Physiological Effectors Modify Voltage Sensing by the Cyclosporin A-sensitive Permeability Transition Pore of Mitochondria*

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This paper reports an investigation on the modulation of the mitochondrial permeability transition pore (MTP) by the membrane potential. Energized rat liver mitochondria loaded with a small Ca^{2+} pulse in sucrose medium supplemented with phosphate favor a high MTP "closed" probability because of the high membrane potential and therefore maintain a low permeability to sucrose. Upon depolarization by the addition of fully uncoupling concentrations of carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) mitochondria favor a high MTP "open" probability and rapidly undergo a process of osmotic swelling following sucrose diffusion toward the matrix. A titration with FCCP reveals that discrete subpopulations of mitochondria with different gating potentials for MTP opening may exist, since increasing concentrations of FCCP increase the fraction of mitochondria undergoing osmotic swelling. We show that physiological effectors (Ca^{2+}, Mg^{2+}, ADP, palmitate) modify pore opening in a mitochondrial population by shifting the fraction of mitochondria with a functionally open pore at any given membrane potential. Many inducers and inhibitors may therefore affect the pore directly through an effect on the MTP voltage sensing rather than indirectly through an effect on the membrane potential. Thus, many effectors may induce pore opening by shifting the MTP gating potential to higher levels, whereas many inhibitors may induce pore closure by shifting the MTP gating potential to lower levels.

Isolated mitochondria can undergo a sudden Ca^{2+}-dependent increase of permeability of the inner membrane (for a review see Ref. 1). Since a low permeability to ions and solutes in general is essential for energy conservation through a chemiosmotic mechanism (2, 3) this phenomenon, termed *permeability transition*, has been widely considered as a damaging event that was unlikely to play a role in mitochondrial physiology (1).

Two main hypotheses have been proposed to explain the mitochondrial permeability transition: (i) formation of permeability defects in the membrane lipid phase following Ca^{2+}-dependent activation of phospholipase A_2 and subsequent accumulation of acyllysophospholipids, as put forward by Pfeiffer and co-workers (4, 5); (ii) opening of an inner membrane pore with a minimum diameter of 2.8 nm (6) activated by Ca^{2+} and inhibited by H^+, Mg^{2+} and adenine nucleotides, as proposed by Hunter and Haworth (7-9).

Besides Ca^{2+} accumulation, the permeability transition requires the presence of one of a surprisingly wide variety of factors collectively termed *inducers* (1). The inducers do not share common functional or structural features, and this observation partly explains why the phospholipase A_2 hypothesis has enjoyed vast popularity for more than a decade. Indeed, it seemed reasonable that a relatively unspecific target, the membrane itself, could better accommodate the large number of unspecific effectors experimentally observed to cause the transition. The pore hypothesis, on the other hand, has recently been reevaluated because of two sets of observations: (i) the permeability transition is inhibited with high specificity by cyclosporin A (10-12), whereas phospholipase A_2 activity is unaffected (12); (ii) the mitochondrial megachannel, a high conductance channel with an estimated diameter of 2.8 nm identified by patch clamp studies of rat liver mitoplasts (13), is inhibited by cyclosporin A (14) and responds to the same effectors in the same way as does the permeability transition (15-17). Thus, the mitochondrial megachannel and the transition pore (MTP)[1] define the same molecular entity (15-18).

Recent studies from our laboratory support the notion that the MTP is modulated by the $\Delta\bar{\mu}H$ and by Me^{2+} ions (15, 19-22). The pore open-closed transition appears to be regulated by three main factors: (i) membrane potential, where membrane depolarization favors MTP opening (19-21); (ii) matrix pH, where matrix acidification below pH 7.0 favors pore closure (15, 16) through reversible protonation of histidyl residues (22); and (iii) Me^{2+} binding to inner and outer sites independently modulating the pore open-closed transition (20). Thus, the complexity of pore modulation in intact mitochondria could be partly explained with a combinatorial effect of some inducers on these basic regulatory events (21). In particular, we have hypothesized that several inducers may be causing MTP opening because they determine membrane depolarization, as is the case for uncouplers (21). Yet, the mechanism of action of many pore effectors remains difficult

[1] The abbreviations used are: MTP, mitochondrial permeability transition pore; $\Delta\bar{\mu}H$, proton electrochemical gradient; $\Delta\psi$, membrane potential; TPMP, triphenylmethylphosphonium; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; Mops, 4-morpholinepropanesulfonic acid.

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to explain on this basis and prompted us to investigate further the mode of pore regulation by the membrane potential.

