance of fluid into the expanding swollen matrix tends to decline because of a reduction in the surface-to-volume ratio and a decrease in the osmolality of the herniated but more voluminous swollen matrix.

Figure 5C shows three type 2 mitochondrial profiles with markedly swollen matrices. These phases precede the ultimate stage of maximal swelling illustrated in Fig. 5D and E. The latter two panels are from a series of ultrathin sections and show that there may be a preponderance of unimembranous mitochondrial profiles during apoptotic bodies formation. Figure 7A shows that swollen mitochondria are frequently the last organelles found in a terminal apoptotic cell that has lost all other membrane bound structures. Note that the cell membrane is still preserved even in this almost completely lysed cytoplasm.

Electron microscopic tomography confirms herniation of the swollen matrix through the ruptured outer membrane

Figure 3C in Frey *et al.* [18] obtained by electron microscopic tomography of a mitochondrion with a PT shows that "the matrix has swollen causing the inner membrane to rupture the outer membrane" and herniate outside of the organelle. A very informative electron microscopic tomography on the herniatiation of the mitochondrial matrix covered by the inner mitochondrial membrane is shown in Fig 6C of Scorrano *et al.* [70]. The transmission electron microscopic images of mitochondria in Fig. 5A from

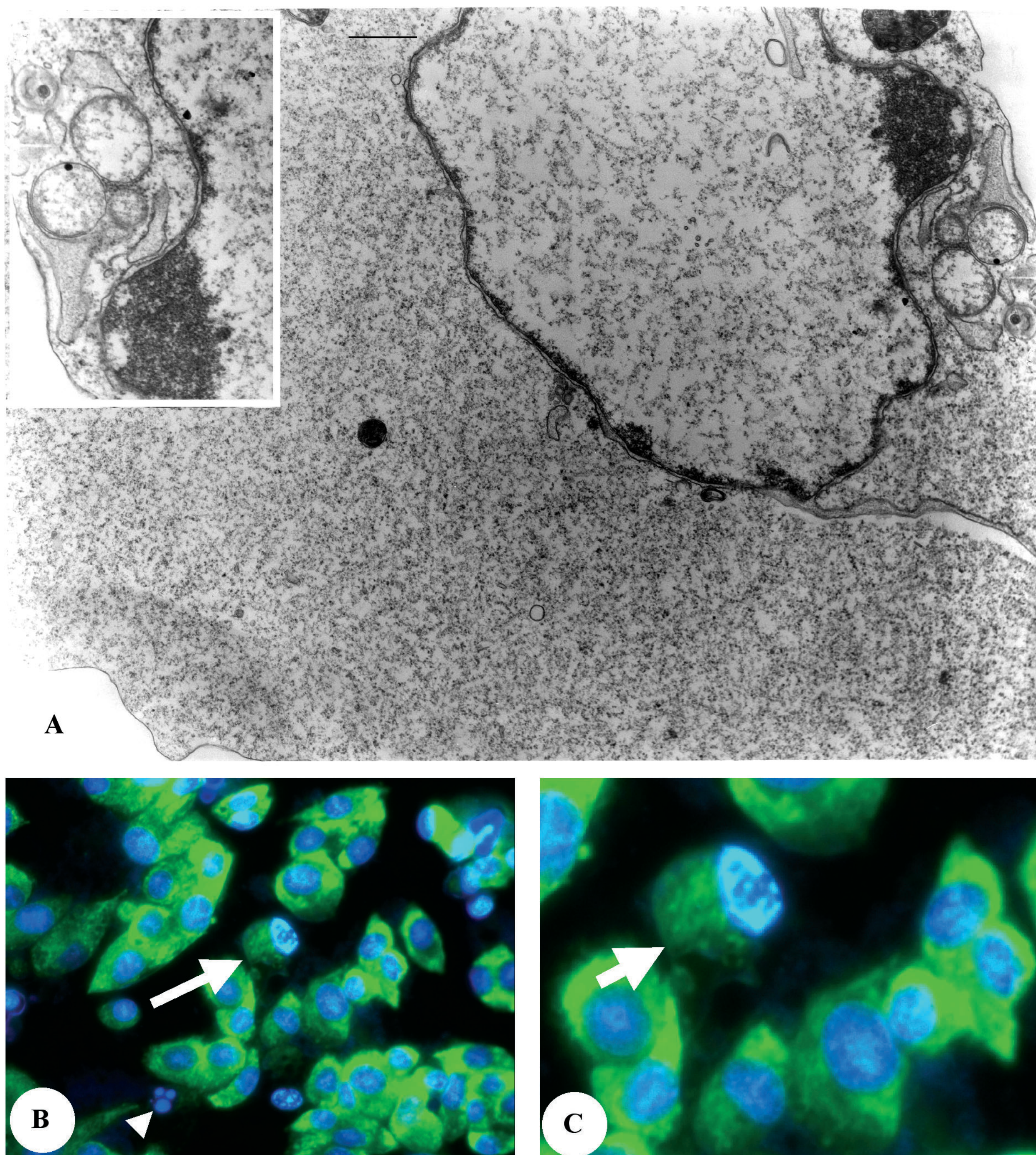

Figure 7. A - WEHI-164 cells exposed to 0.5 µM staurosporine for 16 h. The cell membrane can be seen in the upper right of this panel. **B** and **C** - fluorescence images of BHK cells exposed to 6 µM camptothecin for 16 h. The cells were stained for 25 min with $DiOC_6(3)$ and Hoechst dyes that detect nuclei (**blue** fluorescence) and then stored in a covered well at 37°C for 5 h, during which time more apoptotic cells appeared. These images from live