Ca^{2+}-reversible inhibition of the mitochondrial megachannel by ubiquinone analogues

Silvia Martinucci, Ildikò Szabò, Francesco Tombola, Mario Zoratti*

Centro CNR Biomembrane e Dipartimento di Scienze Biomediche, Università di Padova, Viale G. Colombo 3, 35121 Padua, Italy

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Abstract Ubiquinone 0 and decylubiquinone have been reported to inhibit the mitochondrial permeability transition pore (PTP) [Fontaine, E., Ichas, F. and Bernardi, P. (1998) J. Biol. Chem. 273, 25734–25740], offering a new clue to its molecular composition. In patch-clamp experiments on rat liver mitochondria we have observed that these compounds also inhibit the previously described mitochondrial megachannel (MMC), confirming its identification as the PTP. Inhibition can be reversed by increasing [Ca^{2+}], in analogy to the behavior observed with several other disparate PTP/MMC inhibitors. To rationalize the ability of Ca^{2+} to overcome inhibition by various quite different compounds we propose that it acts via the phospholipid bilayer. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Permeability transition pore; Patch-clamp; Calcium; Ubiquinone; Rat liver mitochondrion

1. Introduction

The mitochondrial permeability transition (PT; [1–3]), proposed to be a key early event in several models of apoptosis [4–10], is due to the opening of the 'permeability transition pore' (PTP) in the mitochondrial membrane system. Despite much work, what proteins form the PTP remains to be established. The adenine nucleotide translocator (AdNT) has long been the leading candidate (e.g. [2,11]). Today most authors in the field however favor the idea that PTP is a supramolecular complex comprising proteins of the matrix (cyclophilin D), of the inner (e.g. the AdNT) and outer (voltage-dependent anion channel (porin) (VDAC), perhaps Bax) mitochondrial membranes and of the periplasmic space (creatine kinase) (e.g. [2,4,5,12,13]). The pore has long been considered to reside in the inner mitochondrial membrane, since its activation leads to efflux of soluble matrix components such as NADH and to swelling due to the influx of osmotically active species. However, since supramolecular assemblies of AdNT, kinases and VDAC are known to be present at sites of contact between the two membranes [14], and, upon isolation and reconstitution, exhibit PTP-like properties [4,15,16], the notion has taken hold that the PTP might be located at these sites. The proposal has also been made that it might be a manifestation of the mitochondrial protein import complex [17,18]. The discoveries that the PTP is modulated by electron flow through Complex I, and that it can be inhibited by ubiquinone analogues [19,20], have led to the hypothesis that the PTP might arise from, or include, this part of the respiratory chain. Indeed, the possibility exists that different variants of the PTP might form, depending on experimental conditions.

A high-conductance (1–1.3 nS in 100 mM KCl), substate-rich pore observed by patch-clamping swollen mitoplasts (the mitochondrial megachannel, MMC) [21,22] has been identified as the PTP on the basis of its properties and of its pharmacological profile [23–25].

The properties and the complex pharmacology of the PTP have been extensively investigated. The PT is induced by a variety of conditions and agents, and inhibited by a similarly imposing array of compounds [1,2]. Two key features are the requirement for matrix Ca^{2+} for PTP activation and its voltage-dependence: opening is facilitated by depolarization [26,27]. The effects of many inducers and inhibitors have been interpreted as shifts of the threshold potential for pore opening [28,29]. A striking feature, still waiting for a satisfactory explanation, is that inhibition by disparate key agents (protons, cyclosporin, divalent cations, local anesthetics) can be counteracted by increases of $[Ca^{2+}]_{matrix}$, in an apparently competitive fashion. Ca^{2+} also antagonizes $\Delta\psi$, in the sense that at higher Ca^{2+} loads lower extents of depolarization are needed to induce the PT. This behavior has been clearly observed in patch-clamp experiments, in which sequential additions of ever-increasing concentrations of Ca^{2+} and divalent cations (Sr^{2+}, Mn^{2+}, Ba^{2+}, Mg^{2+}), protons or CSA result in the correlated activation/inhibition of the PTP [24,25]. Whereas the idea that Ca^{2+} and other divalent cations may compete for the same binding sites on (a) protein(s) is reasonable, it seems unlikely that the same binding sites are involved in the case of other agents mentioned above. An explanation for their competitive behavior must therefore be sought.