In this paper we provide evidence that discrete subpopulations of mitochondria with different gating potentials for MTP opening may exist since increasing concentrations of FCCP increase the fraction of mitochondria undergoing osmotic swelling. We show that physiological effectors (Ca^{2+}, Mg^{2+}, ADP, and palmitate) modify pore opening in a mitochondrial population by changing the fraction of mitochondria with a functionally open pore at any given $\Delta\psi$. Many inducers and inhibitors therefore affect the pore directly through an effect on the MTP voltage sensing rather than indirectly through an effect on the membrane potential. Thus, many effectors may induce pore opening by shifting the MTP gating potential to higher levels, whereas many inhibitors may induce pore closure by shifting the MTP gating potential to lower levels.

MATERIALS AND METHODS

Preparation of rat liver mitochondria and measurements of oxygen consumption were performed as described previously (23). Mitochondrial permeabilization to sucrose was determined either from the absorbance changes at 540 nm, or from the changes of 90° light scattering at 545 nm with a Perkin-Elmer-Cetus Instruments 650-40 spectrofluorometer equipped with magnetic stirring and thermostatic control. In principle, absorbance changes reflecting a variation of mitochondrial volume can be obtained in two ways.

(i) The volume of all mitochondria changes at the same time, *e.g.* by variations of medium osmolarity or by induction of ion fluxes across the inner membrane. In this case, the absorbance changes reflect a homogenous volume change throughout the mitochondrial population. The relation between volume change and absorbance change is nonlinear, and the actual volume change must be obtained with relatively complex calibration curves that may vary in slope in different regions of the osmotic curve (24–27).

(ii) As in the permeability transition, the volume change for the individual mitochondrion is an all-or-nothing event (7–9)in which the $t_{1/2}$ for solute diffusion through the open pore is much faster than the kinetics of swelling for the total population (28). In this case, the absorbance changes reflect the volume change of the fraction of mitochondria that have undergone the transition. The relationship between absorbance changes and fraction of permeabilized mitochondria is not known, and we have therefore devised a simple protocol to obtain this information.

Fig. 1, *panel 1*, shows the typical absorbance increase following addition of 1 mg of mitochondria to an isotonic sucrose medium containing succinate and 1 mM P_i. The absorbance reading stabilized

within a few seconds and then remained constant (*dashed trace*) unless a 150 μM Ca^{2+} pulse was added to initiate the spontaneous transition (Fig. 1, *panel 1*). In Fig. 1, *panel 2*, a similar experiment was carried out with 0.5 mg of mitochondria. Following the addition of Ca^{2+} and completion of the swelling phase, ruthenium red and cyclosporin A were added followed by 0.5 mg of mitochondria. Since the newly added mitochondria cannot open their pores, the final absorbance reading will correspond to a condition in which 50% of the mitochondria have undergone the transition (see also Fig. 4). Thus, the experiment allows a calibration of the absorbance as a function of the fraction of swollen mitochondria, where zero is the value obtained from mitochondria incubated in the absence of Ca^{2+} and 1 is the value obtained after all of the mitochondria have undergone the transition. Fig. 2 summarizes the results of a series of experiments in which the fraction of swollen mitochondria was systematically varied in protocols analogous to that depicted in Fig. 1, *panel 2*. It can be clearly seen that both the absorbance and the 90° light scattering values were a linear function of the fraction of mitochondria that had undergone the transition. Thus, the fraction of swollen mitochondria can be determined precisely by reference to a minimum (before Ca^{2+} addition) and to a maximum (spontaneous swelling after Ca^{2+} addition) under a wide variety of experimental conditions.

For electron microscopy, mitochondria were incubated under the specified conditions and finally centrifuged at 15,000 × g in the presence of 0.85 μM cyclosporin A. The pellets were fixed overnight with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, postfixed with 1% osmic acid in 0.1 M cacodylate buffer, dehydrated, and included in Epon 812. Thin sections were observed with a Philips EM 301 electron microscope.

Measurements of membrane potential were carried out with a $TPMP^+$-selective electrode (initial $[TPMP^+]_0 = 4$ μM) as described by Zoratti *et al.* (29). Solutions of ruthenium red, purified according to Luft (30), were prepared daily, and concentrations were determined spectrophotometrically based on an extinction coefficient of 68 × $mM^{-1} \times cm^{-1}$ at 533 nm. All chemicals were of the highest purity commercially available; cyclosporin A was a generous gift of Sandoz Pharma AG (Basel).

