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Progress on the Mitochondrial Permeability Transition Pore: Regulation by Complex I and Ubiquinone Analogs

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This review summarizes recent progress on the regulation of the mitochondrial permeability transition pore, an inner membrane channel that may play a role in cell death. We briefly cover its key control points as emerged over the last few years from studies on isolated mitochondria; and describe in some detail our recent results indicating that the pore is modulated by the respiratory chain complex I and can be specifically blocked by selected ubiquinone analogs. We discuss the potential relevance of these findings for the structural definition of the permeability transition pore and illustrate the pharmacological perspectives they offer in diseases where mitochondrial dysfunction is suspected to play a key role.

KEY WORDS: Mitochondrial channels; respiratory chain; complex I; ubiquinone; cell death; disease; aging.

INTRODUCTION

As a result of Ca^{2+} accumulation, or simply as a consequence of *in vitro* "aging," mitochondria can undergo a generalized increase of permeability of the inner membrane, which has been termed "permeability transition" (Haworth and Hunter, 1979; Hunter and Haworth, 1979a, b). The permeability pathway has an exclusion limit of approximately 1500 Da, which means that PTP opening leads to collapse of the protonmotive force, disruption of ionic homeostasis, loss of pyridine nucleotides, and hydrolysis of ATP by the F_1F_0-ATPase. The permeability transition has long been considered as an unspecific form of membrane damage, possibly due to breakdown of the phospholipid bilayer (see Gunter and Pfeiffer, 1990; Bernardi *et al.*, 1994; Gunter and Gunter, 1994; Zoratti and Szabo, 1995 for reviews).

In the 1970s, Pfeiffer and co-workers proposed the "membrane" theory of the permeability transition. The defect was traced to the membrane itself, which would undergo major changes of permeability as a result of the accumulation of acyllysophospholipids following activation of Ca^{2+}-dependent phospholipase A_2 (Pfeiffer *et al.*, 1979; Beatrice *et al.*, 1980). This theory accounted for the effects of a variety of inducers and inhibitors, and could also readily explain the lack of selectivity of the permeability pathway. An alternative theory, which had already been proposed in the early 1970s (Massari and Azzone, 1972), considered the permeability transition as linked to reversible opening of a pore. The pore theory was fully developed in the late 1970s (Haworth and Hunter, 1979; Hunter and Haworth, 1979a,b), but it did not gain much consensus. Only with the demonstration that the permeability transition could be inhibited by nanomolar concentrations of cyclosporin A (CsA) did the pore theory become established (Fournier *et al.*, 1987; Crompton *et al.*, 1988; Broekemeier *et al.*, 1989). The electrophysiological demonstration that the mitochondrial megachannel, a high-conductance inner membrane channel (Kinnally *et al.*, 1989; Petronilli *et*

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al., 1989), is inhibited by CsA (Szabo and Zoratti, 1991) and responds to most inducers and inhibitors as does the permeability transition (Szabo *et al.*, 1992; Szabo and Zoratti, 1992) left little doubt that the permeability transition is mediated by opening of a high-conductance channel.

Despite this progress, major issues remained open. First, it was hard to understand how a single channel could be modulated by over 40 classes of unrelated compounds, a finding that still makes the molecular nature of the pore quite enigmatic. Indeed, any candidate PTP protein(s) must account for all inducers and inhibitors. Second, despite intensive investigation, the physiological role of the pore and its very occurrence *in vivo* remain the matter of intense debate. Many experimental observations support the idea that PTP opening plays a key role in the pathways to cell death, either directly, because of disruption of energy metabolism, or indirectly, because of mitochondrial swelling leading to rupture of the outer mitochondrial membrane with release of apoptotic factor(s) located in the intermembrane space (Imberti *et al.*, 1993; Pastorino *et al.*, 1993; Zamzami *et al.*, 1995; Susin *et al.*, 1996; Pastorino *et al.*, 1998). On the other hand, evidence is also accumulating that the PTP may serve the physiological function of providing mitochondria with a fast Ca^{2+} release channel (Bernardi and Petronilli, 1996), which is consistent with recent observations relevant to mitochondrial Ca^{2+} signaling *in situ* (Ichas *et al.*, 1997) and with the finding of reversible pore openings in intact cells (Petronilli *et al.*, 1999).

Here, we briefly review recent work that illustrates how the PTP is regulated, and how a multiplicity of known pore effectors converge on a more limited set of regulatory sites that modulate the pore open–closed transitions *in vitro*. On request of the Editor, the review covers in some detail results obtained in the Bernardi laboratory and it will include recent results suggesting that the PTP is regulated by electron flux through the respiratory chain complex I (Fontaine *et al.*, 1998a). In this context, we will discuss the mechanism by which ubiquinone analogs modulate the pore (Fontaine et al., 1998b), a discovery that may open new perspectives for PTP modulation *in vivo* and possibly for its definition at the molecular level.

