On the Voltage Dependence of the Mitochondrial Permeability Transition Pore

A CRITICAL APPRAISAL

Luca Scorrano‡, Valeria Petronilli, and Paolo Bernardi§

From the Consiglio Nazionale delle Ricerche Unit for the Study of Biomembranes and the Laboratory of Biophysics and Membrane Biology, Department of Biomedical Sciences, University of Padova Medical School, Viale G. Colombo 3, I-35121 Padova, Italy

The mitochondrial permeability transition pore, a cyclosporin A-sensitive channel, can be opened by the addition of protonophoric uncouplers such as carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) after energy-dependent accumulation of Ca2+. We have proposed that the relevant effect of FCCP on the pore is membrane depolarization, suggesting that this channel is voltage-dependent (Bernardi, P. (1992) J. Biol. Chem. 267, 8334–8339). Here, we reconsider this hypothesis in the light of recent observations suggesting that increased production of reactive oxygen species and/or direct effects of FCCP, rather than membrane depolarization, could be the actual triggers of the FCCP-dependent permeability transition. We show that although reactive oxygen species can contribute to the permeability transition, pore opening by FCCP can still be observed under strict anaerobiosis after ATP-dependent Ca2+ accumulation and that the permeability transition can be induced by the addition of valinomycin to respiring mitochondria treated with nigericin in low potassium medium. In this system, pore opening in increasing fractions of mitochondria depends on the concentration of valinomycin, i.e. on the magnitude of the potassium current that determines the extent of membrane depolarization. We conclude that the permeability transition pore is directly modulated by the membrane potential in intact isolated rat liver mitochondria.

The permeability transition (PT) is a permeability increase of the inner mitochondrial membrane most easily observed after matrix Ca2+ accumulation (see Ref. 1 for a review). Although the PT can be favored by a large series of heterogeneous compounds and conditions (see Ref. 2 for a complete list), it is generally agreed that it is mediated by an opening of a complex channel, the CsA-sensitive MTP (1). Recent work indicates that the MTP is highly regulated (see Ref. 3 for a recent review) and that its opening may be instrumental in determining the fate of the cell both in accidental and programmed cell death (4).

We have suggested that one key feature of MTP regulation is its control by the proton electrochemical gradient and that the pore is modulated by both the transmembrane electrical potential difference (where depolarization favors pore opening; see Ref. 5) and by matrix pH (where acidification favors pore closure; see Ref. 6). The conceptual framework of the MTP voltage dependence has proved useful to explain its modulation by a variety of inducers and inhibitors. The basic idea is that the probability of pore opening can be modified either by changes of the membrane potential or by changes of the threshold potential at which pore opening occurs through discrete sites (see Refs. 7 and 8 for reviews on the mechanistic aspects of pore modulation within this framework).

Evidence that the pore is voltage-dependent in intact isolated mitochondria remains indirect, however, and it is entirely based on the effects of the protonophoric uncoupler FCCP (8). Two recent sets of data prompted us to critically reconsider the mechanism by which the addition of uncouplers causes MTP opening and to reevaluate our conclusion that FCCP induces the PT because it induces a collapse of the transmembrane electrical potential. A first reason of concern is that uncouplers can also increase the production of ROS (such as H2O2), particularly in the presence of antimycin A (9) and after Ca2+ accumulation (10). Since ROS have long been implied in the PT (Refs. 10–13, and references therein), it appears essential to establish if membrane depolarization (5) or rather ROS-dependent oxidative stress (10) is the link between FCCP and MTP opening. A second question is posed by the intriguing finding that the uncoupling effect of protonophores like FCCP (but not 2,4-dinitrophenol) can be selectively reversed by nanomolar concentrations of 5α-cholestan-3β-ol-6-one suggesting a protein-mediated mechanism for uncoupling by FCCP (14) and possibly for pore opening.

In this study we have experimentally addressed these issues. We show that although ROS can contribute to the permeability transition in a relatively small fraction of mitochondria, MTP opening by FCCP can still be easily observed under strict anaerobiosis. We also find that the permeability transition can be induced by the addition of valinomycin to respiring mitochondria treated with nigericin in low potassium medium; MTP opening in increasing fractions of mitochondria depends on the concentration of valinomycin, i.e. on the magnitude of the potassium current and on the extent of membrane depolarization. We conclude that the pore is directly modulated by the membrane potential in intact isolated mitochondria and that ROS presumably contribute to the PT by sensitizing the pore to

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depolarization through oxidation of mitochondrial pyridine nucleotides (15) and of a critical dithiol (16), which appears to be in redox equilibrium with matrix glutathione (17).

MATERIALS AND METHODS

Liver mitochondria from albino Wistar rats weighing about 300 g were prepared by standard differential centrifugation as described previously (18). MTP opening was followed as the change of absorbance or of 90° light scattering at 540 nm with an Aminco DW2A spectrophotometer, respectively. Mitochondria (0.5 mg × ml⁻¹) were incubated in thermostatted magnetically stirred cuvettes (final volume of 2 ml), and pore opening was triggered after accumulation of a limited Ca²⁺ load as specified in the figure legends. The data are expressed as the fractional pore opening (Φ) calculated as described in detail in Ref. 16. Incubation medium and further details are specified in the figure legends.