RESULTS

In the experiments illustrated in Fig. 3 mitochondria energized with succinate and supplemented with 1 mM P_i were incubated in a sucrose-based medium, and mitochondrial volume was followed as the 90° light scattering of the mitochondrial suspension at 545 nm. Fig. 3, *panel 1*, shows that the
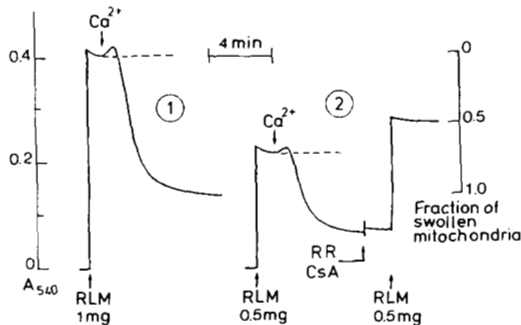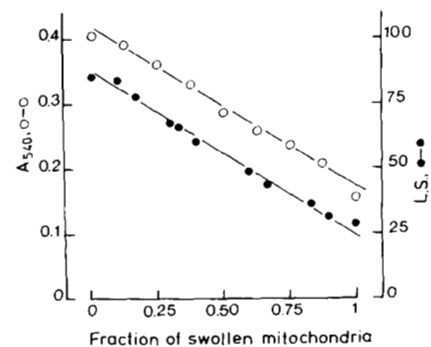

FIG. 1. **Absorbance variations upon swelling of different fractions of mitochondria.** The incubation medium contained 0.2 M sucrose, 10 mM Tris-Mops, pH 7.4, 5 mM succinate-Tris, 1 mM P_i-Tris, 10 μM EGTA-Tris, 2 μM rotenone, 1 μg × ml^{-1} oligomycin. Final volume 2 ml, 25 °C. Additions were (*arrows*): mitochondria as indicated (*RLM*), 150 μM Ca^{2+}, 0.1 μM ruthenium red (*RR*), and 850 nM cyclosporin A (*CsA*). Dashed traces, no Ca^{2+}. The ordinate values on the *right* side of the *panel* refer to the fraction of mitochondria that have undergone the permeabilization process. For further explanation see "Materials and Methods."

FIG. 2. **Relationship between fraction of swollen mitochondria and absorbance or 90° light scattering.** The experiments were carried out as outlined in Fig. 1, *panel 2*. Increasing concentrations of mitochondria were added (up to 0.5 mg × ml^{-1}), and the transition was induced by 150 μM Ca^{2+}. When the swelling process was complete, ruthenium red and cyclosporin A were added followed by enough mitochondrial protein to give a final concentration of 0.5 mg × ml^{-1} in all cases. Values on the *ordinate* refer to the absorbance (*open symbols*) or 90° light scattering readings (*closed symbols*) obtained after the second addition of mitochondria as a function of the mitochondria added before ruthenium red and cyclosporin A, *i.e.* of the swollen fraction. Light scattering (*L.S.*) values are in arbitrary units.
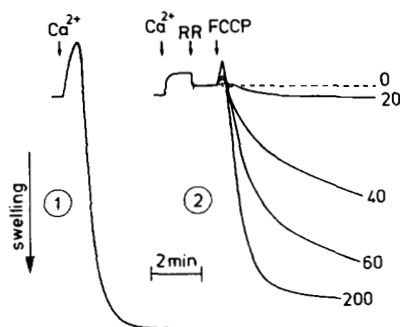
FIG. 3. **Mitochondrial swelling induced by Ca²⁺ or by increasing concentrations of FCCP.** Experimental conditions were as in Fig. 1. The experiments were started by the addition of 1 mg of mitochondria (not shown). Where indicated (*arrows*), 150 μM Ca²⁺ (*panel 1*) or 40 μM Ca²⁺ (*panel 2*) followed by 0.1 μM ruthenium red (*RR*) and the FCCP concentrations labeling each trace (nM) were added. *Dashed trace*, no FCCP was added.

addition of 150 μM Ca²⁺ caused a short contraction phase followed by the expected osmotic swelling resulting from pore opening throughout the mitochondrial population. In the experiments of Fig. 3, *panel 2*, the pore was induced with a different protocol. In this case the Ca²⁺ pulse was 40 μM, *i.e.* a size unable to induce pore opening *per se*. After Ca²⁺ accumulation ruthenium red was added to prevent Ca²⁺ redistribution, and MTP opening was then induced by the addition of increasing concentrations of FCCP (Fig. 3, *panel 2*). Under these conditions Ca²⁺ efflux following the addition of FCCP occurred exclusively through the pore since the accumulated Ca²⁺ was not released if the MTP was inhibited by cyclosporin A (data not shown; see Ref 21).

Fig. 3 shows that even with fully uncoupling concentrations of FCCP the final light scattering decrease was slightly but consistently smaller than that observed in the spontaneous swelling protocol (compare *panel 1* with *panel 2*), suggesting that with limiting Ca²⁺ concentrations a fraction of the mitochondrial population does not undergo pore opening despite full deenergization. A second point of interest is that with limiting concentrations of FCCP the fraction of swollen mitochondria appeared to be a function of the FCCP concentration (*panel 2*). It should be noted that the response to suboptimal FCCP concentrations was biphasic, with a fast decaying initial phase followed by a slower, linear swelling response. It can be appreciated that while the fast phase was proportional to the concentration of FCCP, the slow phase was largely independent of the FCCP concentration above 20 nM, as confirmed by independent titrations (not shown). As should become clear later (see Figs. 7–9) the slow phase was most likely caused by slow leakage of pore inhibitory factors allowing a time-dependent pore opening in mitochondria initially resistant to depolarization.