MECHANISTIC ASPECTS OF PORE REGULATION

Divalent Cations

Ca^{2+} is the single most important factor for opening of the PTP. Addition of Ca^{2+} alone is sufficient to induce a mitochondrial permeability transition (see Zoratti and Szabo, 1995 for review), which can be reverted by addition of excess EGTA (Haworth and Hunter, 1980; Crompton *et al.*, 1987; Petronilli *et al.*, 1994b). The relevant parameter is matrix Ca^{2+} since inhibition of Ca^{2+} uptake by ruthenium red abolishes PTP opening (Hunter and Haworth, 1979a). The amount of matrix Ca^{2+} required to open the pore is decreased in the presence of the so-called "inducers", while it is increased in the presence of PTP inhibitors (see below). Other Me^{2+} ions generally behave as pore inhibitors, and this is true irrespective of whether cations are accumulated (like Sr^{2+} and Mn^{2+}) or not (like Mg^{2+}). The general effects of Me^{2+} ions on the pore can be rationalized with the existence of two separate Me^{2+}-binding sites on the PTP: (i) an external site: occupation of this site (apparent I_{50} = 0.2 m*M*) by any Me^{2+} ion (including Ca^{2+} itself) decreases the probability of pore opening; (ii) an internal site: occupation of this site by Ca^{2+} increases the probability of pore opening, while all other Me^{2+} are inhibitory and apparently compete with Ca^{2+} (Bernardi *et al.*, 1993).

Membrane Potential

The PTP behaves like a voltage-dependent channel, which favors the closed conformation at high membrane potentials and the open conformation after depolarization (see Bernardi *et al.*, 1994 for a review). The voltage dependence appears to be an intrinsic property of the pore. This has been described both in isolated mitochondria, by modulating the membrane potential with uncouplers (Bernardi, 1992) or valinomycin (Scorrano *et al.*, 1997), and in patch-clamp experiments of single channels, by modulating the applied voltage (Szabo *et al.*, 1993; Szabo and Zoratti, 1993). These findings suggest the existence of a sensor that decodes the voltage changes into variations of the probability of pore opening. Highly conserved arginine residues play a key role in voltage sensing by the plasma membrane K^+, Na^+, and Ca^{2+} channels (Catterall, 1988), and recent results with arginine-selective reagents suggest that arginine residues may also play an important role in modulation of PTP opening by Ca^{2+} and depolarization (Eriksson *et al.*, 1997, 1998).

The proposal that the pore also senses changes of the surface potential represents a substantial improvement in the understanding of how PTP responses can be modulated by a variety of heteroge-

neous agents (Broekemeier and Pfeiffer, 1995). Amphipathic anions (like fatty acids) behave as pore activators with an effect that cannot be explained by depolarization (Broekemeier and Pfeiffer, 1995). Conversely, polycations (like spermine, positively charged peptides, and amphipathic cations like sphingosine and trifluoroperazine) inhibit pore opening, the latter acting independently of inhibition of phospholipase A_2 (Lapidus and Sokolove, 1992; Rigobello *et al.*, 1995; Broekemeier and Pfeiffer, 1995). All these data are consistent with the view that a more positive surface potential favors pore closure, while a more negative surface potential favors its opening (Bernardi *et al.*, 1994; Broekemeier and Pfeiffer, 1995).

Matrix pH

Since the early work of Hunter and Haworth, it had been appreciated that the mitochondrial permeability transition is inhibited at acidic pH values (Haworth and Hunter, 1979). Our laboratory has shown that the relevant parameter is matrix pH (Bernardi *et al.*, 1992), and that modulation is exerted through histidyl residues (Nicolli *et al.*, 1993). Reversible PTP closure occurs upon protonation of these residues and this can be prevented by carbethoxylation with diethylpyrocarbonate (Nicolli *et al.*, 1993). Besides a direct effect of pH on histidyl residue(s), it should be kept in mind that pH also affects the pyridine nucleotides redox potential (see below).

Oxidative Stress

Oxidative stress has long been known to favor the permeability transition (see Zoratti and Szabo, 1995 for review). The PTP possesses at least two redox-sensitive sites that both increase the probability of opening after oxidation; (i) the "S-site," a dithiol in apparent redox equilibrium with matrix glutathione; and (ii) the "P-site," in apparent redox equilibrium with the pyridine nucleotides pool (Chernyak and Bernardi, 1996; Costantini *et al.*, 1996).

Cross-linking of the S-site by arsenite or phenylarsine oxide, or its oxidation by diamide can induce the permeability transition under conditions where the pyridine nucleotides pool is demonstrably reduced (Costantini *et al.*, 1996). Oxidized glutathione is probably the immediate oxidant of the S-site and many pore inducers (like organic hydroperoxides and hydrogen peroxide) appear to affect the pore through changes in the level of reduced glutathione rather than by direct oxidation of the S-site (Chernyak and Bernardi, 1996). Pretreatment with monobromobimane (Costantini *et al.*, 1995a) or with low concentrations (10–20 μM) of *N*-ethylmaleimide (Petronilli *et al.*, 1994a), or reduction with dithiothreitol fully prevent the effects of oxidants that act at the S-site (Petronilli *et al.*, 1994a).

