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Review

Mitochondrial permeability transitions: how many doors to the house?

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Abstract

The inner mitochondrial membrane is famously impermeable to solutes not provided with a specific carrier. When this impermeability is lost, either in a developmental context or under stress, the consequences for the cell can be far-reaching. Permeabilization of isolated mitochondria, studied since the early days of the field, is often discussed as if it were a biochemically well-defined phenomenon, occurring by a unique mechanism. On the contrary, evidence has been accumulating that it may be the common outcome of several distinct processes, involving different proteins or protein complexes, depending on circumstances. A clear definition of this putative variety is a prerequisite for an understanding of mitochondrial permeabilization within cells, of its roles in the life of organisms, and of the possibilities for pharmacological intervention.

Keywords: Permeability transition; Adenine nucleotide transporter; Bax; Cyclosporin A; Protein import; Mitochondria

1. Introduction

The mitochondrial permeability transition (PT) (reviews: Refs. [1–8]) consists in the opening of a permeation pathway allowing the diffusion of solutes up to about 1500 Da from the mitochondrial matrix to the extramitochondrial space, and vice versa. The phenomenon, characterized in landmark studies by Hunter and Haworth in the late 1970s [9–11], has recently attracted renewed attention because of its proposed roles in the release of cytochrome *c* and other pro-apoptotic factors in many models of apoptosis (reviews: Refs. [7,12–17]) including ischemia/reperfusion-induced tissue damage (reviews: Refs. [8,18–27]). The permeability transition is a complicated process, with many recognized inducers, modulators and inhibitors, some of which are mentioned below. Its most evident characteristic is a requirement for Ca^{2+} accumulation in the mitochondrial matrix (but see discussion below). In most cases, the PT has been studied using isolated mitochondria (particularly rat liver) and methods (mitochondrial swelling, depolarization, Ca^{2+} release) that can report on the opening and operation of the "pore" (PTP), but are not well suited to provide detailed information on the nature and properties of this pathway. The same can be said of the techniques used for the detection and characterization of the PT within intact cells or in tissues, which involve tracking the release from or entrapment in mitochondria of Δψ-indicating dyes or of PTP-permeant molecules [24,28–33]. These technical limitations may have hampered full appreciation of the long suspected (e.g.: Refs. [2,34]) multiplicity of biochemical species and processes which may lead to non-lytic permeabilization of the inner mitochondrial membrane. Here we present a brief review of the evidence suggesting such a multiplicity, with particular attention to the hypothesis that one of the forms of the

Abbreviations: ANT, adenine nucleotide translocator; CATR, Carboxyatractyloside; CK, creatine kinase; CSA, cyclosporin A; CypD, cyclophilin D; GSK-3β, glycogen synthase kinase 3β; HK, hexokinase; MCC, multiple conductance channel; MMC, mitochondrial megachannel; PA_2, phospholipase A_2; PhAsO, PhenylArsineOxide; PT, permeability transition; PTP, permeability transition pore; PK, protein kinase; ROS, reactive oxygen species; TFP, Trifluoroperazine; VDAC, voltage-dependent anion channel (mitochondrial porin)

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permeability transition pore may correspond to (a) complex(es) of the protein import machinery.

2. The PTP as ANT-centered complex(es)

Current ideas about the molecular nature of the PTP are varied, perhaps reflecting reality. For a period it was thought to be a membrane "defect" due to the production of lysophospholipids by a Ca^{2+}-dependent mitochondrial phospholipase A_2 [1,2]. This model has now been largely abandoned (but see below), but it remains true that both Ca^{2+}-dependent [35] and -independent [36] PA_2s are present in mitochondria, and produce free fatty acids which are powerful co-activators of the Ca^{2+}-induced PT [37,38]. Due largely to the discovery of a potent PT inhibitor which does not affect PA_2 activity, Cyclosporin A (CSA), and to the identification of the permeability transition pore with a gating channel observed by patch clamp, today the PTP is generally believed to be formed by proteins.

