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Regulation of the permeability transition pore, a voltage-dependent mitochondrial channel inhibited by cyclosporin A

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Abstract

Mitochondria from a variety of sources possess a regulated inner membrane channel, the permeability transition pore (MTP), which is responsible for the 'permeability transition', a sudden permeability increase to solutes with molecular masses ≤ 1500 Da, most easily observed after Ca^{2+} accumulation. The MTP is a voltage-dependent channel blocked by cyclosporin A with K_i in the nanomolar range. The MTP open probability is regulated by both the membrane potential and matrix pH. The probability of pore opening increases as the membrane is depolarized, while it decreases as matrix pH is decreased below 7.3 through reversible protonation of histidine residues. Many physiological and pathological effectors, including Ca^{2+} and ADP, modulate MTP operation *directly* through changes of the gating potential rather than *indirectly* through changes of the membrane potential (Petronilli, V., Cola, C., Massari, S., Colonna, R. and Bernardi, P. (1993) J. Biol. Chem. 268, 21939–21945). Here we present recent work from our laboratory indicating that (i) the voltage sensor comprises at least two vicinal thiols whose oxidation-reduction state affects the MTP gating potential; as the couple becomes more oxidized the gating potential increases; conversely, as it becomes more reduced the gating potential decreases; (ii) that MTP opening is fully reversible, as mitochondria maintain volume homeostasis through several cycles of pore opening/closure; and (iii) that the mechanism of MTP inhibition by cyclosporin A presumably involves a mitochondrial cyclophilin but does not utilize a calcineurin-dependent pathway.

Key words: Mitochondrion; Channel; Voltage; Cyclosporin; Membrane permeability; Permeability transition pore

1. Introduction

Mammalian mitochondria possess an inner membrane channel, the permeability transition pore (MTP), which is blocked by the immunosuppressant cyclosporin A (see Ref. [1] and references therein). The MTP appears to coincide with the mitochondrial megachannel, a 1.3 nS inner membrane channel which responds to most effectors in the same direction and concentration range as does the permeability transition [2–4]. As first proposed in the pioneering studies of Hunter and Haworth, opening of the MTP is responsible for the 'permeability transition', a sudden increase in permeability to solutes with molecular masses $\leq$ 1500 Da most easily observed after Ca^{2+} accumulation in the presence of a variety of so-called 'inducing agents' (see Ref. [1] for references).

A low membrane permeability to protons, anions and cations generally is a basic feature of chemiosmotic energy conservation [5]. Given (i) the uncoupling with collapse of the $\Delta\tilde{\mu}_{H^+}$ and cessation of ATP synthesis following the permeability transition; (ii) the lack of selectivity of the permeability pathway itself; and (iii) the lack of common structural or functional features among the inducers, it is not surprising that in the past this phenomenon has been widely considered as a deleterious event, largely referred to as an 'unspecific membrane damage' or a 'permeability defect' leading to irreversible mitochondrial dysfunction (see discussion in [1]). This may not be the case, however, as MTP opening in vitro can be reversed by Ca^{2+} chela-

Abbreviations: MTP, mitochondrial permeability transition pore; $\Delta\tilde{\mu}_{H^+}$, proton electrochemical potential gradient; EGTA, ethylenebis(oxoethylenenitrilo)tertraacetic acid; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone; NEM, *N*-ethylmaleimide.

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tion with recovery of mitochondrial energy-linked functions after pore closure [6], while the action of many inducers has now been traced to the basic effectors of pore regulation [7–9]. Taken together, these data indicate that the MTP is a *regulated* channel serving an as yet undefined function.

This concept is strongly supported by recent work from our laboratory aimed at identifying the fundamental effectors of MTP regulation. It appears that the proton electrochemical gradient itself modulates the probability of pore opening (e.g. [9]). Here we present recent developments in our understanding of MTP modulation by the membrane potential, of its reversibility, and of its inhibition by cyclosporin A after briefly reviewing the basic features of pore regulation as defined in our laboratory over the last few years.