Oxidation of the P-site by oxidized pyridine nucleotides can induce the permeability transition under conditions where the glutathione pool is demonstrably kept in the fully reduced state, or when the S-site is reacted with arsenite (Chernyak and Bernardi, 1996; Costantini *et al.*, 1996). At variance from the S-site, the P-site cannot be blocked by monobromobimane or dithiothreitol, while it is sensitive to *N*-ethylmaleimide in the same concentration range as the S-site.

Although these experiments demonstrate that the PTP open–closed transitions can be independently modulated at the S- and P-sites, it must be considered that their relative contribution to PTP opening under conditions of oxidative stress may be extremely difficult to assess because the mitochondrial levels of reduced pyridine nucleotides and glutathione are connected through energy-linked transhydrogenase and glutathione reductase (Hoek and Rydström, 1988). A key role of NADH as the source of reducing equivalents for mitochondrial antioxidant defenses through transhydrogenase is supported by the finding that paraquat induces PTP opening through GSH oxidation in succinate-energized rat liver mitochondria only in the presence of rotenone, while it becomes totally ineffective when NAD(P)H is kept reduced by reverse electron flux through complex I when rotenone is omitted (Costantini *et al.*, 1995b).

A third redox-sensitive site has recently been described. Treatment of mitochondria with low concentrations of the sulfhydryl oxidant, copper-*o*-phenanthroline promotes pore opening (Costantini *et al.*, 1998). At variance from all reagents acting at the S-site, with this inducer pore opening is promoted rather than inhibited by low (micromolar) concentrations of *N*-ethylmaleimide, while pore closure is still observed upon reduction with dithiothreitol or β-mercaptoethanol (Costantini *et al.*, 1998). Because of the lack of inhibition by *N*-ethylmaleimide and because copper-*o*-phenanthroline is membrane-impermeant (and, therefore, unlikely to directly affect matrix glutathione), this site appears to be distinct from the S-site.

Phosphate

H^+ extrusion by the mitochondrial respiratory chain builds up both a transmembrane electrical potential and an inside alkaline pH gradient, which can be as high as −100 mV in the absence of weak acids (see, e.g., Bernardi and Pietrobon, 1982). Subsequent addition of P_i leads to its accumulation with matrix pH decrease and proportional increase of membrane potential, both conditions that favor PTP closure and should, therefore, prevent the permeability transition. Yet, P_i is a potent inducer of the permeability transition through a mechanism that remains unclear. There is no compelling evidence that P_i directly binds to the pore and it is generally assumed that P_i acts by modulating other PTP regulators. It has been proposed that P_i may favor PTP opening, in part at least, by causing a decrease of intramitochondrial free [Mg^{2+}] (Petronilli *et al.*, 1993) or [ADP] (Kunz *et al.*, 1981; Lapidus and Sokolove, 1994).

Ligands of the Adenine Nucleotide Translocator

Together with Mg^{2+} and H^+, adenine nucleotides were the earliest PTP inhibitors to be identified. ADP is more effective than ATP, while AMP has only marginal effects (see Zoratti and Szabo, 1995 for review). Agents known to deplete the matrix adenine nucleotides pool (e.g., PP_i or phospho enol pyruvate) favor the permeability transition (Peng *et al.*, 1974; Hunter and Haworth, 1979a; Asimakis and Sordahl, 1981; Vercesi and Lehninger, 1984), and patch-clamp experiments confirmed that ADP acts on the matrix side of the pore (Szabo and Zoratti, 1992). It is generally assumed that the inhibitory action of ADP involves interactions with the adenine nucleotide translocator (ANT). This proposal is supported by the finding that exogenous ANT ligands such as carboxyatractylate and bongkrekate also regulate the permeability transition. Although both carboxyatractylate and bongkrekate inhibit ADP–ATP exchange, the former favors PTP opening while the latter inhibits it. Since there is no relation between ANT activity and the PTP open–closed state, it has been proposed that the PTP may rather be regulated by the ANT conformational changes resulting from atractylate and bongkrekate binding. Indeed, the PTP inducer carboxyatractylate stabilizes the ANT in the "C" (for cytosol-facing) conformation while the PTP inhibitor bongkrekate stabilizes the ANT in the "M" (for matrix-facing) conformation (Schultheiss and Klingenberg, 1984). A causal link between the M ANT conformation and the PTP closed state is strengthened by the observation that ADP also stabilizes the ANT in the M conformation (Scherer and Klingenberg, 1974). This issue will be further discussed below.

Cyclosporin A and Cyclophilin D

In 1987, Fournier *et al.* reported that, in the presence of Cyclosporin A (CsA), mitochondria accumulate large Ca^{2+} loads that would otherwise collapse the membrane potential and cause Ca^{2+} efflux because of a "membrane damage" (Fournier *et al.*, 1987). Inhibition of PTP opening by CsA was subsequently demonstrated (Crompton *et al.*, 1988; Broekemeier *et al.*, 1989). Today CsA is used as a diagnostic tool for the characterization of the PTP in isolated mitochondria and in living cells and organs. However, it must be kept in mind that CsA does not selectively act on mitochondria, but also inhibits other cellular functions.