and apoptotic cells were generated by superimposing the **blue** image of nuclei on the **green** fluorescence of mitochondria. The **arrowhead** in **B** shows an apoptotic cell with collapse of the inner intermembrane potential; no mitochondrial fluorescence can be seen. The **arrow** indicates an apoptotic cell that is enlarged in **C.** In this figure, individual mitochondria were stained, indicating either these mitochondria suffered no dissipation of the inner transmembrane potential or that staining with $DiOC_6(3)$ may ocassionally stain functionally inert mitochondria. The lack of cytoplasmic staining in apoptotic cells (**arrowhead**) does not support the latter possibility.

Scorrano *et al.* [70] (classes III and IV) and Fig. 7A-D of Mootha *et al.* [59] are comparable to our figures of ruptured outer membrane (Figs. 2-5). Following the addition of truncated (t) BID (tBID) the mitochondria shift from class I to class II, a change that temporarily correlates with the redistribution of cytochrome c. The mitochondrial structure is remodelled and ~85% of the total intracristal stores of cytochrome c are mobilized and become available for a more rapid discharge through the widened cristal junction. Scorrano *et al.* [70] claim that type II mitochondria "were prominent in cells following several intrinsic death stimuli (Figure 7)". In the apoptotic cells that we have thoroughly analyzed [73], we have been unable to identify the type II mitochondria described by Scorrano *et al.* [70]. The mitochondrial profile shown as class II in Figs. 5 and 6 is not seen in ordinary electron micrographs of these organelles.

Marked expansion of the inner mitochondrial membrane may cover the swollen matrix in mitochondria with a PT (Figs. 2B and 5C,D). In our early studies, we recognized this as possibly the best means for the cytoplasmic exposure of intermembrane proteins stored in cristae. Nevertheless, it is