Since potential PTP precursor complexes can be readily isolated by biochemical means, they must be relatively abundant (and stable), although a quantitative estimate is difficult. Their transition to the unselective pore is relatively difficult: even under very favourable experimental conditions, in vitro, the PT takes several seconds to spread through a population of even the most susceptible mitochondria. In patch-clamp experiments, the pore thought to correspond to the PTP rarely appears in more than one copy per patch. This is of course to be expected for a process which needs to be tightly controlled and which requires only one or a few pores to open to disable a mitochondrion. The number of open pores per mitochondrion may, however, increase with increasing inducer concentration [39].

What proteins form the PTP is still open to question. A widespread consensus model envisions a supramolecular complex spanning the double membrane system of mitochondria, localized at contact sites [7,40–42]. Components of all mitochondrial compartments have been proposed to participate (e.g.: Refs. [43,44]; reviews: Refs. [8,45]). While the mitochondrial *cis–trans* peptidyl-prolyl isomerase Cyclophilin D (CypD) (matrix), the adenine nucleotide translocator (ANT) (inner membrane) and porin (VDAC) [46–49] presumably form the core of the complex (reviews: Refs. [8,50]), creatine kinase [46,51,52] (periplasmic space), the peripheral benzodiazepine receptor [53–57] (outer membrane) and VDAC-associated hexokinase (cytoplasm) [46,52,58–61] are also thought to have roles. The pro-apoptotic Bcl-2 family protein Bax might be part of the assembly (see below) and other proteins such as the BH3-only Bcl-2 family protein NOXA [62], anti-apoptotic members of the same family [63–65], and kinases such as PKA [66], PKG [67], a PKC isoform [68] and GSK-3β [69] may have roles as regulators.

For some of these putative components, data exist which suggest their presence may be optional or depend on tissue or circumstances, and that, therefore, a variety of permeability transition pores may exist. The PTP-forming complexes probably are transient, capable of disassembling and reassembling—perhaps with a different composition—with a relatively high frequency and/or in response to appropriate stimuli. Whether VDAC itself is a necessary component is debated [8]. The contact sites comprising ANT and VDAC are known to be under metabolic control, i.e., to be reversible [70–72]. A key component of the PTP, Cyclophilin D, is a matrix protein, although it can bind to the ANT (e.g.: Ref. [73]). This implies the existence of a dynamic association/dissociation equilibrium. It has been suggested that ANT-VDAC interaction may depend on CypD binding to the former [41]. Association of hexokinase (HK) to mitochondrial VDAC is known to depend on the cytoplasmic concentration of Glucose-6-phosphate [74–76]. It has been proposed, on the basis of biochemical (complex isolation) data, that HK may bind to a particular conformation of VDAC, induced by interaction between VDAC and the ANT in its "c" (atractyloside-stabilized) conformation (ANT1 was the isoform in the relevant experiments) [46,61]. HK association would be in competition with the formation of complexes between the ANT, octameric mitochondrial creatine kinase (mtCK), and VDAC. mtCK, located in the periplasmic space and interposed between ANT and VDAC, would act to prevent the PT (provided creatine is present) [46,51,77]. This type of interplay would be tissue-specific, since CK is not expressed in mammalian liver.

Let us now consider some of the major components of the "classical" PTP complex, the ANT, CypD and Bax.

2.1. ANT

Adenine nucleotide translocator ligands can induce (atractyloside) or repress (bongkrekate, adenine nucleotides) the PT (e.g: Refs. [78,79]). For these and other good reasons, many researchers have long considered the ANT (presumably as a dimer) to form the PTP, or to be the centerpiece of the complex forming it (reviews: Refs. [5,8,80]). The recently published structure of the ANT–CATR complex [81] is broadly compatible with the detailed model developed mainly by Halestrap's group [8]. For example, ANT Pro61, thought to be essential for CypD binding and action, is indeed appropriately located in a matrix-exposed surface helix. Purified ANT can be converted by Ca^{2+} into a high-conductance channel [82–84] bearing some similarity to channels observed by patch-clamping mitoplasts and assigned to the PTP (see below). The reconstituted Pi carrier can also form channels [85]—although with characteristics quite different from those expected for the PTP—and so might other transporters as well [86,87]. A recent paper has shown that a Ca^{2+}-induced PT takes place also in the mitochondria of mouse liver lacking both ANT genes [88]. Modestly (threefold) higher loads of Ca^{2+} were required to induce swelling of the mutant

mitochondria than of control ones, and in the former the phenomenon was no longer sensitive to ANT ligands. *t*-Butylhydroperoxide and diamide, two well-known PT inducers believed to operate by reacting with SH groups (proposed to belong to the ANT Refs. [89,90]), were still able to facilitate the PT in ANT-less mitochondria, although their effectiveness was reduced. (See also the comment in Ref. [91]).