Most biological effects of CsA are mediated by its binding to a family of intracellular receptors, the cyclophilins, which all possess peptidyl-prolyl-*cis*–*trans*-isomerase activity (reviewed by Galat and Metcalfe, 1995). Immunosuppression is due to the Ca^{2+}–calmodulin-dependent binding of the CsA–cyclophilin complex to calcineurin, a cellular phosphatase, which becomes inhibited. Calcineurin inhibition, in turn, prevents dephosphorylation of transcriptional activators that cannot translocate to the nucleus in the phosphorylated form, preventing transcription of specific genes of activated T cells (Emmel *et al.*, 1989). Calcineurin inhibition is not involved in pore modulation since *N*-MethylVal-4-cyclosporin, a derivative that binds cyclophilin but not calcineurin (Schreier *et al.*, 1993), is as effective as CsA at PTP inhibition (Nicolli *et al.*, 1996).

Mitochondria possess a unique matrix cyclophilin, cyclophilin D (Woodfield *et al.*, 1997), but apparently no other cyclosporin-binding proteins (Nicolli *et al.*, 1996). The most plausible hypothesis concerning pore inhibition by CsA is that cyclophilin binding to the matrix side of the PTP favors its opening and that CsA indirectly causes pore closure through unbinding of cyclophilin D after formation of the CsA–cyclophilin D complex (Connern and Halestrap, 1996; Nicolli *et al.*, 1996). The peptidyl-prolyl-*cis*–*trans*-isomerase activity of cyclophilins is inhibited as a result of CsA binding, but this enzymatic activity is

probably not essential for PTP modulation by cyclophilin D, since diethylpyrocarbonate promotes pore opening at concentrations that fully inhibit the peptidyl-prolyl-*cis–trans*-isomerase activity of cyclophilin D (Nicolli *et al.*, 1993).

Ubiquinones and Electron Flux Through Complex I

We have recently described a novel mechanism of PTP regulation related to respiratory chain complex I activity. An increased probability of pore opening is observed in skeletal muscle mitochondria when electron flux increases through complex I (but not through complex II, III or IV), with an effect that is not related to hydrogen peroxide production (Fontaine *et al.*, 1998a). The inducing effect of complex I activity can be clearly dissociated from oxidation of pyridine nucleotides. Indeed, for comparable redox and membrane potentials PTP opening in the presence of P_i is reached after accumulation of much lower Ca^{2+} loads when mitochondria are energized with complex I rather than complex IV substrates (Fontaine *et al.*, 1998a). Similar results can be observed after Ca^{2+} loading followed by oxidation with either diamide or *tert*-butylhydroperoxide (E. Fontaine and P. Bernardi, unpublished observations). PTP modulation by electron flux through complex I is most easily observed in skeletal muscle mitochondria, but this type of regulation is also present in liver mitochondria (Fontaine *et al.*, 1998a,b).

We have recently discovered that some quinones, such as ubiquinone 0 and decylubiquinone, are potent PTP inhibitors (Fontaine *et al.*, 1998b). Inhibition is observed irrespective of the method used to induce the permeability transition, indicating that these quinones are general pore inhibitors (Fontaine *et al.*, 1998b). Based on the Ca^{2+} retention capacity of rat liver mitochondria, ubiquinone 0 is as effective as CsA when mitochondria are energized with succinate and more potent than CsA when mitochondria are energized with glutamate and malate. Decylubiquinone is somewhat less potent but, unlike ubiquinone 0, it does not inhibit respiration. The optimal concentration for PTP inhibition is about 50 μM for ubiquinone 0 and 100 μM for decylubiquinone (corresponding to 50–100 nmol $\times$ mg protein^{-1}) when these compounds are added directly to the assay medium (Fontaine *et al.*, 1998b), but much lower amounts are required (down to less than 10 nmol $\times$ mg protein^{-1}) when quinones are added to mitochondria before dilution in the incubation medium (E. Fontaine *et al.*, unpublished observations).

Importantly, ubiquinone 5 (which does not affect the PTP *per se*), specifically counteracts the inhibitory effect of ubiquinone 0 or decylubiquinone, but not that of CsA (Fontaine *et al.*, 1998b). This is a critical result that can be most easily explained by postulating the existence of a quinone-binding site on the pore, which would be shared by active and inactive ligands, separate structural features being required for binding and for pore inhibition. The scheme of Fig. 1 depicts our current model to explain how quinones may regulate the PTP open–closed transitions. We hypothesize that the PTP can exist in a liganded state with either inactive or inhibitory quinones, which, in turn, would confer different conformations to the pore resulting in a different accessibility to Ca^{2+}, the Ca^{2+}-binding sites being less accessible when the PTP is liganded with inhibitory quinones. These conformations are in equilibrium according to the relative membrane concentration of quinones and to their binding affinities, which remain undefined. In this scenario, addition of a limited Ca^{2+} load is more likely to open the PTP in the conformation liganded with inactive quinones, while a higher Ca^{2+} load is required to access the Ca^{2+}-binding site(s) in the conformation liganded with inhibitory quinones. Thus, the PTP open–closed transitions can be modulated by either increasing the Ca^{2+} load or by displacing the inhibitory quinone(s).