Measurements of pore opening under anoxic conditions (Fig. 3) were carried out in stopped cuvettes equipped with two small ports for the inflow and outflow of gas, the latter also being used for the additions. The medium was equilibrated with pure N₂ gas in the closed cuvettes, and the gas flow was maintained throughout the experiment. The O₂ concentration was measured in parallel with a Clark O₂ electrode in a chamber with identical volume and surface area. The chamber was sealed with parafilm, and the medium was equilibrated with N₂ gas through a small hole snugly fitting the gas inlet tubing, while additions were made through a small hole also allowing gas outflow. The N₂ gas flow was maintained throughout the experiment.

Mitochondrial membrane potential and matrix pH were calculated from the distribution of triphenylmethylphosphonium ion with a triphenylmethylphosphonium ion-selective electrode and from 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein fluorescence measurements exactly as described previously in the presence of 1 μM CsA to prevent depolarization following pore opening (Refs. 5 and 6, respectively).

The Ca²⁺ and K⁺ content of the incubation medium was determined by atomic absorption spectroscopy. Adventitious Ca²⁺ was always taken into account when calculating the size of the Ca²⁺ load, and the K⁺ concentration stated in the legend to Fig. 4 refers to that measured by atomic absorption spectroscopy of the 30,000 × g supernatants obtained after treatment of mitochondria with nigericin alone.

All chemicals were of the highest purity commercially available. Catalase (C-40) was purchased from Sigma and prepared daily. Its activity was assessed by measuring the production of oxygen from H₂O₂ with the Clark electrode.

RESULTS AND DISCUSSION

Relative Role of Depolarization and ROS in FCCP-dependent MTP Opening—We have recently exploited a protocol for induction of the PT based on the addition of FCCP to mitochondria that have accumulated a Ca²⁺ load not able to cause the PT per se (16). This protocol has been successfully used by several laboratories (10, 19, 20). We (16) as well as others (19, 20) have interpreted the FCCP effect as an indication that the pore can be opened by depolarization, but a different explanation has been offered by Vercesi and co-workers (11). They reported that the effects of FCCP as a PT inducer can be completely prevented by the addition of 2 μM catalase (11). Matrix Ca²⁺ can increase the production of ROS by mitochondria (9, 11) possibly via mobilization of intramitochondrial iron stores able to stimulate the Fenton reaction of H₂O₂ (11). This would yield OH⁻ then acting as the oxidant of a critical pore dithiol (11). In support of this scheme, catalase can prevent pore opening in models based on the addition of exogenous peroxides (11) or on the endogenous production of H₂O₂ (12).

The experiments of Fig. 1 illustrate our protocols and report the effects of 4 μM catalase on FCCP-triggered pore opening at three different Ca²⁺ loads. In the presence of catalase (traces a) the rate and extent of FCCP-induced pore opening were smaller than in its absence (compare traces a and b). Note that at the lowest Ca²⁺ load the inhibitory effect of catalase was proportionally larger (panel A). These findings suggest that only a fraction of mitochondria is affected by catalase and that the sensitivity of the PT to ROS may critically depend on the Ca²⁺ load.

The Ca²⁺ dependence was studied in a range of Ca²⁺ concentrations (Fig. 2, panel A). The inhibitory effect of catalase was only partial, and importantly, it did not increase at increasing catalase concentrations (Fig. 2, panel B). This suggests that the lack of inhibition is not due to a kinetic imbalance between the rate of Ca²⁺-dependent production of ROS and their rate of scavenging by catalase. It should be noted that in this experiment we choose the Ca²⁺ load (10 nmol × mg of protein⁻¹) giving maximal sensitivity to inhibition by catalase (see Fig. 1). These experiments indicate that catalase-sensitive and -insensitive mechanisms may exist in the FCCP-triggered PT. Why only a fraction of mitochondria appears to be sensitive to catalase remains unclear and may relate to the iron content of individual mitochondria. However, given that the pores appear to be heterogeneous in size (21), ROS might instead slightly increase the average pore dimensions, thus apparently recruiting a larger fraction of mitochondria in the sucrose permeation assay.

To further test whether ROS are important in uncoupler-dependent pore opening, we have carried out experiments under normoxic and anoxic conditions. ATP was used as the energy source for Ca²⁺ accumulation in protocols otherwise similar to those reported in Fig. 1, except that antimycin A was added to maximize ROS production (9, 10). Fig. 3 shows that FCCP-de-
A

pendent pore opening could be readily observed after Ca\(^{2+}\) accumulation under N\(_2\) anoxia (panel B), although the rate and the final degree of swelling were somewhat reduced relative to the normoxic incubations (compare traces b in panels A and B).