Here we report that the ubiquinone analogues and PTP inhibitors ubiquinone 0 (Ub_0) and decylubiquinone (decylUb) also inhibit the MMC in patch-clamp experiments, thus confirming the identity PTP ≡ MMC. Also in the case of these compounds, and of ADP, inhibition can be counteracted by increasing [Ca^{2+}]. We propose that Ca^{2+}-induced modifications of the properties of the lipid bilayer may underlie this antagonistic behavior.

2. Materials and methods

Patch-clamp experiments on rat liver mitoplasts were conducted essentially as previously described [21,23–25]. The standard experi-

*Corresponding author. Fax: (39)-49-8276049.
E-mail: zoratti@civ.bio.unipd.it

Abbreviations: AdNT, adenine nucleotide translocator; decylUb, decylubiquinone; MMC, mitochondrial megachannel; PT, permeability transition; PTP, permeability transition pore; RLM, rat liver mitochondria; Ub_0, ubiquinone 0; Ub_5, ubiquinone 5; VDAC, voltage-dependent anion channel (porin)

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mental medium (the same in both bath and pipette) was 150 mM KCl, 0.1–1.0 mM $CaCl_2$, 20 mM HEPES/K^+, pH 7.2. In most experiments 1 or 2 s long voltage pulses, separated by 0.1 s intervals at zero potential, were sequentially applied to the pipette electrode. The polarity of the voltage was inverted after each pulse, and low potentials (most often ±10 or ±20 mV) were used. This type of protocol ensures that the megachannels will not inactivate because of their voltage dependence; there was no evidence of 'channel rundown'. In experiments involving inhibition by ubiquinone analogues, stationary megachannel activity was recorded for a few minutes and 50–200 μl of medium containing 10–20 μg/ml BSA to help solubilization and the desired amount of inhibitor (from a stock solution in DMSO) were then added to the bath by pipetting. To re-activate the pores, $CaCl_2$ was added as a few μl of stock solution. In most (11/13) reversal-of-inhibition experiments with Ub_5 (see Section 3), mitoplasts were incubated with Ub_0 for about 15 min to inhibit the MMCs, a tight seal was then established in the presence of the inhibitor, and Ub_5 was added after verifying that megachannel activity was absent. The potentials reported in this paper are those applied to the pipette. Current (cations) flowing from the pipette to the ground electrode are considered positive and plotted upwards. Ub_0, Ub_5, decylUb and ADP were from Sigma (Milan, Italy).

3. Results

Typical MMC activity was observed in nearly all attempts after establishment of tight seals on mitoplasts. In the experiments reported here most patches exhibited activity by more than one MMC (up to 10). We tested the inhibitory effect of Ub_0 on the MMC by adding the compound at a final concentration of 50 or 200 μM to the solution bathing a pipette-attached whole mitoplast (Fig. 1). This experimental configuration presumably accounts for the fact that inhibition was slow, requiring a few minutes to reach its maximum extent, since the inhibitor had to diffuse to the patch held by the pipette. The active MMCs were inhibited in an apparently sequential manner, disappearing 'one after the other' over a period of minutes. Total or partial inhibition of the active MMC channels in the patch by 50 μM Ub_0 took place in 12 out of 17 experiments, in which the seals lasted 2–19 min after the addition and mixing. In five experiments there was no clear inhibitory effect. However, in these five cases the seals lasted 1–3.5 min after addition, and this shorter lifetime probably accounts at least partially for the lack of inhibition. In 30 other experiments Ub_0 was added at a final concentration of approximately 200 μM. Extensive inhibition took place in 22 cases. In the remaining eight the seals lasted less than 2 min after addition of the inhibitor, again presumably accounting for the lack of effect.

Re-activation of MMC activity by increasing the concentration of Ca^{2+} in the bath was attempted in four cases in which inhibition had been achieved by 50 μM Ub_0 and in six of the experiments in which the channels had been inhibited by 200 μM Ub_0, $[Ca^{2+}]_{bath}$ was shifted from 0.5–1 to 4–11 mM. In all cases channel activity was re-established after a delay. Reactivation apparently occurred in a stepwise manner, analogous to the inhibition process.