This model can also easily explain why increased electron flux through complex I favors PTP opening. Indeed, if we assume that endogenous ubiquinones bind the PTP, stabilizing it in the closed conformation, increasing electron flux within complex I may displace ubiquinones from this site, increasing, in turn, the pore open probability. If such a hypothesis is confirmed, endogenous ubiquinones should be viewed as endogenous PTP regulators (see below).

NATURE OF THE PERMEABILITY TRANSITION PORE

The molecular nature of the PTP remains unsolved. The existence of more than one permeability pathway remains a clear possibility, although the general dependence on voltage and matrix pH, and the inhibition by CsA, suggest that the PTP may be a single molecular entity. We think that any candidate PTP proteins should impart to membrane permeabilization in reconstituted systems all of the basic regula-

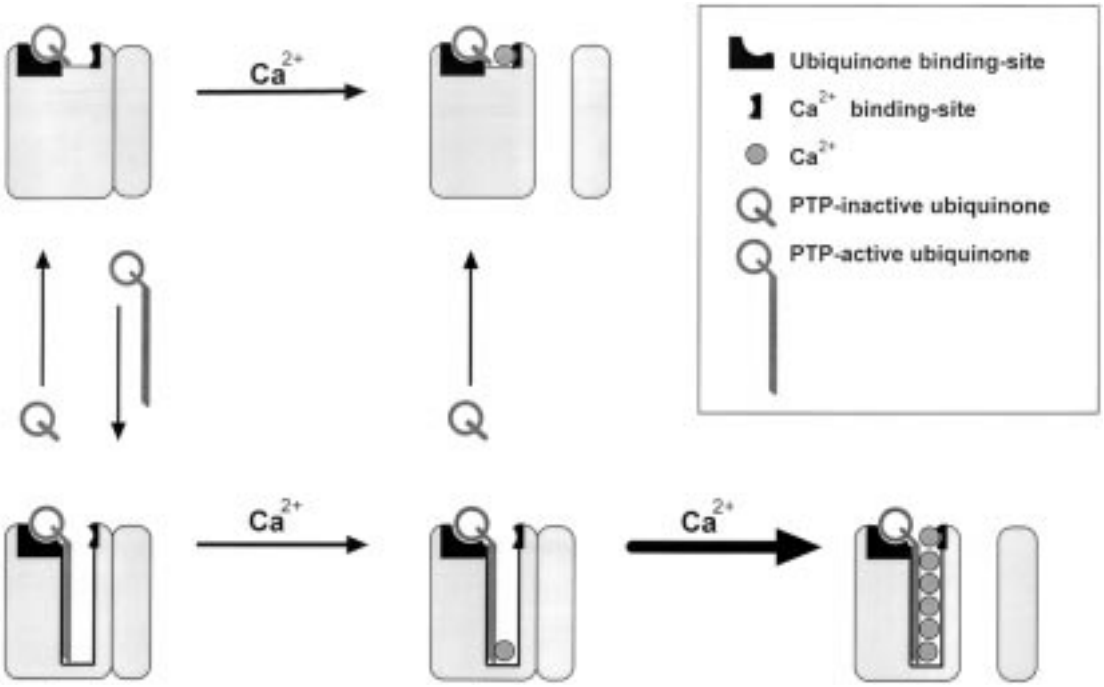

Fig. 1. Model for PTP regulation by ubiquinones. The open space between the two parts of the PTP denotes the open state of the pore. For an explanation, see text.

tory features established with isolated mitochondria, a condition that has thus far not been met. Below we discuss some results suggesting involvement of the ANT, of respiratory chain complex I, and of multiple protein–protein interactions in the regulation of permeability transition.

The Permeability Transition and the Adenine Nucleotide Translocator

Based on the effects of selective ANT inhibitors (carboxyatractylate and bongkrekate) and of ADP, it has been suggested that the pore might be formed by the ANT itself (Halestrap and Davidson, 1990). Direct electrophysiological evidence shows that ANT reconstituted in giant liposomes exhibits a high-conductance channel activity that is stimulated by Ca^{2+} and displays the gating effects and voltage-dependence expected of the PTP (Brustovetsky and Klingenberg, 1996). It must be mentioned, however, that such reconstituted channel is not inhibited by CsA (Brustovetsky and Klingenberg, 1996). Proteinaceous complexes prepared by extraction of mitochondria with low concentrations of detergent, and enriched in hexokinase, porin, and ANT, exhibit Ca^{2+}-dependent and CsA-sensitive high-conductance channel activity when reconstituted in planar lipid bilayers. These preparations also catalyze Ca^{2+}-dependent and CsA-sensitive ATP and malate diffusion after incorporation in proteoliposomes (Beutner *et al.*, 1996). It must be mentioned, however, that these fractions contain a number of other proteins and that the most active among them were not enriched in ANT or porin (Beutner *et al.*, 1996).