Thus, a pore allowing sucrose diffusion can be formed with or without ANT involvement. Presumably other mitochondrial carriers can substitute for the latter (e.g., the phosphate carrier, given the phosphate sensitivity of PTP), although this remains to be demonstrated. The properties of these pores seem to be at least superficially similar: both types are formed upon application of inducers believed to have physiological significance (Ca^{2+}, oxidative stress), both allow sucrose permeation, and both are inhibited by Cyclosporin A. The relative contribution of ANT-containing and ANT-less forms of the PTP to the PT in isolated mitochondria and in cells clearly is, at this point, a relevant question, e.g., for the development of PTP-directed drugs. The former type seems to be the most physiologically important, because of the protection against cell death afforded by presumably specific ANT-interacting compounds both in vitro and in vivo. The effectiveness of BGK against apoptosis and necrosis in several model systems is particularly relevant (e.g.: Refs. [92–94]). One should keep in mind, however, that BGK inhibits mitochondrial adenine nucleotide transport as well as the permeability transition. Furthermore, protection is generally partial; intervention of non-ANT-based PTP variants may contribute to this less-than-optimal effectiveness.

The probable prevalence of ANT-containing form(s) of the PTP may be due at least in part to the abundance of this protein: beef heart [95–97] and rat liver [95] mitochondria contain about 1100–1300 and 150–200 nmol of ANT per gram of mitochondrial protein, respectively. By comparison, in RLM the phosphate [98], carnitine [99] and tricarboxylate [100] carriers are in the 20–30 nmol/g prot. range. Mammalian heart mitochondria contain approximately 50 nmol/g prot. of α-oxoglutarate [101] and aspartate/glutamate carrier [102], respectively. The data presented in Ref. [103] for RLM lead to estimates of approximately 80 and 200 nmol/g prot. for complex II or cytochrome aa_3 and F_0F_1 ATPase, respectively. VDAC content of RLM has been estimated at approximately 100 nmol/g prot. [104].

A point of interest in this context is whether there may be ANT isoform specificity in PTP formation. That this may be the case is suggested by the intriguing observation that overexpression of mouse or human ANT1 or hANT3, but not that of ANT2, induces cell death in a variety of cultured cell lines [105–107]. Since this effect could be counteracted by co-transfection with Cyclophilin D (see discussion below) or by treating the cells with CSA+TFP or with BKG, death was considered to be mediated by the PTP, which would therefore not involve ANT2. Such choosiness in PTP complex formation, if real, would presumably extend to the selection of other carriers. Differences in protein–protein interactions [108] might account for this putative specificity, which might help to explain tissue-dependent variations in mitochondrial susceptibility to PT induction. Notably, however, in the mouse and rat ANT1 is the major form in brain, kidney and muscle, while ANT2 predominates in all other tissues, including the liver [109–111]. Mouse or rat liver mitochondria are well known to be among the most PT-prone, suggesting that the above-mentioned effects might not be directly related to the PT (see also the discussion in Ref. [8]).