DecylUb behaved analogously (Fig. 2). In 14 experiments the compound was added at a final concentration of 200–450 μM. In 11 cases the channels were extensively or completely inhibited within 1–10 min. In the three other experiments, conducted with 200 or 330 μM decylUb, the tight seal held for less than 3 min after the addition. Increasing $[Ca^{2+}]_{bath}$ to 5 mM resulted in the re-establishment of channel activity in all seven attempts made.

In experiments with suspensions of rat liver mitochondria

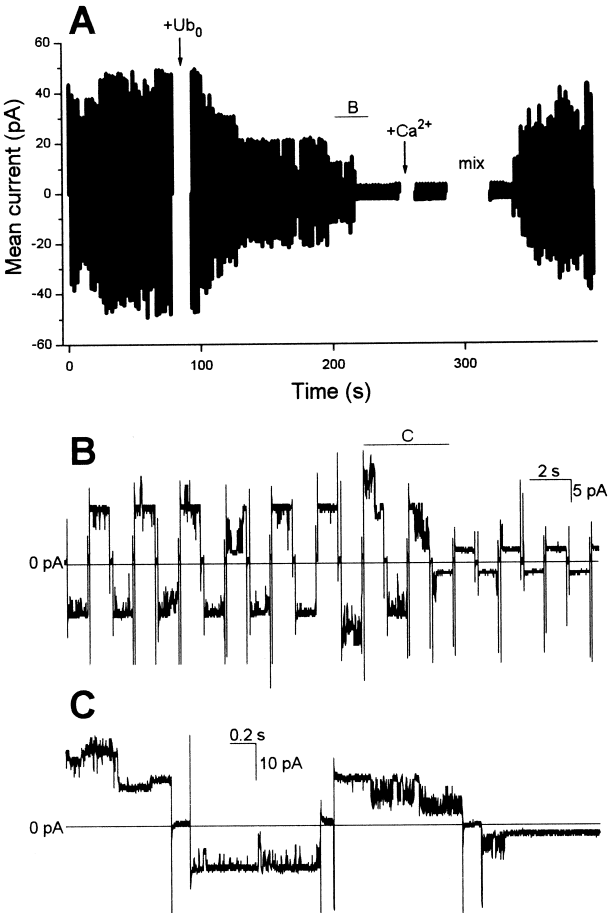

Fig. 1. Ca^{2+}-reversible inhibition of MMC activity by Ub_0. A representative experiment is illustrated. A: A mean current plot: consecutive 1 s ±10 mV voltage pulses, separated by 100 ms periods at 0 mV, were applied to a mitoplast-attached patch. The current flowing during each pulse was averaged and plotted as a function of time. The medium contained 1 mM Ca^{2+}. Additions to the chamber (where indicated): Ub_0: 250 nmol (final concentration: 216 μM); $CaCl_2$: 5.4 μmol (final concentration: 5.5 mM). The segment indicated is plotted below as part B. B: A segment of the current record comprising the Ub_0-induced closure of the last active MMC. Sampling: 1 kHz. Filter: 0.5 kHz. The section denoted by 'C' is plotted on an amplified time scale in part C. C: The closure of the last MMC to be inhibited by Ub_0. Sampling: 5 kHz. Filter: 1 kHz. Capacitive current spikes, corresponding to voltage changes to/from 0 from/to ±10 mV, have been reduced for clarity in these and all other current traces presented.

(RLM), Ub_5 did not prevent the onset of the PT in Ca^{2+}-loaded mitochondria, and it relieved instead the inhibition caused by Ub_0 or decylUb [20]. In patch-clamp experiments this compound was similarly unable to inhibit the MMC at concentrations up to 100 μM (not shown; $n=5$). To verify whether it would revert the effects of Ub_0, after inhibiting MMC activity with 50–240 μM Ub_0 we applied 120–450 μM Ub_5 to pipette-attached mitoplasts. In 11 out of 13 experiments the addition was followed, after a variable delay, by the development of transmembrane currents. However this activity only sporadically resembled MMC gating. Instead, it generally exhibited fast, flickering gating which might or might not be due to MMCs operating in a kinetic mode originated by a competition between Ub_0 and Ub_5 (Fig. 3).