Halestrap and co-workers have found that the ANT binds to cyclophilin D both in detergent extracts of mitochondria and after purification (Woodfield *et al.*, 1998), but the relevance of these observations to PTP regulation remains questionable. We note that: (i) in these experiments, cyclophilin D also bound a large number of other proteins, which could equally well represent PTP components; (ii) after most proteins had been released by high concentrations of detergent and NaCl, several proteins other than the ANT remained bound to cyclophilin D in a CsA-inhibitable manner (see Fig. 3A in Woodfield *et al.*, 1998); (iii) once bound to cyclophilin D, the ANT could not be eluted with CsA, an observation that is hard to reconcile with the ready inhibition of the PTP by CsA in mitochondria; (iv) binding of the ANT to cyclophilin D was

reduced by treatment with both carboxyatractylate and bongkrekate, which affect the PTP in opposite directions; and (v) cyclophilin D bound equally well ANT purified from rat liver or yeast, yet the permeability transition is not inhibited by CsA in yeast mitochondria (Jung *et al.*, 1997).

On balance, we think that a direct–or exclusive–involvement of the ANT in PTP formation has not been convincingly demonstrated. Furthermore, one should wonder why, besides carboxyatractylate and bongkrekate, no other pore inducers and inhibitors (including CsA) affect the activity and the conformation of the ANT. In our opinion, an indirect effect of the ANT on the pore appears more likely. Since the ANT is the single most abundant inner membrane protein, its "C"–"M" transition may profoundly affect general membrane properties like the surface potential. Thus, it remains possible that the carrier conformation affects the pore indirectly through modulation of the putative PTP voltage sensor (Bernardi *et al.*, 1994).

The Permeability Transition and Respiratory Complex I

Our recent results on PTP regulation by complex I and its inhibition by specific quinones suggest an involvement of complex I in the permeability transition (Fontaine *et al.*, 1998a,b). Composed of 42 subunits, complex I is the largest respiratory chain complex in mammals. Its regulation is far from understood, to the point that Vinogradov considers it likely that not all of its functions are presently known (Vinogradov, 1993). Of particular relevance to the present discussion is the interconversion between inactive and active forms of the enzyme that can be easily observed in submitochondrial particles (Kotlyar and Vinogradov, 1990; Kotlyar *et al.*, 1992). The major determinant in the process of inactivation is temperature, in the sense that incubation at increasing temperatures between 25 and 40°C is accompanied by a rapid decrease of the rotenone-sensitive NADH-ubiquinone reductase activity but not of the rotenone-insensitive NADH-ferricyanide or NADH-hexammineruthenium (III) reductase activities (Vinogradov, 1993, 1998). The inactive enzyme can be converted back to the active form by NADH when ubiquinone is the electron acceptor, indicating that the enzyme is activated by its own turnover (Vinogradov, 1993 and references therein). NADH binding modifies the proteolytic pattern of complex I, suggesting that NADH binding modifies complex I conformation (Yamaguchi *et al.*, 1998). Also the transition between the active and inactive states of the enzyme is accompanied by changes of conformation that make the inactive form very sensitive to Ca^{2+}, alkaline pH, and *N*-ethylmaleimide (Kotlyar *et al.*, 1992), a finding that suggests the existence of a regulatory Ca^{2+}-binding site in complex I.

To assess whether Ca^{2+} regulates complex I activity in intact mitochondria, we measured the rate of oxygen consumption by rat liver mitochondria incubated in a sucrose-based medium supplemented with 1 μM CsA and 2 mM Mg^{2+} to prevent PTP opening (which would be followed by Ca^{2+} release). Mitochondria were incubated with either succinate plus rotenone (Fig. 2, panel A) or with glutamate plus malate (Fig. 2, panel B). In the case of succinate oxidation, the addition of a Ca^{2+} pulse was followed by a respiratory stimulation of the same magnitude of that elicited by the addition of uncoupler (compare traces a and b). Upon completion of Ca^{2+} accumulation (not shown), respiration returned to basal levels and could be stimulated by the addition of uncoupler in the presence of the uniporter inhibitor ruthenium red (trace b) to the same final rate as that maintained by mitochondria that had not accumulated Ca^{2+} (trace a). A completely different picture was observed when glutamate and malate were oxidized. In this case, (i) the rate of respiration measured after the addition of Ca^{2+} was significantly slower than the uncoupled rate in the absence of Ca^{2+} accumulation (compare traces d and c); and (ii) the uncoupler-stimulated respiration remained well below the rate measured in the absence of Ca^{2+} accumulation. This decreased activity of complex I was not due to a leak of NADH through open pores, since no PTP opening took place in these protocols, as assessed by lack of swelling (Figure 2, inset). Furthermore, respiratory inhibition could not be prevented by added NADH (not shown). These experiments indicate that complex I is inhibited by Ca^{2+} in intact mitochondria, an effect that could be due to transition to the "inactive" form of the enzyme. Whether or not this interconversion will prove relevant to PTP regulation, we find it remarkable that Ca^{2+}, pH, *N*-ethylmaleimide, NADH, and complex I turnover are also well-characterized modulators of the PTP.