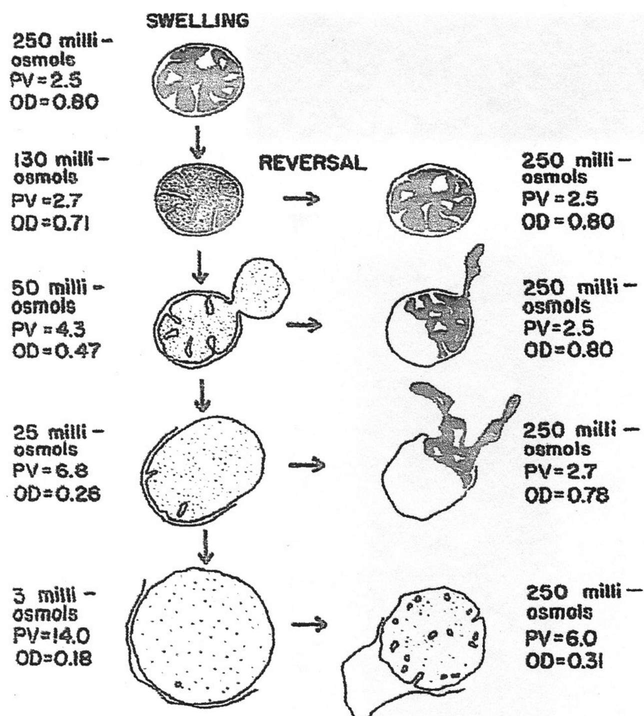

Figure 8. Effect of lowering the osmolality on the morphology of isolated mitochondria. Reproduced from Figure 12 of the paper by Stoner and Sirak [78] (*J. Cell Biol.* **48,** 521-538, 1969), with permission from the *Journal of Cell Biology* and the Rockefeller University Press.

IN APOPTOTIC CELLS THE INNER MITOCHONDRIAL MEMBRANE WICH HERNIATES TO THE CYTOPLASM THROUGH THE RUPTURED OUTER MEMBRANE IS COMPOSED OF DOMAINS DERIVED BOTH, FROM THE INNER MEMBRANE AND THE CRISTAE

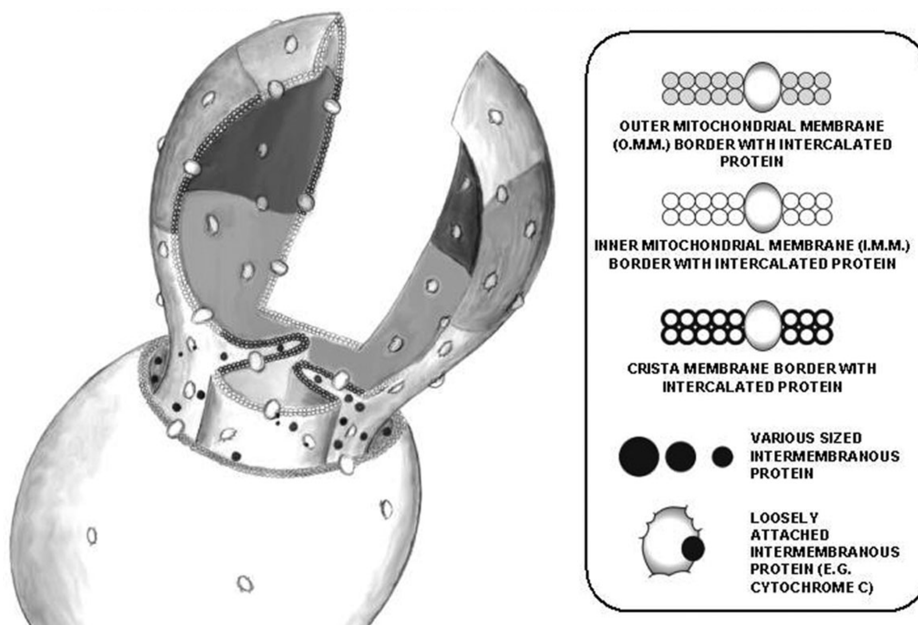

Figure 9. Schematic representation of a mitochondrion with a ruptured outer membrane. The ballooning inner membrane covering the mitochondrial matrix is formed by portions of the peripheral inner membrane and the inner membrane of the cristae. Note that the cristal junctions are shown widened, as proposed by Scorrano *et al*. [57].

unclear how the release of these proteins would be facilitated by widening of the cristal junction [70]. If these proteins are not squeezed out of the mitochondria, then one possibility is that they somehow make use of the permeabilization of the outer mitochondrial membrane. How can these proteins passively transpose the mitochondrial outer membrane? The channels involved must of necessity be shown by electron microscopy.

Since first proposed, the concept of outer membrane permeation through large pores in this membrane has been seriously hampered by the lack of convincing structural evidence for membrane channels that can allow the passage of very large intermembrane molecules. Since TEM has shown that gap junctions, which allow the passage of molecules up to 1 kDa, have a diameter of 1.5 nm, it is difficult to understand why the predictably larger channels that allow the passage of cytochrome c (~14 kDa) and AIF (~50 kDa) have so far not been identified microscopically. The size of these channels is within the working resolution of ordinary transmission electron microscopes.