These experiments suggest that mitochondria may be heterogeneous in their response to membrane depolarization, in that the gating potential at which pore opening occurs may be different for different mitochondrial subpopulations. This hypothesis was investigated further, and Fig. 4 shows an electron microscopy study of the morphology of mitochondria under several experimental conditions. Fig. 4a shows that the majority of freshly isolated mitochondria had the typical orthodox configuration, with prominent inner membrane christae, whereas swollen mitochondria were found only occasionally. After treatment with Ca²⁺ plus phosphate and completion of the absorbance changes (see Fig. 3, *panel 1*), on the other hand, all mitochondria appeared swollen and had

lost the typical pattern of inner membrane folding (Fig. 4b). When mitochondria were treated with 40 nM FCCP after uptake of a small Ca²⁺ pulse (see Fig. 3, *panel 2*) a fraction of the mitochondria appeared to maintain an orthodox configuration while many mitochondria appeared swollen, and no intermediate states could be detected (Fig. 4c). This pattern was in perfect match with the picture obtained after mixing equal amounts of preswollen and intact mitochondria in the presence of cyclosporin A (Fig. 4d). Thus, increasing FCCP concentrations determine pore opening in increasing fractions of the mitochondrial population.

The experiments of Fig. 5 show the effect of ADP on the mitochondrial swelling induced by increasing concentrations of FCCP. Fig. 5, *panel 1*, shows an experiment in which the MTP was induced in mitochondria that had accumulated a 40 μM Ca²⁺ pulse in the presence of oligomycin. As expected, increasing fractions of mitochondria underwent the transition, reaching a maximal value of 0.8 at fully uncoupling FCCP concentrations. In the experiments of *panel 2*, 20 μM ADP was added before FCCP, and the same titration with FCCP was carried out. It can be seen that a much smaller fraction of mitochondria opened up their pores at any given FCCP concentration in the presence of ADP (compare *panel 1* and *panel 2*). It must be stressed that the experimental conditions were chosen so that the state 4 membrane potential preceding the addition of FCCP was the same irrespective of whether or not ADP was present (not shown).

Parallel measurements of $\Delta\psi$ were carried out on mitochondria treated with cyclosporin A (to prevent pore opening) to test whether the dose-response curve of membrane depolarization by FCCP was affected by ADP. Fig. 6, *panel A*, shows that this was not the case and that membrane depolarization by increasing concentrations of FCCP was identical in the presence or absence of ADP and was not affected by the size of the Ca²⁺ load *per se*. Thus, the differential effects of FCCP on the fraction of swollen mitochondria (Fig. 5) cannot be explained by differing levels of $\Delta\psi$ before the addition of uncoupler or by differing levels of uncoupler-induced depolarization. Rather, ADP appears to be acting through a modulation of the fraction of mitochondria that open up their pores at a given concentration of FCCP.

To assess how general this mechanism might be, we carried out experiments of similar design in the presence of another pore inhibitor (Mg²⁺) or under conditions that lead to increased probability of pore opening (higher Ca²⁺ load, addition of palmitate or *t*-butylhydroperoxide, and *in vitro* aging). Also in this case conditions were chosen so that spontaneous pore opening did not take place. A titration of the membrane potential with FCCP, also shown in Fig. 6, revealed that none of these agents or conditions affected the membrane potential response to uncoupler (Fig. 6, *panels B–D*), whereas only with palmitate the expected (31) 15 mV decrease of state 4 membrane potential could be observed (Fig. 6, *panel C*). In an effort to relate the fraction of swollen mitochondria to the membrane potential, parallel $\Delta\psi$ and permeabilization determinations were carried out at increasing FCCP concentrations. The results obtained with Ca²⁺, palmitate, Mg²⁺, and ADP are summarized in Figs. 7 and 8. As expected, the rate of permeabilization was linearly related to the fraction of swollen mitochondria under all of the conditions tested (not shown). In these experiments we have therefore measured the rate of permeabilization to reduce the incubation times and therefore the *in vitro* aging of mitochondria (see Fig. 9).