Endogenous ubiquinone is involved in redox reactions in complex III (Q cycle), and acts as an electron shuttle between complexes I, II, and III (see Ernster and Dallner, 1995 for review). The total number of ubiquinone-binding sites in the respiratory chain has not been defined with certainty, but in the case of

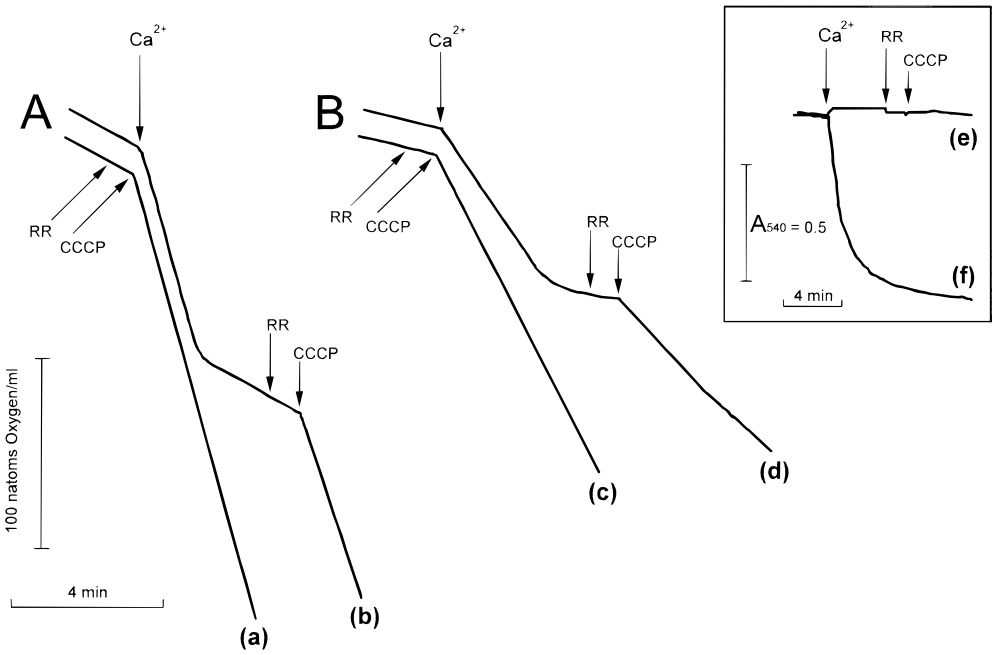

Fig. 2. Effect of Ca^{2+} on the maximal rate of respiration with Complex II or Complex I respiratory substrates. The incubation medium contained 250 m*M* sucrose, 1 m*M* P_i–Tris, 10 m*M* Tris–MOPS, 2 m*M* $MgCl_2$, and 1 μ*M* CsA in traces a-e, while CsA was omitted in trace f. Final volume, 2 ml, pH 7.35, 25°C. In Panel (A), the substrate was 5 m*M* succinate–Tris plus 2 μ*M* rotenone, while in Panel (B) and in Inset the substrates were 5 m*M* glutamate–Tris and 2.5 m*M* malate–Tris. Experiments were started by the addition of 2 mg mitochondria (not shown). Where indicated, 500 μ*M* Ca^{2+}, 1 μ*M* ruthenium red (RR), and 400 nM CCCP were added. Panel (A) and (B), mitochondrial oxygen consumption; Inset, represents mitochondrial absorbance at 540 nm.

complex I, at least two bindings sites have been identified (Di Virgilio & Azzone, 1982; Degli Esposti *et al.*, 1996). At the level of the first binding site, ubiquinone is reduced in a rotenone-sensitive manner during a process that couples electron flux to proton extrusion. At the level of the second binding site, ubiquinone is reduced in a rotenone-insensitive manner during a process that is not coupled to proton translocation. Whether PTP inhibition by ubiquinone 0 or decylubiquinone is due to binding to either or both of these sites remains unknown, but there can be little doubt that complex I represents an obvious candidate for the pore inhibitory effects of these quinones.

In summary, we note (i) that both PTP and complex I are affected by matrix pH and electron flux in complex I, and both possess NADH-, ubiquinone-, and divalent cation-binding sites; (ii) that the transmembrane voltage—a major parameter regulating the PTP open–closed transitions—is obviously a key element in the control of the complex I proton pumping activity and of the coupled electron transfer reactions; and (iii) that a role for cyclophilin D in the proper assembly and functioning of the 42-subunit complex I, and possibly in the interconversion between its active and inactive conformations, should be investigated. We find it striking that *Saccharomyces cerevisiae* mitochondria, which lack a classical Ca^{2+}- and CsA-sensitive permeability transition (Jung *et al.*, 1997) also lack complex I, but not the ANT (de Vries and Marres, 1987).