One way by which intermembrane proteins can be exposed to the cytoplasm is depicted schematically in Fig. 8, which shows the extrusion of the inner membrane. The changes in configuration that affect the inner membrane originate from osmotically driven movements caused by the difference in osmolality between the mitochondrial matrix and the cytoplasm. The scheme illustrates how the intermembrane proteins are progressively exposed to the cytoplasm after rupture of the outer membrane. Note that the route used by incoming fluid that swells the mitochondrial matrix is not shown.

Identification of the mpt *in vivo* and in pellets by using permeable fluorescent probes that are retained in mitochondria with a transmembrane potential

Permeable fluorescent probes are used to monitor the beginning of the MPT. Widely used probes include tetramethylrhodamine methylester (red fluorescence) [49], $DiOC_6$ [64] (green fluorescence) and JC-1, which also fluoresces green at low concentrations or at a low membrane potential. The uptake of these probes by the mitochondria of non-apoptotic cells depends on the membrane potential ($\Psi\Delta_m$) of these organelles; the greater the mitochondrial $\Psi\Delta_m$, the more stain will be accumulated in the mitochondrial matrix. In these conditions, JC-1 forms aggregates that fluoresces red in the state of PT, and dissipation of this potential renders the organelle non-fluorescent in medium with this probe. Lemasters *et al.* [49] used laser scanning confocal fluorescent microscopy and tetramethylrhodamine methylester to study the mitochondrial $\Psi\Delta_m$ during the onset of apoptosis in cultured rat hepatocytes. During the first 1-3 h, different mitochondria successively expressed PT such that cells entering apoptosis had a mixed population of red fluorescing normal mitochondria and non-fluorescent mitochondria, the latter expressing a PT. Similarly, Hüser *et al.* [37] used a voltage-sensitive fluorescent dye to monitor the membrane potential in single mitochondria from heart (see also [49]).

Our results shown here agree with this description. Figure 5A shows the beginning of this process, with panels A and B being compatible with inner membrane of the type 2 profile (entirely permeable membranes). However, examination of our sequence of mitochondrial profiles from the minute aperture in the outer membrane (Fig. 3 and subsequent figures) raises the question of whether the mitochondria in the PT stage shown in Figs. 2 and 3A-C still have some inner transmembrane potential ($\Psi\Delta_m$) and, consequently, some fluorescence in the membrane-bound compartment that contains most of the surface area of the inner membrane. It remains unclear whether in these very early stages of apoptosis the complete dissipation of this potential is or is not limited to the small region of inner membrane corresponding to the ruptured outer membrane. Although we currently have no answer for these questions (also raised by the data in Fig. 7B,C), they nevertheless justify the use of TEM to monitor experiments involving the MPT. Indeed, TEM is an unsurpassed analytical tool for identifying and analyzing the MPT in tissues. Figure 4A,B in [73] shows the presence of an MPT in apoptotic and non-apoptotic pancreatic acinar cells. Which other approach could have revealed these two occurrences in the same fixed preparation?

These considerations indicate that TEM is a more informative means of assessing the incidence of MPT than the use of fluorescent mitochondrial probes or monitoring the decrease in the optical density of isolated mitochondria undergoing PT (this decrease, commonly measured at 540 nm, results from the entrance of water into swelling mitochondria).

How should the incidence of mitochondrial swelling in apoptotic cells be defined?

The importance of the opening of the mitochondrial pore as a consequence of maximal mitochondrial swelling in promoting apoptosis is widely recognized (for example, see [11,28,29]). Apoptosis has been observed in the absence of mitochondrial swelling or without collapse of the inner mitochondrial transmembrane potential [16,17,22,23,51,56,71,93], and the preservation of potential-sensitive fluorescent dyes is either absent in apoptotic cells or occurs after the activation of caspases [8,14,46,86]. Some of these experiments were monitored using TEM and indicate that the lack of mitochondrial swelling in apoptotic cells may even be frequent. Although normal mitochondria are much more frequent than swollen mitochondria in apoptotic cells [12], our findings mentioned below, as well as comments by others [50], do not endorse an uncritical acceptance of this view.