Fig. 7 shows that both increasing the Ca²⁺ load (*panel 1*) and the addition of palmitate (*panel 2*) determined a shift to
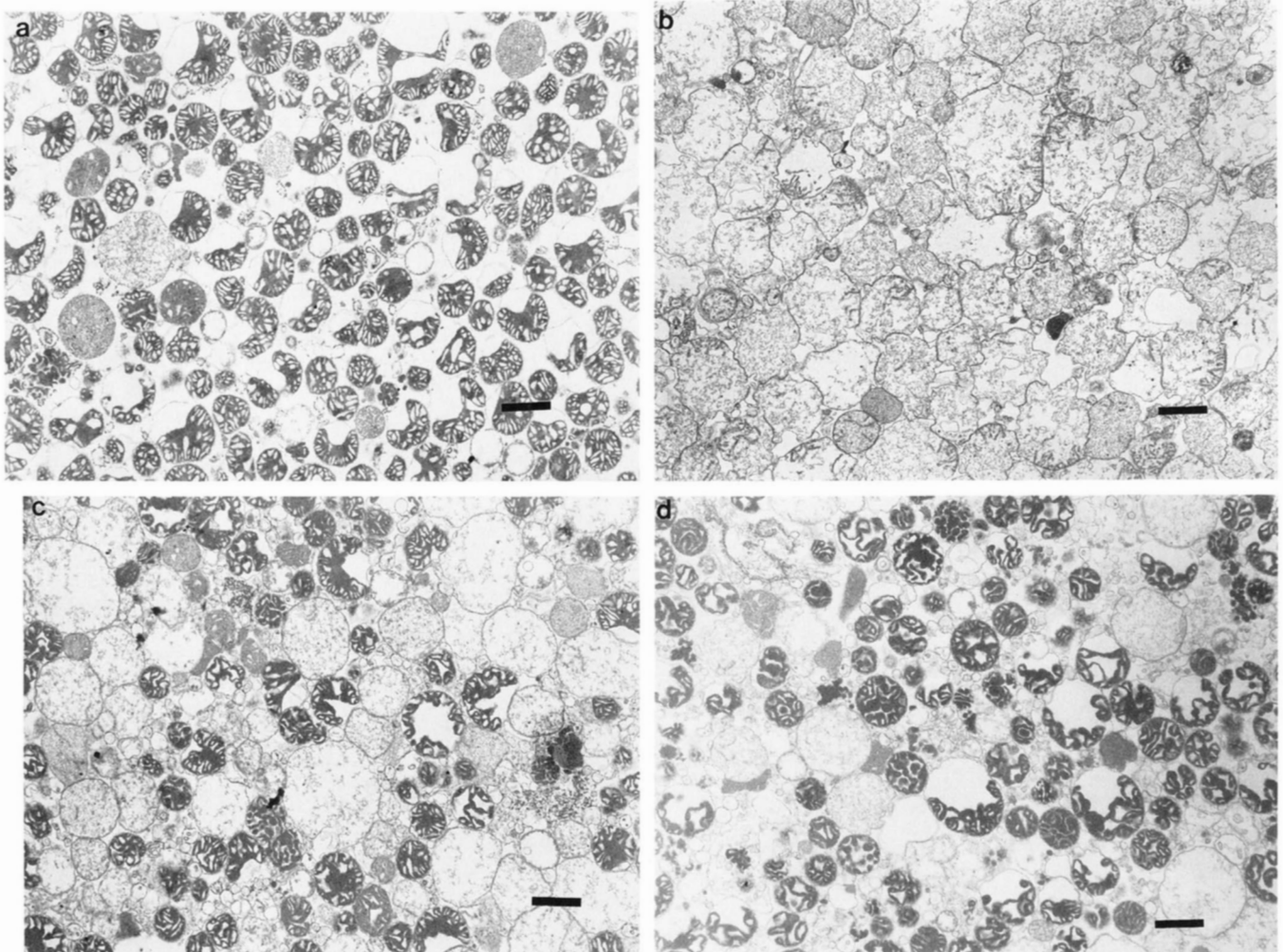
FIG. 4. **Effects of Ca²⁺ and FCCP on mitochondrial morphology.** Mitochondria were processed for electron microscopy as described under "Materials and Methods" after incubation at 0.5 mg × ml⁻¹ in the medium described in Fig. 1. *Panel a*, no further additions; *panel b*, the medium was supplemented with 150 μM Ca²⁺, and the sample was centrifuged after completion of the process of absorbance decrease (see Fig. 3, *panel 1*); *panel c*, the medium was supplemented with 40 μM Ca²⁺; after 2 min of incubation, 0.1 μM ruthenium red and 40 nM FCCP were added, and the sample was centrifuged after a further 2 min (see Fig. 3, *panel 2*); *panel d*, mitochondria were treated as in *panels a* or *b* and mixed in equal proportions after the addition of 0.1 μM ruthenium red and 0.85 μM cyclosporin A. *Bar*, 1 μm.
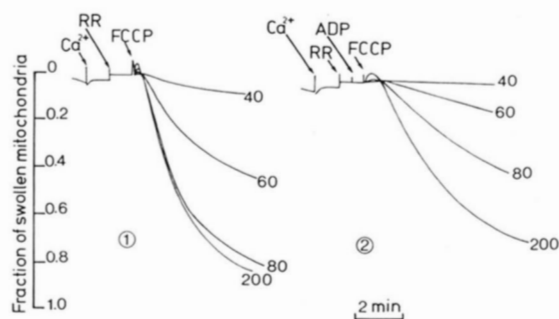