2.2. Cyclophilin D

The finding that the PT in ANT-less mitochondria is inhibited by CSA is significant in itself: CSA is believed to function by competing CypD, its binding partner, away from a binding site on the PTP complex [73,112–114], or by inhibiting its PPIase activity while at this binding site [115]. In either case, the binding site was thought to be on the ANT, but clearly the interaction must be less specific. As already mentioned, CypD has been found to repress apoptosis induced by ANT-1 overexpression, and to be up-regulated in several tumors. These observations have been interpreted in terms of an inhibitory interaction of CypD with the pore formed, within the PTP complex, by the ANT [105,107]. CypD would thus oppose PTP opening and therefore apoptosis. This interpretation seems, however, to contradict the currently favoured binding model of CypD-CSA action, which envisions a PT-permissive role for ANT-bound CypD. Crompton's group [116] in fact has reported that CypD overexpression in a neuronal cell line made isolated mitochondria more susceptible to Ca^{2+} and redox stress-induced PT, and cells more susceptible to necrosis, but, in agreement with the studies just mentioned, reinforced cells against NO- and staurosporine-induced apoptosis, believed to involve the mitochondrial pathway. The conclusion was that in the system considered the PT is involved in necrosis, but not in apoptosis.

Indeed, the literature on the inhibitory effects of CSA strongly suggests that matters may be complicated. Insufficient attention has been devoted so far to the possibility that some of the effects of CSA may be due to interaction with something other than the PTP or calcineurin. A recent study reports for example that CSA inhibits mitochondrial Ca^{2+} uptake [117]. The apparently fickle behavior of this supposedly diagnostic inhibitor has been reviewed elsewhere [2,34,118–123]. Although CSA does inhibit the permeability transition induced by a variety of agents, the inhibitory effect is often partial (or absent) and/or transient, or it may require a synergic co-inhibitor. In several instances, its characteristics have been shown to depend on the method of inducing the PT and on the tissue or cell line of origin of the mitochondria. Apparently contradictory results can be found in the literature. For example, in studies

with non-synaptosomal rat brain mitochondria suspended in KCl-based media, CSA has been recently reported to inhibit efficiently the PT of cortex mitochondria [124] or not at all that of forebrain (i.e., mainly cortex) mitochondria [125]. Another study indicates that Ca^{2+} handling by rat cortex mitochondria is sensitive to CSA only in sucrose/mannitol-based media [126]. Such variability can be attributed only in part to the recognized heterogeneity of mitochondria isolated from different brain regions [127,128]. The mitochondria isolated from some immortal cell lines, in particular, exhibit Ca^{2+}/Pi-induced, CSA-insensitive swelling (Fontaine, E., personal communication; De Marchi and Zoratti, unpublished). In one case, that of mitochondria from a baby mouse kidney (BMK) cell line, we have verified that this is not due to a lack of Cyclophilin D expression (not shown). These observations strengthen the idea that CypD-dependent and -independent mechanisms of permeabilization may exist [129]. It may be that, depending on cell type and other circumstances, other chaperons may intervene in the mechanistic pathway that involves CypD. He and Lemasters [130] have recently concluded that an increased expression of mitochondrial Hsp25 antagonizes (rather than facilitating, as might have been expected) permeabilization. Preliminary results [131] indicate that the PT of mitochondria from CypD knock out mice [132] differs from that of wild-type organelles only in that it requires higher Ca^{2+} loads and is insensitive to CSA. These various and apparently discrepant observations point to a nonessential role of CypD as a promoter of the Ca^{2+}-dependent PT, as proposed by Halestrap [8]. Sanglifherin—a recently discovered immunosuppressant peptide which also inhibits the PTP via CypD [115,133]—and two newly reported CSA analogs [134] may therefore be expected to exhibit the same sort of variability.