In a study of 15 cell lineages from established cultures and rat tissue, only L 929 cells exposed to TNFα did not show any apoptosis [73]. Apoptosis was seen in all of the other cell types examined and, in some cases, swollen mitochondria with a ruptured outer membrane were seen in cells that have not yet reached the stage of explicit apoptosis. Type 2 mitochondria were seen in apoptotic type II human pneumocytes [5], and in BHK, MDCK and Vero cells, in primary cultures of human fibroblasts, and in lung cancer, NCI-H-292 (human bronchial tumoral epithelial cells) and HT-29 (human colon adenocarcinoma) cells (unpublished data). In some of the lineages examined [73], type 2 mitochondria were only seen in apoptosis promoted by certain drugs. Thus, even in cell types in which apoptotic cells often exhibit swollen mitochondria with ruptured outer membrane, certain apoptogenic agents do not produce mitochondrial swelling. Considering that we did not choose or preselect the cells to be examined by TEM after the induction of apoptosis in any of the foregoing cases, the results favor the appearance of a ruptured outer mitochondrial membrane as a common feature of these cells.

Eight of the 23 cell types that we examined and which contained mitochondria with a ruptured outer membrane, were of human origin. A rapid survey of the literature to assess the incidence of mitochondrial swelling in human apoptotic cells revealed the occurrence of this phenomenon in umbilical vein endothelial cells [62,63], human hepatocytes [30], fetal liver [88], liver and the hepatocellular carcinoma HepG2 [44], the pneumocytic cell line U937 [84], H520 lung cancer cells [43], the lung adenocarcinoma A549 [77], esophageal carcinoma [74], the oral carcinoma KB-3-1 [52], cervical cancer [87], MCF7 breast cancer cells [10], Jurkat T leukemic cells [53], B lymphoma [45] and colon carcinoma cells [54]. Swollen mitochondria were also found in apoptotic neurons and in endothelial, renal and myocardial cells of rats and mice. These observations suggest that mitochondrial swelling may prevail in apoptotic cells of human origin and possibly also in mouse and rat cells.

Another aspect closely related to mitochondrial swelling in apoptotic cells involves the temporal relationship between the collapse of the inner transmembrane potential ($\Delta\Psi m$) and the release of cytochrome c. The release of cytochrome c may occur prior to, during, or after the appearance of $\Delta\Psi m$. Determining which of these possibilities, including the incidence of mitochondrial swelling in apoptotic cells, is the most frequent, would require a detailed analysis of numerous published studies, a task that is complicated by the rapid appearance of new papers in this field. Can this task be done?

The examination of several hundred of the thousands of published papers on programmed cell death revealed that that the key signaling events of apoptosis, such as the onset of the MPT, mitochondrial swelling and cytochrome c release to the cytoplasm, are influenced by factors such as the cell type and presence of apoptogenic agents. These two factors need to be examined in any assertion that non-swollen mitochondria are more frequent than swollen mitochondria in apoptotic cells after opening of the mitochondrial transition pore. Let us consider an attempt to evaluate the incidence of mitochondrial swelling or of the main time sequence for the onset of MPT, mitochondrial swelling and cytochrome c release in apoptotic cells. Two approaches can be envisaged and derive from the choice of cells from which the contrasting events (e.g., swollen versus non-swollen mitochondria) will be evaluated. One approach would involve an actual assessment of the cases of swelling versus non-swelling extracted from numerous, often unrelated publications. The other approach would be to select the cells to be analyzed, while taking into account the actual or potential biomedical interest of the cell type and the apoptogenic agent.

Our work done mostly in human and rat apoptotic cells has shown a high prevalence of swollen mitochondria with a ruptured outer membrane. An adult human weighing 80 kg has ~10^{17} cells, depending on the average cell size used for the estimation, and of these 1-6 x 10^{13} are endothelial cells. Since ~10^{11} cells die daily by programmed cell death [13], human cells are a rich source for studying cell death by apoptosis. Documentation of the morpho-functional events normally involved in the sorting of apoptotic human cells is of practical relevance since a clear understanding of these events could allow them to be used as a standard for determining the initial and subsequent apoptotic events in cells destined to die by apoptosis in normal and diseased organs of human embryos and adults. The resulting information could be complemented with other relevant data from similar normal and diseased organs in laboratory animals.

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