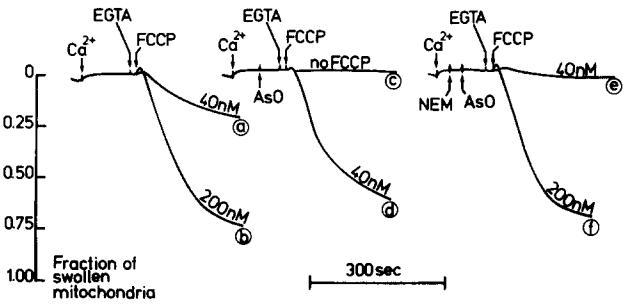

Fig. 1. Shifts of the MTP gating potential induced by arsenite and NEM – The incubation medium contained 0.2 M sucrose, 10 mM Tris-Mops (pH 7.4), 5 mM succinate-Tris, 1 mM P_i-Tris, 0.5 $\mu g \times ml^{-1}$ oligomycin, 2 μM rotenone and 10 μM EGTA-Tris. Final volume 2 ml, 25°C. The experiments were started by the addition of 1 mg of rat liver mitochondria (not shown). Where indicated, 10 μM Ca^{2+}, 0.5 mM EGTA-Tris, 10 μM NEM (traces *e* and *f*), 0.1 mM arsenite (AsO, traces *a*, *c*, *e* and *f*) and the concentrations of FCCP labeling each trace were added.

2. Regulation of the MTP by the proton electrochemical gradient

The MTP is a voltage-dependent channel [10] modulated by (i) the membrane potential, with an increase of the pore open probability upon depolarization [7–10]; and (ii) matrix pH, with a decrease of the open probability as matrix pH decreases below 7.3 [3] because of reversible protonation of histidyl residue(s) [11]. Thus, one way to induce MTP opening in a mitochondrial population is to collapse the membrane potential below the gating value by the addition of excess uncoupler while preventing matrix acidification [8].

Many effectors, however, modify the pore open probability by affecting the MTP voltage sensing rather than by modifying the membrane potential per se [9]. Thus, matrix Ca^{2+} and *tert*-butylhydroperoxide shift the gating potential to higher levels, accounting for their overall effect of pore activators, while Mg^{2+} and ADP shift the gating potential to lower levels, accounting for their overall effect of pore inhibitors [9]. These findings prompted us to further investigate the nature of the MTP voltage sensor and its mode of regulation by physiological and pathological effectors.

3. Regulation of the MTP voltage sensing by dithiols

The permeability of the inner mitochondrial membrane can be modified by sulfhydryl group reagents. Both relatively high concentrations of NEM, a substituting agent, and thiol oxidants or cross-linkers have been reported to increase membrane permeability, suggesting that a link between the status of membrane thiol groups and probability of MTP opening may exist [1].

In the experiments of Fig. 1 rat liver mitochondria energized with succinate and supplemented with 1 mM Pi were incubated in a sucrose-based medium, and the kinetics of pore opening was followed as the 90° light scattering of the suspension at 545 nm (see [9] for a quantitative treatment of the relationship between scattered light and fraction of mitochondria that have opened the MTP). After addition of a small Ca^{2+} pulse, addition of 40 nM FCCP (causing a 40 mV depolarization from the resting level of −200 mV) was followed by MTP opening in less than 20% of the mitochondrial population, indicating that under these conditions a membrane potential of −160 mV was still above the gating potential for the vast majority of individual mitochondria (Fig. 1, trace a). If, however, the addition of FCCP was of 200 nM (causing maximal depolarization) fast pore opening ensued in about 75% of the mitochondrial population (Fig. 1, trace b). When arsenite, a vicinal dithiol cross-linker, was added after Ca^{2+} at such a low concentration (0.1 mM) that neither the membrane potential nor the MTP was affected (Fig. 1, trace c), the addition of 40 nM FCCP, causing again a 40 mV depolarization, was now followed by a fast decrease of light scattering, indicative of MTP opening in about 60% of the mitochondria (Fig. 1, trace d). These experiments indicate that after reaction with arsenite the gating potential of many mitochondria has been shifted above −160 mV. Interestingly, the effect of arsenite was completely offset by pretreatment with NEM at concentrations which did not affect either the membrane potential or the pore. Under these conditions 40 nM FCCP (i.e., a 40 mV depolarization) was unable to open the pore (Fig. 1, trace e), while again 200 nM FCCP (i.e., a full depolarization) was required for MTP opening in about 70% of the mitochondria (Fig. 1, trace f, compare with trace