Once inhibited by Ub_0 or decylUb, the channels seldom re-

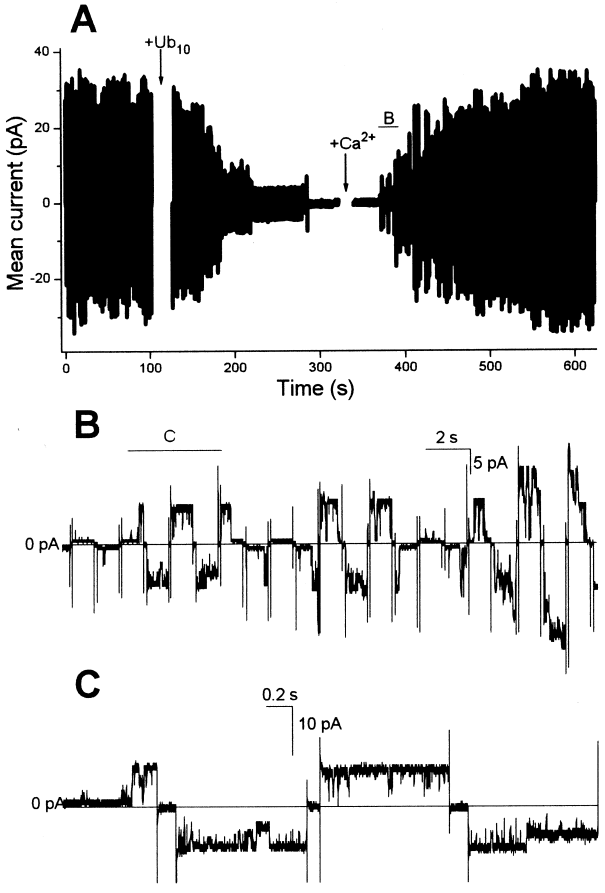

Fig. 2. Ca^{2+}-reversible inhibition of MMC activity by decylUb. A: A mean current plot analogous to the one in Fig. 1A. Conditions as in Fig. 1A. $[Ca^{2+}]_{medium}$: 0.5 mM. Additions: decylUb: 0.5 μmol (final concentration: 450 μM). Ca^{2+}: 5.4 μmol (final concentration: 5.3 mM). The current trace of the segment denoted by 'B' is plotted below. B: The initial stages of channel reactivation after Ca^{2+} addition. Sampling: 1 kHz. Filter: 500 Hz. C: A segment of the record in B showing the Ca^{2+}-induced opening of the first MMC, plotted on an amplified time scale. Digital sampling: 5 kHz. Filtering: 1 kHz.

opened completely unless $[Ca^{2+}]_{bath}$ was increased. When they did, they remained active for seconds, and displayed the normal pattern of transitions to substates (favored by pipette-negative potentials). Thus, the presence of these inhibitors appears not to result in marked alterations of the behavior of the open channels. Most full closing or re-opening events took place via brief sojourns in substates, generally in the fast-gating 'half-conductance' substate already described [21,25]. MMCs closing or opening 'spontaneously' or under the influence of voltage often display the same behavior. Patches in which MMC activity had been practically eliminated by these inhibitors also exhibited, besides gating by 107 pS channels [30], rare bursts attributable to MMCs operating in a fast-gating subconductance state. Fig. 4 presents an exemplary gallery of events recorded from inhibited patches.

Our attempts at re-inhibiting the Ca-reactivated channels by increasing the concentration of Ub_0 or decylUb invariably failed due to loss of the tight seal. It is therefore unclear whether repetitive inhibition/reactivation cycles can be carried out with these ubiquinone analogues in analogy to the behavior with other inhibitors, exemplified in Fig. 5 in the case of ADP.