2.3. Bax

Another very interesting putative component of the PTP is Bax. Its presence has been reported in complexes isolated from rat brain which could behave in PTP-like fashion upon reconstitution [64,135,136]. Bax has been reported (see discussion in Ref. [8]) to interact with the ANT [65,136–140] and with VDAC ([141,142]; review: Ref. [143]; but see Ref. [144]), and with the complex they form [50,61] (BclxL also interacts with VDAC [142,145]). If Bax and the ANT indeed come in contact in in situ mitochondria, presumably they do so through residues of each protein protruding into the periplasmic space, after Bax has incorporated into the outer membrane. Bax overexpression has been reported to induce cyclosporin A-inhibitable (and thus presumably PT-mediated) Jurkat cell death [146], and the addition of high doses (>100 nM) of Bax to isolated mitochondria was found by some authors to induce the PT in a Cyclosporin- [141,147] and hexokinase II- [148] sensitive manner (contra.: Refs. [149,150]). Other data suggest that Bid may also interact with the PTP [151,152]. These findings suggest that Bax (and perhaps its homolog Bak and Bid) may induce the mitochondrial PT and cytochrome *c* release by interacting with the PTP to either form or regulate it (or both). This idea has been strengthened by the observation that anti-apoptotic Bcl-2 family members antagonize the permeability transition (review: Ref. [153]) and inhibit the activity of a reconstituted complex thought to correspond to PTP [64,135]. This antagonistic action, however, takes place regardless of the presence of Bax in the complex. On the other hand, some authors [154,155], at variance from others [156,157], have reported that Bax cannot be detected in Western blots of rat brain and rat liver mitochondria, two prominent models for PT studies. Several studies have concluded that Bax-mediated release of cytochrome *c* is independent of the PTP (review: Ref. [158]) and takes place without permeabilization of the inner membrane [150,154,159,160]. Others have concluded that the PT may serve to make cytochrome *c* available for release by remodeling inner membrane folding [161], or that it may serve as a signal for Bax migration to the mitochondria [162]. The results in Ref. [136] are particularly significant in this context. The authors found that the presence of Bax strongly favoured the opening of the purified/reconstituted PTP complex by atractyloside, but that it was not needed when permeabilization was induced by oxygen radicals or thiol cross-linking. We have concluded that it is also not involved in Ca^{2+}-induced PT in isolated mitochondria [163]. These observations seemingly imply that the permeabilization process differs depending on the inducing agent, i.e., again, different types of permeability transition can occur.

2.4. A role for respiratory chain complex I?

Further complexities stem from the reports [164–166] that Ca^{2+}-induced, CSA-sensitive swelling is facilitated by electron flow through the mitochondrial respiratory chain Complex I, and that ubiquinone analogs can inhibit or induce it, probably via direct interactions rather than through redox reactions [164,165,167–170].

S. cerevisiae mitochondria do not exhibit an analogous Ca^{2+}-induced, CSA-sensitive permeabilization, but their membrane can become semipermeable under conditions quite different from those effective for mammalian organelles [171], a fact that, again, points to the possible existence of several distinct possible permeabilization mechanisms. As mentioned by Fontaine and Bernardi [164], it is intriguing that yeast mitochondria have no complex I (and no Bax).

3. "Non classical" pores

Insensitivity to CSA is one of the defining features of a "non-classical" permeability transition induced by the combination of saturated fatty acids and divalent cations

(Sr^{2+} and Mn^{2+}, "classical" PTP inhibitors, as well as Ca^{2+}) [122,123], which may be related to the formation of Ca^{2+}/fatty acid complexes and of "lipid pores" in the mitochondrial membrane [172,173]. The production of fatty acids by mitochondrial PA_2 may thus account for some of the phenomenological complexities mentioned above.

Another "must" of the permeability transition, the requirement for Ca^{2+} accumulation in the matrix for its onset, may not be universal. High doses of thiol reagents such as Hg^{2+} [34], diamide [174] or phenylarsineoxide [175], of the ganglioside GD3 [176], of peptides such as mastoparan [177] and perhaps of Bax (see above) can induce permeabilization (as detected by the swelling assay) without a requirement for exogenous Ca^{2+} and often in a CSA-insensitive manner. Most of these agents act at lower doses in Ca^{2+}-requiring and CSA-sensitive fashion. While the effects of high doses might just reflect a specific disruption of the lipid bilayer, solute exclusion experiments suggest that this is not the case, since size limits are close to those determined for the "classical" phenomenon [34,177]. It should be noted that different mechanisms may underlie the permeabilization induced by the agents just mentioned. While thiol reagents presumably modify proteins, acting as "sensitizers" or perhaps inducing cluster formation (see next paragraph), GD3 and mastoparan might function as membrane-perturbing agents. GD3 and/or other lipid metabolites may also directly regulate ion channel activities.