Protein Interactions in the Regulation of the Permeability Transition

Although the permeability transition is primarily an inner membrane event, this does not exclude interactions with other proteins not located in the inner membrane. It is striking that overexpression of Bcl-2 (an antiapoptotic member of the Bcl protein superfamily that largely localizes to the outer mitochondrial membrane, Riparbelli *et al.*, 1995) appears to prevent induction of the permeability transition following specific stimuli (Susin *et al.*, 1996); and conversely, that overexpression of bax (a proapoptotic member of the

same family) promotes cell death, which appears to correlate with onset of the permeability transition (Pastorino *et al.*, 1998). In general, it appears conceivable that the permeability transition may be affected by complex and dynamic interactions between inner and outer membrane components; and that complex I (and/or the ANT) may either play a structural role in channel formation or a regulatory role in its open–closed transitions. It is of interest that the PTP may also be regulated by protein–protein interactions from the matrix (through cyclophilin D, Connern and Halestrap, 1996; Nicolli *et al.*, 1996), the intermembrane space (through creatine kinase, O'Gorman *et al.*, 1997), and the cytosol (through hexokinase, Beutner *et al.*, 1996).

UBIQUINONES, DISEASE, AND AGING

The discovery that ubiquinones can regulate the PTP may open new perspectives in the pharmacological modulation of the PTP *in vivo*. This finding can also shed new light on experimental results showing that ubiquinones play a role in aging and disease.

The content of endogenous ubiquinones (mainly ubiquinone 45 in rodents and ubiquinone 50 in man) decreases with aging both in human tissues (Kalen *et al.*, 1989; Soderberg *et al.*, 1990; Edlund *et al.*, 1992) and in rat liver and heart mitochondria (Genova *et al.*, 1995). Furthermore, the level of ubiquinone 50 is significantly lower in platelet mitochondria obtained from Parkinson patients than from age- and sex-matched control subjects (Shults *et al.*, 1997), suggesting that endogenous ubiquinones may delay cell degeneration.

On the other hand, ubiquinone 50 protects cultured neurons against glutamate toxicity (Favit *et al.*, 1992) and against degeneration induced by mumps virus (Edlund *et al.*, 1994). Pretreatment of rats or rabbits with ubiquinone 50 protects hearts against ischemia/reperfusion damage (Takeo *et al.*, 1987; Crestanello *et al.*, 1996), and improves cardiac function in rats with chronic heart failure (Sanbe *et al.*, 1994). Oral administration of ubiquinone 50 to rats also markedly attenuates the striatal lesions produced by systemic administration of 3-nitropropionic acid (Matthews *et al.*, 1998) or by intrastriatal administration of aminooxyacetic acid (Brouillet *et al.*, 1994) or malonate (Beal *et al.*, 1994); reduces the loss of striatal dopamine and dopaminergic axons induced by 1-methyl-4-phenyl-1,2,3-tetrahydropyridine in aged mice (Beal *et al.*, 1998); and increases the life-span in a transgenic mouse model of familial amyotrophic lateral sclerosis (Matthews *et al.*, 1998). It is important to note that feeding rats a diet supplemented with ubiquinone 50 leads to a significant increase in the concentration of ubiquinone 50 in cerebral cortex mitochondria (Matthews *et al.*, 1998).

It is generally proposed that the protective effects of ubiquinone 50 are related to its free radical scavenging activity in the mitochondrial inner membrane. If PTP opening plays a key role in cell death, particularly in neurodegenerative diseases (Beal, 1998; Schapira, 1998), the hypothesis that endogenous ubiquinones may regulate PTP opening *in vivo* should also be considered. Our recent finding that exogenous ubiquinones increase the Ca^{2+} retention capacity in isolated mitochondria (Fontaine *et al.*, 1998a,b) indicates that the PTP–ubiquinone-binding sites are not saturated and, therefore, that the PTP can, in principle, be affected by dietary quinones *in vivo*. In this context, we also note that the ubiquinone 50 content is significantly higher in human colorectal neoplasms than in normal colorectal mucosa (Palazzoni *et al.*, 1997). Thus, ubiquinone 50 increases in immortalized cells, while it decreases in aging and in degenerative diseases. These data suggest that the content of ubiquinone 50 correlates with the cell's life-span, and we feel that it is worth exploring the possible contribution of the PTP status to this correlation.

PERSPECTIVES

In this short review, we have discussed the regulation of the PTP by pathophysiological effectors, with special emphasis on the regulatory role of complex I as well as on modulation by endogenous and exogenous ubiquinones. The development and screening of novel pore modulators based on the quinone structure is currently under way in our laboratories. Testing their effects in models of cell death *in vitro* and *in vivo* should improve our understanding of pore modulation and provide further clues to its role in cellular life and death.

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