FIG. 5. **Effect of ADP on mitochondrial swelling induced by increasing FCCP concentrations.** Experimental conditions were as in Fig. 1. The experiments were started by the addition of 1 mg of mitochondria (not shown). Where indicated 40 μM Ca²⁺, 0.1 μM ruthenium red (*RR*), and 20 μM ADP were added. Swelling was induced by the addition of the FCCP concentrations labeling each trace (nM).

the right of the curves relating the rate of permeabilization to the level of $\Delta\psi$, indicating that increasing numbers of mitochondria opened up their pores at higher membrane potential.

Fig. 8 shows that both ADP (*panel 1*) and Mg²⁺ (*panel 2*) had the opposite effect on MTP opening and shifted the curves to the left, indicating that decreasing numbers of mitochondria opened up their pores despite membrane depolarization. In other words, Ca²⁺ and palmitate increased the gating potential toward the state 4 level, accounting for their overall effect of pore inducers, whereas Mg²⁺ and ADP decreased the gating potential below the state 4 level, accounting for their overall effect of pore inhibitors.

It is well known that *in vitro* aging is accompanied by a decreased stability of the mitochondria, which undergo the transition more easily and tend to lose their energy-coupled functions (1). The experiments of Fig. 9 show that *in vitro* aging had the effect of shifting the gating potential of increasing fractions of mitochondria to higher levels. The shift was relevant, reaching a difference of about 20 mV within 8 h of isolation and could therefore account for the increased tendency of aged mitochondria to undergo the transition despite
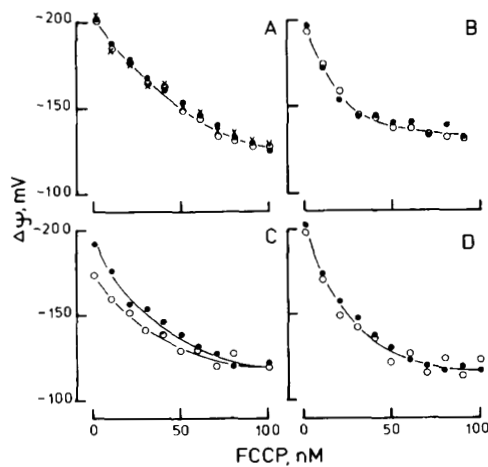
FIG. 6. **Effect of FCCP on mitochondrial membrane potential.** The incubation medium was the same as that described in Fig. 1, except that 4 μM TPMP$^+$ and 0.85 μM cyclosporin A were added. Final volume, 4 ml, 25 °C. The experiments were started by the addition of 2 mg of mitochondria. After a steady-state distribution of TPMP$^+$ had been reached, Ca^{2+} was added (concentrations for each *panel* are specified below). After a steady-state $\Delta\psi$ level had been reestablished, 0.1 μM ruthenium red was added, followed by the indicated concentrations of FCCP. *Panel A*, the Ca^{2+} addition was 20 μM (*closed symbols*) or 40 μM (*open symbols* and *crosses*); the experimental points denoted by *crosses* were obtained from incubations in which 20 μM ADP was added before FCCP. *Panel B*, the Ca^{2+} addition was 40 μM in the absence (*closed symbols*) or presence (*open symbols*) of 0.5 mM Mg^{2+} added before FCCP. *Panel C*, the Ca^{2+} addition was 30 μM in the absence (*closed symbols*) or presence (*open symbols*) of 2 μM palmitate added before FCCP. *Panel D*, the Ca^{2+} addition was 20 μM, and the experiments were performed either immediately after isolation of mitochondria (*closed symbols*) or after 8 h of aging *in vitro* (*open symbols*).
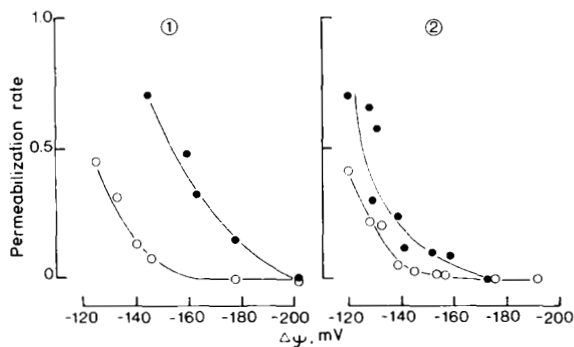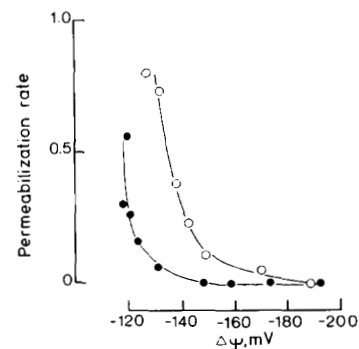