The groups of Vercesi (e.g.: Refs. [178,179]) and of Lemasters [34] have emphasized the role of free radical oxygen species (ROS) in the induction of the PT, presenting data which imply their involvement downstream of the classical inducers Ca^{2+}+Pi as well as of redox agents. According to this mechanistic hypothesis, ROS would induce, via thiol oxidation and cross-linking, misfolding and clustering of membrane proteins, which would expose hydrophilic residues to form a membrane-spanning aqueous pore. Chaperonins, including CypD, would bind to the cluster. Increased Ca^{2+} would perturb the protein/chaperonin complex and cause opening of the PTP, an effect antagonized by CSA. CSA-insensitive permeabilization would result from the formation of clusters in excess of the capacity of chaperonins to block the potential pores [34]. The great abundance of ANT and VDAC in the mitochondrial membranes would explain both their frequent (but not exclusive) involvement and the relative reproducibility of PTP properties.

The formation of protein clusters may be one way to rationalize the formation of ion-permeable, sucrose-impermeable "narrow" pores which respond (or not: Ref. [174]) to most pharmacological agents which act on the large, "classical" PT, although the ANT may be involved as well (e.g.: Ref. [180]). Convincing evidence (revs: Refs. [2,119,181] has been gathered that such pores develop under conditions of oxidative stress (including Ca^{2+} overload). Their formation appears to depend on matrix alkalinization [181], and they may progress to full-size PTP [120,182]. These selective states of the PTP have recently been credited with responsibility for ischemic preconditioning-induced protection against ischemia/reperfusion damage. Their transient opening during the preconditioning protocol has been proposed to result in limited uncoupling, ROS production, reduced Ca^{2+} load and impaired subsequent opening of the full-size PTP [183] (see Ref. [184]). These pores have been characterized mainly in in vitro experiments with isolated organelles, under conditions different from those normally employed in experiments involving the "classical" PT. Whether they are obligatory precursors of the larger pore is uncertain: in some electrophysiological experiments we (unpublished) and others [185] have observed what appeared to be a gradual development of the PTP channel from smaller conductances, but much more frequently (in our hands) the MMC materialized in a previously silent patch as a fully formed large channel. Notably, however, a kinetic analysis of PhAsO- or Ca^{2+}+Pi-induced swelling [39] indicated that in a substantial fraction of mitochondria sugar entry was restricted, as if occurring through a relatively narrow pore. The study concluded that permeability transition pores are heterogeneous in size. In patch-clamp experiments the conductance of channels believed to be the PTP also varies to some extent (see below), but "narrow", sucrose-impermeable pores have yet to be characterized by electrophysiological techniques.

4. May protein-importing complexes form permeability pores?

The suggestion that the PTP may in some cases coincide with one of the protein import complexes is based on electrophysiological data, along with the permeabilization of isolated mitochondria by leader peptides [186–188], and on the recently observed phenotype of Tim50 deletion [189].

In the early 1990s our group characterized a high-conductance channel of the membrane of rat liver mitoplasts which we then identified as the PTP on the basis of its characteristics (size, voltage dependence) and, mainly, pharmacological similarities, i.e., activation by Ca^{2+} and inhibition by CSA, Mg^{2+}, Mn^{2+}, Sr^{2+}, H^+, ADP and quinone analogs [2,167]. The channel, dubbed mitochondrial megachannel, MMC, is characterized by a maximal conductance in the 0.9–1.5-nS range (150 mM KCl) and by the presence of multiple substates, including a "half-conductance", fast-gating state whose presence strongly suggests a dimeric structure [47], and which is evident in Fig. 1A. What appears to be the same channel has been observed in mitoplasts from human hepatoma [190] and colon cancer (Campello, S. et al., unpublished) cell lines. A similar multiple conductance channel (MCC) activity has been studied by Kinnally's group (e.g.: Refs. [191,192]). While the relationship between the MCC and the channel studied by us and between MCC and PTP has not yet been fully