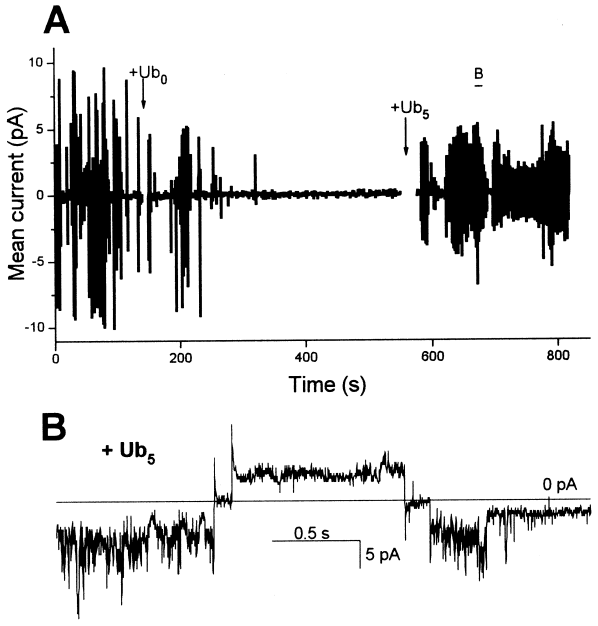

Fig. 3. Effect of Ub_5. A: A mean current plot analogous to those in Figs. 1A and 2A. Conditions as in Fig. 1A. Additions to the chamber, when indicated: Ub_0: 50 μM; Ub_5: 200 μM (final concentrations). A record segment at the position denoted by 'B' is plotted below. B: A segment of the current record after Ub_5 addition. Digital sampling: 1 kHz. Filtering: 0.5 kHz.

4. Discussion

The results presented above demonstrate that the MMCs observed in patch-clamp experiments are inhibited by Ub_0 and decylUb, and not by Ub_5, in analogy with the inhibition of the PTP in isolated, suspended mitochondria [19,20]. This

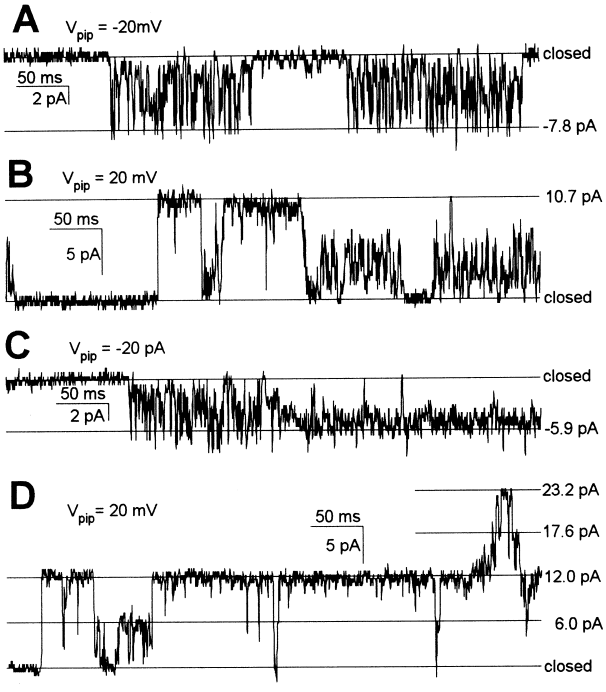

Fig. 4. Current traces illustrating bursts of MMC activity in Ub_0-inhibited patches. Sampling: 5 kHz. Filtering: 1 kHz.