b). Thus, substitution by NEM prevented the shift of MTP gating potential induced by arsenite, suggesting that the MTP voltage sensor comprises at least two vicinal thiols whose status can influence the pore open probability: when the couple forms a complex with arsenite the gating potential increases and the MTP favors the open state; conversely, after substitution by NEM the dithiol cannot undergo cross-linking or oxidation, the gating potential decreases and the MTP favors the closed state. A similar behaviour was obtained with a variety of thiol oxidants (including *tert*-butylhydroperoxide, menadione, diamide) and reductants [12]. The effect of NEM appears to be specific, since mersalyl was totally ineffective at concentrations which fully inhibit phosphate transport, ruling out the possibility that the effect of NEM is secondary to its inhibition of phosphate transport itself. From these experiments and the existing literature it appears that the MTP can be modified at two separate classes of thiols, which can be easily distinguished based on the effects of NEM. (i) One class is revealed after substitution with relatively high concentrations (typically of the order of 50 μM or more) of NEM or other substituted maleimides, and is not dealt with here. With this class of thiols *substitution leads to an increased probability of pore opening* [1]. (ii) The second class is revealed after reaction of mitochondria with thiol oxidants or cross-linkers, which increase the probability of pore opening [1]. As shown here (Fig. 1), with this class of thiols *substitution by NEM counteracts the increased probability of pore opening*.

4. MTP opening is fully reversible and, per se, does not impair mitochondrial function

After Ca^{2+}-dependent MTP opening in sucrose medium, it is possible to achieve mitochondrial resealing by addition of excess EGTA [6]. Sucrose will be entrapped inside the swollen matrix, but mitochondria will largely regain their ability to form a $\Delta\tilde{\mu}_{H^+}$ [6]. The experiments of Fig. 2 show that this recovery extends to a fundamental mitochondrial property, i.e. the maintenance of volume homeostasis. In this protocol, mitochondria were incubated in isotonic ammonium chloride and MTP opening was induced by excess Ca^{2+} in the presence of P_i (panel 1). After MTP-dependent swelling, MTP closure was achieved by addition of EGTA. It can be seen that a fast phase of contraction ensued reestablishing the same volume preceding MTP opening. The electron micrographs shown in panel 2 display the mitochondrial morphology before MTP opening (a), after maximal swelling (b), and at the end of the contraction phase (c). Quite remarkably, after one cycle of pore opening/closure mitochondria are indistinguishable from the starting population (compare a and c in panel 2), and this could be repeated for several cycles (not shown). These findings indicate that

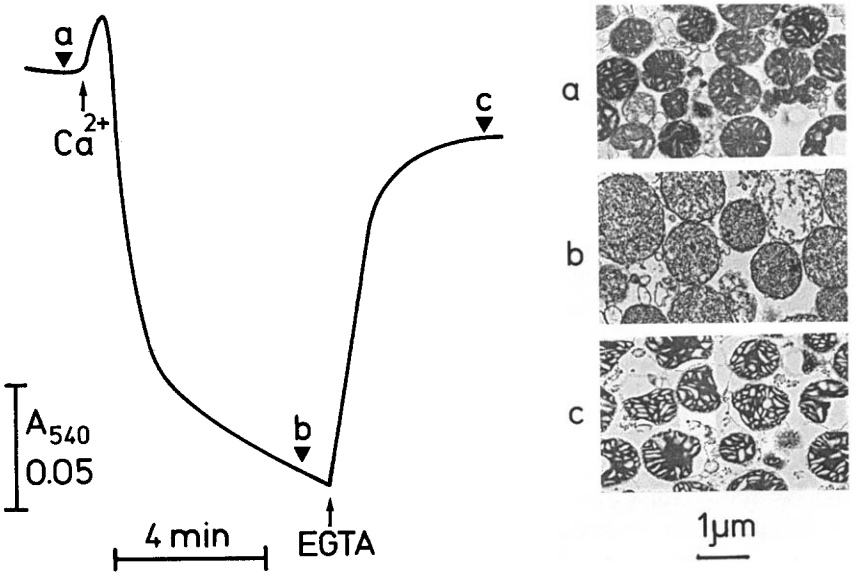

Fig. 2. Recovery of mitochondrial volume after reversible MTP opening – Experimental conditions were as in Fig. 1, except that Sucrose was replaced with 0.1 M NH_4Cl and the medium was supplemented with 5 μM cytochrome *c*. Where indicated, 200 μM Ca^{2+} and 400 μM EGTA were added. Mitochondria were processed for electron microscopy after centrifugation at the time points indicated by letters a–c, as described in Ref. [9].