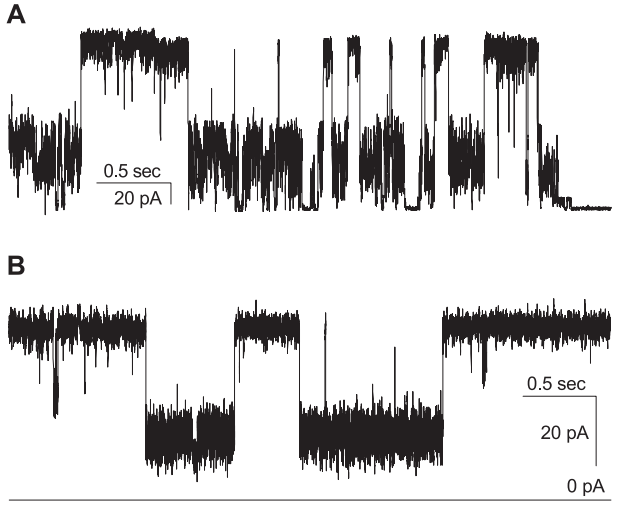

Fig. 1. The "half-conductance" substates. Exemplificative current traces showing activity by a rat liver MMC (A), and by a reconstituted yeast Tim22 complex (B). (A) Patch-clamp recording from a mitoplast in symmetrical 500 mM KCl medium (unpublished data by Szabò, I. and Zoratti, M.). Pipette voltage: 30 mV. (B) From a planar bilayer experiment in 200:20 (*cis*/*trans*) mM KCl, in the presence of P2 peptide (internal address sequence of the mitochondrial Pi carrier) (unpublished data by P. Kovermann, R. Wagner and coworkers). Conditions as in Ref. [201]. *V*: 125 mV. Both traces were filtered at 1 kHz and sampled at 10 kHz.

clarified, they may be the same, as they have similar conductance ranges and can visit multiple substates, including the "half-conductance" one, and the MCC displayed some Ca^{2+} dependence [193] which was abrogated by Bcl-2 overexpression [194]. MCC activity was observed also in yeast mitoplasts (yeast mitochondria do not exhibit a "classical" PT), and was found to be independent of ANT expression [195]. Since leader peptides induced fast block, and the activity was inhibited by antibodies raised against Tim23, and it was altered in yeast carrying mutations in Tim23, the authors concluded that MCC was a manifestation of the Tim23 protein import complex [196].

The outer and inner membrane protein import complexes Tom40 [197–199] and Tim22 [200,201], and the Tim23 protein [202] have been isolated and characterized biochemically and electrophysiologically. Remarkably, Tom40 and Tim22 behave as twin pores, gating with a degree of coordination, and appear as dimers of pore-forming units in electron micrographs. The current records bear a definite resemblance to those assigned to the MMC in that both can display a fast-gating substate of approximately half conductance (compare traces A and B in Fig. 1). Tim23 records suggest either a three-unit [202] or a two-unit [203,204] structure. Electromicrographs of this complex have not yet been published.

A closer comparison reveals differences as well as analogies, and some uncertainties. The latter are due in part to discrepancies in the reports in the literature, and in part to a certain variability of the properties of (at least) the MMC from experiment to experiment. Besides conductance, the variable characteristics include the propensity to enter the half-conductance substate and the strength of the voltage dependence. This variability may be an intrinsic characteristic of the channel, or it may indicate that various high-conductance channels might actually be detected, or that their properties may be influenced by modifications (e.g., phosphorylation) or by interactions with other membrane components. Often the channel is "slowly" but persistently inactivated at voltages of unphysiological polarity above approximately 30 mV; at the opposite polarity a fast-gating behavior is elicited by moderate potentials (see Fig. 2 in Ref. [47]). This behavior is qualitatively similar to examples reported for Tom40 (compare, e.g., Fig. 3 in Ref. [199]). In other experiments the channel closes more reluctantly at voltages of either polarity, without entering the fast gating mode. This behavior resembles that reported for Tom40 in [197], and that shown for activity assigned to *S. cerevisiae* Tim23 and Tom40 channels in Fig. 2 of Ref. [205]. These latter authors found Tim23 and Tom40, studied in proteoliposomes reconstituted from purified inner or outer membranes, to have overall similar properties. On the other hand, purified Tim23 (not the whole complex) has been reported to be rapidly activated, but then slowly inactivated, by increasing voltages of either sign [202]. Purified Tim22 protein and Tim22 complex, reconstituted into planar bilayers, are also activated by voltage of either sign [201]. The reconstituted channels all appear to have lower conductances than the MMC, although different experimental conditions may undermine comparisons. Tom40 again appears to be the most similar to the MMC in terms of conductance: 1.25 nS for the highest state of the twin pore (both channels open) in 150 mM KCl [199]. Inner membrane pores seem smaller: Tim22 exhibits a conductance of some 540 pS in 250 mM KCl [201]; reconstituted Tim23 is reported to gate as triplets of 450 pS conductances in 250 mM KCl [202]. These latter properties do not match those reported by Kinnally's group for the putative Tim23 complex in membranes, but only limited relevance should probably be attached to quantitative agreements or disagreements among such reports, since they concern different experimental situations as well as, in some cases, species of origin. In particular, the reconstituted proteins and complexes all derived from *S. cerevisiae* or *N. crassa*, while the electrophysiological properties of the MMC/PTP were studied in rat liver mitochondria. In porin-less yeast mitoplasts our group observed activity by cation-selective channels whose conductance, as well as open probability, depended on voltage, and which may well be related to protein transport systems [206].