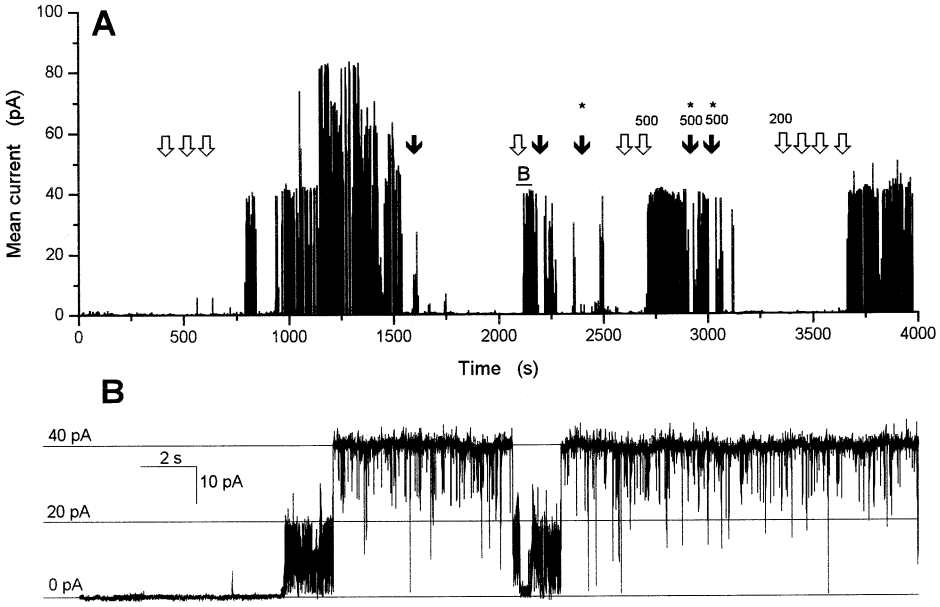

Fig. 5. Competition between Ca^{2+} and ADP. A: A mean current plot. In this experiment V was held at +40 mV. The current (leaks subtracted) was averaged over 5 s consecutive intervals, and the means are plotted sequentially. The medium contained 0.1 mM Ca^{2+} and the bath volume was approximately 1 ml. When indicated by the arrows, Ca^{2+} (⇓) or ADP (↓) was added to the bath. The amount added was 100 nmol unless otherwise specified by the numbers above the arrows. The patch initially displayed only 107 pS channels. Addition of a total of 300 nmol of Ca^{2+} elicited, after a lag, activity by two MMCs, which was promptly inhibited upon addition of 100 nmol of ADP. One of the channels was reactivated by the addition of more Ca^{2+}, again inhibited by ADP, reactivated by Ca^{2+}, reinhibited by ADP and reactivated once more by Ca^{2+} before loss of the tight seal. Where indicated by an asterisk gaps appear in the record because of excessive noise introduced by the additions. The current trace corresponding to the segment marked 'B' is shown below. B: The opening of one MMC upon readdition of Ca^{2+} after the first inhibition by ADP in the same experiment illustrated above. The current record corresponding to the segment marked by a 'B' in Fig. 5A is shown. The channel opens and closes via sojourns in the 'half-conductance state', as it typically does. Sampling: 1 kHz. Filter: 0.5 kHz.

confirms the identification of the MMC as the electrophysiological counterpart of the PTP. Furthermore, our observations confirm the conclusion that inhibition by these compounds is due to direct interactions rather than to an inhibitory effect on electron flow, since in patch-clamped mitoplasts (osmotically shocked, permeabilized by PTP opening and incubated in a respiratory substrate-free medium) little electron transport ought to take place. The channels inhibited by Ub_0 and decylUb are the same that are closed by an array of other PTP inhibitors. They are therefore, presumably, formed by the same proteins, whose identity remains uncertain. The action of ubiquinone analogues and PTP modulation by electron flow through Complex I suggest that the latter may be involved [19,20]. On the other hand, other data strongly implicate the AdNT as well as other proteins, including VDAC. It may be that the PTP is even more complex than imagined so far, including, but probably not limited to, components of the respiratory chain as well as the AdNT. Ligand-induced changes in the conformation of one or the other of these components may result in its closure/inactivation/disassembly.

One interesting aspect is the fact that inhibition by Ub_0, decylUb and ADP could be reverted by increasing the $[Ca^{2+}]$ in the medium. These compounds thus join the list of the MMC/PTP inhibitors which can be competitively antagonized by Ca^{2+}. The present results and previous work with other inhibitors ([25]; Szabò and Zoratti, unpublished observations) suggests that the activating Ca^{2+} binding sites are not saturated in the mM concentration range. Since the closest thing to an unsaturable Ca^{2+} binding site in mitochondria are the membrane phospholipids, it seems reasonable to suggest that at least part of the Ca^{2+} effects, at high Ca^{2+} concentrations, may be exerted at the membrane level.

Ca^{2+} is known to form ion pairs with the negatively charged groups of acidic lipids, which are abundant in mitochondrial membranes [31]. These interactions modify the surface charge and potential of the membrane, leading to a corresponding variation of the electrical field sensed by membrane-embedded charged groups in proteins. Since the effects of several PTP inhibitors have been ascribed to variations of the sensitivity of the pore complex to depolarization (a shift of the threshold potential for opening to more depolarized values) [28,29], this might be considered to explain why Ca^{2+} binding to the inner surface of the inner membrane can reverse their effect. Indeed, for example, the action of AdNT ligands (atractyloside, bongkrekate, ADP) has been proposed to be mediated by variations of surface potential secondary to AdNT conformational changes [32–34]. However, in our electrophysiological experiments very low potentials (± 10 or 20 mV) were routinely applied, i.e. the mitochondrial membrane was always essentially depolarized. Furthermore, while Ca^{2+} could re-activate inhibited channels, this effect was not produced by the application of matrix-positive potentials, which should in principle be equivalent to make the potential of the matrix-side inner membrane surface more positive by Ca^{2+} binding.