FIG. 7. **Shifts of the pore gating potential by Ca^{2+} and palmitate.** Experimental conditions were as in Fig. 1. The experiments were started by the addition of 1 mg of mitochondria (not shown). After 1 min of incubation, the following additions were made. *Panel 1*, 20 μM (*open symbols*) or 40 μM (*closed symbols*) Ca^{2+}, followed after 1 min by 0.1 μM ruthenium red and after 1 further min by FCCP (0–100 nM). *Panel 2*, 30 μM Ca^{2+} followed by the same additions as *panel 1* in the absence (*open symbols*) or presence (*closed symbols*) of 2 μM palmitate added 30 s before FCCP. Values on the *ordinate* refer to the rate of the linear swelling phase following the addition of FCCP. Values on the *abscissa* report the membrane potential values determined on parallel incubations in the presence of 0.85 μM cyclosporin A. Since aging affects pore gating (see Fig. 9) each experimental point was obtained from incubations carried out in parallel at exactly the same time.

their initial ability to develop a high membrane potential.

It must be noted that to study the effects of membrane depolarization on pore opening, in the experiments of Figs. 7–9 we chose conditions that do not lead to spontaneous pore
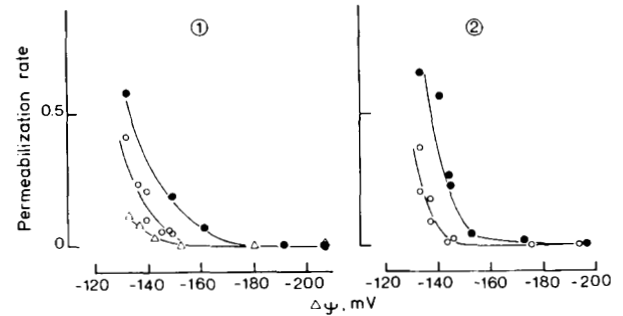
FIG. 8. **Shifts of the pore gating potential by ADP and Mg^{2+}.** The experiments were carried out exactly as described in Fig. 7, with a Ca^{2+} load of 30 μM (*panel 1*) or 40 μM (*panel 2*). *Panel 1*, 30 μM (*open circles*) or 60 μM ADP (*triangles*) were added 30 s before FCCP. *Panel 2*, 0.5 mM Mg^{2+} (*open circles*) was added 30 s before FCCP. In both *panels*, *closed circles* denote the addition of FCCP alone.

FIG. 9. **Shifts of the pore gating potential by *in vitro* aging.** The experiments were carried out exactly as described in Fig. 7, with a Ca^{2+} load of 20 μM. The experiments were performed immediately after isolation of mitochondria (*closed circles*) or after 8 h of aging *in vitro* (*open circles*).

opening. This is the reason why sizable fractions of the mitochondria opened up their pores only when the $\Delta\psi$ dropped well below the state 4 level. As the Ca^{2+} or the inducer concentration was raised the pore could be opened at much higher membrane potentials (not shown), but this in turn prevented a full titration with FCCP from being carried out.

DISCUSSION

Mitochondrial Heterogeneity and MTP Opening—Isolated mitochondria do not behave as a homogeneous population in their response to pore opening, as first shown by Beatrice *et al.* (32) for the transition induced by Ca^{2+} plus *t*-butylhydroperoxide or Ca^{2+} plus oxaloacetate (32). The results presented in this paper suggest that this property may extend to the mitochondrial response to uncoupler. Indeed, increasing concentrations of FCCP increase the fraction of mitochondria undergoing the transition (Figs. 3 and 4). Under the assumption that at any given FCCP concentration the $\Delta\psi$ is uniform throughout the mitochondrial suspension, this is evidence that different mitochondrial subpopulations possess different gating potentials. Indeed, if all mitochondria possessed the same gating potential one would expect an all-or-nothing behavior, with pore opening in all mitochondria when the concentration of FCCP is sufficient to lower the membrane potential below the gating value. Under the opposite assumption, *i.e.* that the $\Delta\psi$ is not uniform and that the mitochondrial suspension does not respond homogeneously to FCCP, one should conclude that all pores have the same gating potential, but only depolarized mitochondria can undergo the transition

at limiting FCCP concentrations. Although some heterogeneity in membrane potential and/or FCCP distribution may occur, we favor the first explanation for one compelling reason: when the Ca^{2+} load is increased or palmitate is added, or mitochondria have undergone an *in vitro* aging process, the same concentrations of FCCP determine pore opening in a larger fraction of the mitochondria (Fig. 7); when, on the other hand, ADP or Mg^{2+} is present the same FCCP concentrations cause pore opening in a smaller fraction of the mitochondrial population (Fig. 8). Since none of these factors modifies the response of the mitochondrial membrane potential to depolarization (Fig. 6), we consider it extremely unlikely that Ca^{2+}, palmitate, aging, Mg^{2+}, or ADP may vary the degree of homogeneity of FCCP distribution or of the mitochondrial response to uncoupling. This consideration is particularly striking in the case of *in vitro* aging, in which the conditions of the experiment are otherwise identical, yet a large shift in the responding population can be easily demonstrated (Fig. 9).