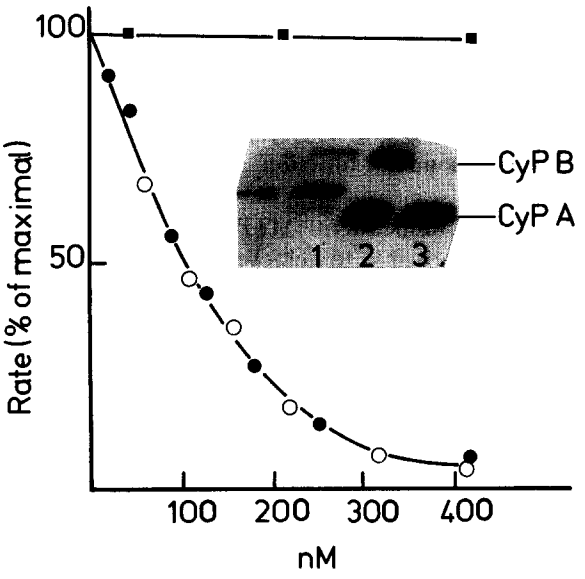

Fig. 3. Purification of mitochondrial cyclophilin and effect of cyclosporin A derivatives on MTP opening – Experimental conditions were as in Fig. 1, and MTP opening was triggered by the addition of 200 nM FCCP after accumulation of a 30 μM Ca^{2+} pulse in the presence of the indicated concentrations of cyclosporin A (closed circles), SDZ 220–384 (open circles) or cyclosporin H (closed squares). Data on the ordinate refer to the rate of permeabilization following the addition of FCCP. *Inset.* Identical protein amounts (125 mg) of mitochondria, microsomes and of a 100000 × *g* supernatant (cytosol) from a rat liver homogenate were chromatographed on an 8-ornithine cyclosporin A affinity matrix after extraction with 2% Triton X-100 in 0.15 M NaCl, 10 mM sodium phosphate and 115 μM PMSF and clarification by centrifugation. After extensive washings, each column was eluted with 0.5 mg ml^{-1} cyclosporin A. After concentration of the eluates by precipitation with 10% Trichloroacetic Acid, the samples were boiled in Laemmli's gel sample buffer, separated by SDS-PAGE on a 15% gel and stained with Coomassie blue. Lane 1, mitochondria; lane 2, microsomes, lane 3, cytosol. The arrow indicates mitochondrial cyclophilin. CyP, cyclophilin.

pore operation per se is fully compatible with maintenance of mitochondrial function, and is not accompanied by structural evidence of mitochondrial damage.

5. MTP inhibition by cyclosporin A presumably involves mitochondrial cyclophilin but not the calcineurin signalling pathway

The MTP is blocked by nanomolar concentrations of cyclosporin A. Since most known effects of cyclosporin A are mediated by a complex with one of a family of cyclosporin-binding proteins, the cyclophilins (reviewed in Ref. [13]), we purified putative cyclosporin-binding mitochondrial proteins by affinity chromatography on immobilized cyclosporin A. Fig. 3 shows that a 20 kDa mitochondrial cyclophilin could be specifically eluted (inset, arrow in lane 1), which could be easily distinguished by cyclophilins A (inset, lane 3) and B (inset, lane 2). The N-terminal sequence was identical to that of the mitochondrial peptidyl-prolyl-*cis-trans* isomerase purified by ion exchange chromatography [14]. These data suggest that mitochondrial cyclophilin may be involved in pore inhibition by cyclosporin A (Nicolli, A. and Bernardi, P., unpublished data). To test this hypothesis, we compared the inhibitory potency of cyclosporin A with that of cyclosporin H, which does not bind cyclophilin, and of *N*-methyVal-4-cyclosporin A (SDZ 220–384), a cyclosporin A derivative which binds cyclophilin but not calcineurin, and is therefore devoid of immunosuppressive activity [15]. While cyclosporin H was totally ineffective, SDZ 220–384 inhibited the pore with the same apparent K_i as cyclosporin A. These findings strongly argue in favor of a role for mitochondrial cyclophilin in modulation of MTP activity, and indicate that calcineurin is not involved in pore inhibition by cyclosporin A.

6. Conclusion and perspectives

The MTP is a regulated mitochondrial channel finely tuned by the $\Delta\tilde{\mu}_{H^+}$. Its location in the energy-conserving membrane, its expression in virtually all mammalian tissues, the complexity of pore regulation, and its involvement in the cyclophilin signalling pathway suggest a fundamental role in mitochondrial function. The increasing availability of conceptual and molecular tools to address its role in the intact cell should soon provide an answer to this fascinating problem of mitochondrial physiology.

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