The Ca^{2+} effect is thus likely to have other explanations. Ca^{2+} binding to negatively charged phospholipids (PS, PA, cardiolipin) induces 'rigidification' and phase separation (e.g. [35–40]) which may well influence protein activity. Changes in membrane order and/or fluidity might favor the aggregation of the inner membrane proteins needed to form the PTP com-

plex, or the conformational changes needed to open the pore. PTP inhibitors are generally considered to act by hindering pore assembly or the relevant changes in protein structure rather than as blockers. PTP components may therefore be considered to exist as unproductive species, stabilized by bound inhibitors or by the lack of some other partner in the complex, or as PTP-producing forms, favored, in this hypothesis, by Ca^{2+}-induced membrane modifications. The system would therefore be described as an equilibrium, whose position would obviously be regulated by thermodynamic factors. Increasing the concentration of inhibitors would drive it towards 'closed-pore' state(s) simply by mass action. Increasing [Ca^{2+}] would drive it towards 'open-pore' states by making the membrane environment more and more favorable for PTP activity. Some inhibitors and activators (e.g. trivalent cations, polyamines, local anesthetics, peptides like mastoparan [41]) probably act directly through alterations of the membrane state.

Another hypothesis might envision Ca^{2+}-induced hemifusion of the outer and inner mitochondrial membranes. High [Ca^{2+}] has long been known to be aggregating and fusogenic (e.g. [42]), finding application for example in the fusion of mitoplasts [43]. Fusion of model phospholipid bilayers [44] as well as protein-mediated fusion (e.g. [45–47]) are believed to proceed via the formation of hemifusion intermediates which then progress to fusion pores. The fusion process can be stopped at the hemifusion stage using mutagenized fusion proteins [48,49]. Mitochondria can undergo fusion with other mitochondria in vivo (e.g. [50,51]) and with liposomes in vitro (e.g. [52,53]) as well as lipid exchange between organelles [54,55]. Their outer membrane has been recently demonstrated to contain fusogenic proteins [56–58] and they might harbor a v-SNARE [59]. Mitochondria form contact sites between the inner and outer membranes for both protein import (e.g. [60]) and energy conservation [14] purposes. Morphological evidence has been presented that Ca^{2+}-induced organizational changes of the mitochondrial membranes promote the formation of contact sites, proposed to be sites of hemifusion between the two membranes ([14] and references therein; [39]). At these locations the mitochondrion would be bound by a single bilayer, or by a non-bilayer lipidic structure, in which proteins of both inner and outer membranes might mingle, forming the MMC/PTP. It might even be imagined that the PTP is actually a mitochondrial version of fusion pore which does not proceed to enlarge infinitely. Fusion pores have been observed to behave like gating channels (e.g. [45]) and their formation appears to be reversible (e.g. [61]). It is however more difficult to explain the antagonistic effect of increases in the concentration of inhibitors within this mechanistic scheme unless the assumption is made that, e.g. ADP or cyclosporin can induce the reversal of the hemifusion process. While this may sound far-fetched, it should be mentioned that contact site formation is reportedly regulated by multiple factors, including whether the mitochondria are engaged or not in phosphorylation [14,62], an effect believed to be mediated by the AdNT [63] (to which cyclophilin D binds [64]).

In summary, our observations strengthen the hypothesis that the first segment of the mitochondrial respiratory chain may contribute to form the PTP. Furthermore, they suggest that the phospholipid bilayer itself may be involved in Ca^{2+}-induced MMC/PTP activation. These conclusions are relevant for studies on cellular processes involving PTP activation, especially since apoptosis in some models has been associated with a direct and early interference with mitochondrial respiratory chain function (e.g. [65–67]).

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