The heterogeneity in gating potential, in turn, may either reflect structural differences in the pores or subtle differences in the concentration of effector molecules (*e.g.* H^+, Ca^{2+}, Mg^{2+}, ADP) within mitochondrial subpopulations or both. Although this issue cannot be resolved at present, this concept has practical implications independent of the precise mechanism by which the heterogeneity is accomplished. Indeed, the gating potential may approach a continuum when the transition is considered for a mitochondrial population, and this in turn may contribute to explain why this important regulatory parameter and its role in the transition have proven so elusive to define in the past.

Regulation of Voltage Sensing by the MTP—The membrane potential is only one of the factors modulating the pore open-closed transition, as realized for many years from the multiplicity of conditions affecting the permeability transition (1). Yet, it appears that several known pore effectors primarily affect the gating potential of mitochondria (Figs. 7 and 8). Agents that are known to promote the transition (Ca^{2+}, palmitate, *t*-butylhydroperoxide) all increase the fraction of mitochondria undergoing the transition at a given membrane potential. Conversely, agents that are known to inhibit the transition (Mg^{2+}, ADP) all decrease the fraction of mitochondria undergoing the transition for the same level of depolarization. This finding confirms the central role of the $\Delta\bar{\mu}H$ in the regulation of the MTP (19–22) and defines a novel mechanism by which physiological effectors can modulate the mitochondrial permeability transition pore. This can be described as the modulation of voltage sensing by the pore, whereby the MTP open-closed transition can be regulated independently of the absolute magnitude of the membrane potential.

Thus, whereas uncoupling agents affect the pore open-closed transition indirectly by decreasing the membrane potential rather than by an effect on the pore itself, a variety of pore effectors appear to modulate pore opening by a direct effect on the pore, possibly by modifying its voltage sensing machinery. Although novel in a mitochondrial setting, this is a likely regulatory mechanism in Na^+, K^+, and Ca^{2+} voltage-gated channels, in which voltage sensing occurs through the highly conserved S4 segment (33, 34) and can be modified by neurotransmitters and pharmacological agents (*e.g.* Refs. 35 and 36).

The concept of modulation of MTP voltage sensing is well suited to describe the autocatalytic nature displayed by the permeability transition within a mitochondrial population *in vitro*. As Ca^{2+} is taken up, the mitochondria with the highest gating potential will open their pores first. As Ca^{2+} is released by this permeabilized fraction, increasing cohorts of pores will be recruited by the increased Ca^{2+} load of the mitochondrial fractions originally possessing a lower gating potential. This wavelike process would continue until all mitochondria favor a high MTP open state, with no need for concomitant membrane depolarization after Ca^{2+} uptake. Although the open state is not irreversible *per se* (mitochondria regain a high $\Delta\bar{\mu}H$ upon addition of EGTA, Ref. 37), it is stabilized *in vitro* because of the loss of Mg^{2+}, adenine nucleotides, and perhaps regulatory proteins occurring once the pore opens (1). The loss of adenine nucleotides appears to be particularly important since ADP favors the open-to-closed transition (38), and ADP-depletion dramatically affects the subsequent response to cyclosporin A (39). Also the effect of *in vitro* aging could be easily explained within this framework. As mitochondria slowly lose their Mg^{2+} and adenine nucleotide content, their gating potential is shifted toward higher levels; and since the spontaneous opening is autocatalytic, even the shift of a small fraction could be sufficient for an increased inducibility for the total population.

Crompton and co-workers were the first to show unequivocally that pore opening is a fully reversible process (37) and to propose that pore opening in a mitochondrial population maintaining a high membrane potential may be a constitutive process permitting continual, nonspecific solute permeation (38). Our findings are in good agreement with this concept and suggest that the fraction of energized mitochondria undergoing the transition at high $\Delta\psi$ may be controlled by the concentration of physiological effectors.

Pore opening may therefore occur with two not mutually exclusive modes: (i) membrane depolarization below the gating potential (19–21), and (ii) increase of the gating potential to levels allowing pore opening (this paper). In the former case pore opening is the *consequence* of membrane depolarization; in the latter, its *cause*. Although much more work will be needed to understand the structural basis for pore modulation by the $\Delta\bar{\mu}H$ and cyclosporin A, it appears that a coherent picture of pore operation *in vitro* is now beginning to emerge.

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