At variance from these findings, Kowaltowski et al. (10) have reported full inhibition of FCCP-dependent pore opening by catalase and anoxia under apparently very similar conditions. In their work, however, the Ca\(^{2+}\), catalase, or uncoupler dependence of this effect has not been systematically addressed. We suspect that these or other factors (e.g. the endogenous iron content of mitochondria) might account for this difference. However, our ability to observe pore opening in the presence of saturating concentrations of catalase (Fig. 2, panel B) and under anoxia (Fig. 3, panel B) indicates that production of H\(_2\)O\(_2\) and ROS is not essential for MTP opening induced by FCCP. It should be noted that the effect of ROS can be easily accommodated within the framework of the MTP voltage dependence, since there is general consensus that ROS facilitate dithiol oxidation (11), which in turn critically sensitizes the pore to the effects of membrane depolarization (15, 16).

Opening of the MTP by a Valinomycin-induced K\(^{+}\) Current—Uncouplers of the FCCP class depolarize the inner membrane through a protonophoretic action. Shuttling of the unprotonated form to the outer surface of the inner membrane and of the protonated neutral form toward the matrix surface would account for the short-circuiting of the protonmotive force (22). It is noteworthy, however, that lower concentrations of FCCP are needed to collapse the proton gradient in mitochondria than in artificial lipid membranes (22). This raises the possibility that the effects of FCCP on mitochondria might be mediated, in whole or in part, by interactions with protein(s), possibly the proton pumps themselves. Support for this view has recently come from Skulachev and co-workers (14), who have demonstrated that the uncoupling effects of protonophores like FCCP, carbonyl cyanide 3-chlorophenylhydrazone, and SF 6847 can be selectively reversed by concentrations of 5α-cholenol-3β-ol-6-one as low as 2–10 nM. This in turn raises the possibility that FCCP might be reacting directly with pore components rather than indirectly through its effects on the membrane potential. We therefore decided to put the hypothesis of pore regulation by the membrane potential to an additional test by inducing mitochondrial depolarization with the classical K\(^{+}\) ionophore, valinomycin.

In the experiments of Fig. 4, panel A, succinate-energized mitochondria were given a Ca\(^{2+}\) pulse of 5 \(\mu\)M followed by the addition of 900 nm nigericin. Under these conditions the exter-
The addition of increasing concentrations of valinomycin to nigericin-treated mitochondria was then followed by a process of absorbance decrease indicative of osmotic swelling, which was virtually unaffected by the subsequent addition of 45 nM valinomycin; data not shown). The transient nature of the nigericin-dependent changes of the protonmotive force in these experiments appears to depend on the uptake of Tris following hyperpolarization (see Ref. 23 for a detailed study on the transient nature of the effects of nigericin on mitochondrial membrane potential, ΔpH, and on the nature of compensatory cation and anion fluxes).

The addition of increasing concentrations of valinomycin to nigericin-treated mitochondria was then followed by a process of absorbance decrease indicative of osmotic swelling, which increased at increasing valinomycin concentrations between 4.5 and 45 nM (traces a–d). Due to the presence of excess nigericin and to the low K⁺ concentration, this swelling process did not depend on active K⁺ accumulation but rather on entry of sucrose, which is the main osmotic support of the incubation medium. Indeed, swelling induced by 45 nM valinomycin was completely prevented by 1 μM CsA (trace f), and no swelling occurred when 45 nM valinomycin was added in the absence of Ca²⁺ (trace g). These data indicate that valinomycin-dependent swelling depends on opening of the MTP. Both the fraction of mitochondria responding with pore opening and the rate of swelling increased with the concentration of valinomycin, and this was matched by increased levels of depolarization in the range expected to modulate the pore (Fig. 4, panel B). The voltage dependence of the MTP in mitochondria treated with valinomycin under these conditions is thus similar to that obtained with uncoupler titrations (5, 15, 16). It must be mentioned that in the experiments of Fig. 4 pore opening was unaffected by 4 μM catalase (data not shown). Results similar to those reported in Fig. 4 were obtained if 45 nM valinomycin was added first, followed by increasing concentrations of nigericin, although the permeabilization rate was slower due to the transient matrix acidification following the addition of nigericin (data not shown).

Conclusions—The data reported in this study provide strong support to the idea that the MTP is modulated by the membrane potential in isolated liver mitochondria, as suggested in previous papers from this laboratory (5, 15, 16). They also confirm that, as suggested by Vercesi and co-workers (10–13), ROS generated after Ca²⁺ uptake and FCCP addition can favor pore opening, possibly through a dithiol-disulfide interconversion (8, 11–13, 15–18). It is clear, however, that pore opening by FCCP can occur in the absence of ROS and therefore that membrane depolarization is a primary cause of the PT (5). It is reassuring that the open-closed transitions of the mitochondrial megachannel (measured as single channel events in electrophysiological experiments on rat liver mitoplasts) are strongly affected by the applied voltage with decreasing probabilities of pore opening at increasing potentials of either sign (24). Thus, the present data further support the suggestion that the megachannel is the electrophysiological counterpart of the PT (6, 24–26).

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