The possibility of a participation of the protein import machinery in the (or rather a) permeability transition is also suggested by the fact that leader peptides can on the one hand induce fast block of the MCC [187,207], and on the other cause mitochondrial swelling and activation of a conductance assigned to the MCC [186,187]. These latter

phenomena take place only in media of low ionic strength, reflecting presumably the need for electrostatic interaction between the positively charged peptides and the import proteins. The pharmacological pattern is different from that of the "classical" PT; in particular, CSA, ADP, bongkrekate (an inhibitor of the ANT and of the PT) and Ca^{2+} chelators have only a partial or no effect [186,188], while trifluoperazine, dibucaine and propanolol, positively charged compounds which inhibit protein import as well as the Ca-induced PT, are good inhibitors. Matrix Ca^{2+} reportedly hinders, rather than promotes, the phenomenon [121]. The pore induced by leader peptides seems somewhat larger than that of the Ca^{2+}/Pi-induced pore according to polymer exclusion experiments [186]. These discrepancies suggest that the pores formed by protein import complexes may well be molecularly different from the "classical" Ca^{2+}-dependent pore. On the other hand, since the modes of induction are obviously different, it cannot be excluded that they are in fact formed by the same—but differently altered or differently organized—proteins. This reasoning obviously also applies to much of what has been said above.

Finally, Guo et al. [189] have shown that the down-regulation of a component of the Tim23 inner membrane protein import complex, Tim50, results in an increased sensitivity of cells to death stimuli, mediated by a facilitation of cytochrome *c* release. These effects are precisely what one would expect of a mutation facilitating PT development, provided of course that the PT is indeed involved in apoptotic cell death.

5. Conclusions

The relevance of the permeability transition(s) to cell life and death, long assumed, is still a controversial topic, but few would today dismiss the PT as completely irrelevant. A more thorough understanding of the phenomenon as manifested by isolated mitochondria in vitro seems a prerequisite for its adequate investigation and assessment at the cellular and organismal level. Evidence gathered by pharmacological, genetic and biochemical approaches leads to the conclusion that the impermeability of the inner mitochondrial membrane to high-MW compounds can be lost by different mechanisms, i.e., by processes involving different (sets of) proteins and/or different modifications/alterations of the same proteins or protein complexes. A confirmation or rebuttal of this idea, and an understanding of the underlying multi-faceted biochemistry, will require a detailed characterization of the pores formed under various circumstances and in different organs or species, and, if at all possible, their differentiation on pharmacological grounds. SiRNA technology may provide a tool to assess the involvement of the various putative components. The mitochondrial membrane system contains ready-made large pores, namely those of the protein import system. Their opening—whether accidental or regulated in the context of cellular processes—would seem the easiest way of inducing the PT. If cells have any use at all for the PT, they would be expected to have exploited this obvious possibility. But under what circumstances would they do so? Would, for example, Ca^{2+} overload transform a functional Tim22 complex into a PT? All this remains to be investigated first in vitro, then, if warranted, in vivo.

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