We have investigated the regulation of the permeability transition pore (PTP), a cyclosporin A-sensitive channel, in rat skeletal muscle mitochondria. As is the case with mitochondria isolated from a variety of sources, skeletal muscle mitochondria can undergo a permeability transition following Ca\(^{2+}\) uptake in the presence of P. We find that the PTP opening is dramatically affected by the substrates used for energization, in that much lower Ca\(^{2+}\) loads are required when electrons are provided to complex I rather than to complex II or IV. This increased sensitivity of PTP opening does not depend on differences in membrane potential, matrix pH, Ca\(^{2+}\) uptake, oxidation-reduction status of pyridine nucleotides, or production of H\(_2\)O\(_2\), but is directly related to the rate of electron flow through complex I. Indeed, and with complex I substrates only, pore opening can be observed when depolarization is induced with uncoupler (increased electron flow) but not with cyanide (decreased electron flow). Consistent with pore regulation by electron flow, we find that PTP opening is inhibited by ubiquinone 0 at concentrations that partially inhibit respiration and do not depolarize the inner membrane. These data allow identification of a novel site of regulation of the PTP, suggest that complex I may be part of the pore complex, and open new perspectives for its pharmacological modulation in living cells.

The permeability transition is an in vitro increase of the inner mitochondrial membrane permeability to solutes, which is favored by Ca\(^{2+}\) uptake. This phenomenon is now interpreted as due to the opening of a proteinaceous but yet unidentified large conductance channel, the PTP.\(^1\) The PTP open-closed transitions are modulated by the transmembrane electrical potential, by matrix pH, by redox potential, by adenosine nucleotides, and by Me\(^{2+}\). Finally, the permeability transition can be induced or inhibited by a variety of drugs (1–4). It has been proposed that the PTP may provide mitochondria with a fast Ca\(^{2+}\) release channel (4), and recent evidence indeed indicates that transient PTP opening in a low conductance mode (i.e., a state permeable to ions but not to larger molecules such as sucrose) may contribute to physiological Ca\(^{2+}\) signaling through amplification of inositol 1,4,5-trisphosphate-dependent Ca\(^{2+}\) release (5).

PTP opening in vitro leads to collapse of the proton-motive force, disruption of ionic homeostasis, mitochondrial swelling, and massive ATP hydrolysis by the F\(_0\)F\(_1\) ATPase. This sequence of events has drawn considerable attention to the permeability transition as a potential player in the cellular pathways to cell death through at least four mechanisms, i.e., decreased levels of cellular ATP (6, 7), increase of cytosolic Ca\(^{2+}\) (6, 8, 9), release of apoptosis-inducing factor, a mitochondrial caspase (10), and release of cytochrome c (11, 12).

With the long term goal of defining the role of mitochondria in the pathways to muscle cell death, we carried out a characterization of PTP regulation in isolated skeletal muscle mitochondria. Unexpectedly, we found that the Ca\(^{2+}\) sensitivity of the permeability transition was strongly affected by the respiratory substrate used for energization, in that PTP opening was much easier with electron donors at complex I than with electron donors at complex II or IV. This increased Ca\(^{2+}\) sensitivity of PTP opening did not depend on differences in proton electrochemical gradient, Ca\(^{2+}\) uptake rates, oxidation-reduction status of P, or production of H\(_2\)O\(_2\), but rather on the rate of electron flux through complex I. Consistently, we find that PTP opening is inhibited by Ub0. These findings suggest that complex I may be part of the PTP and open new perspectives for its pharmacological modulation in living cells.

**MATERIALS AND METHODS**

Rat skeletal muscle mitochondria were prepared according to Maden et al. (13) with slight modifications. Albino Wistar rats weighing 250–350 g were killed by decapitation, and the gastrocnemius muscles were rapidly excised and transferred into isolation medium (150 mM sucrose, 75 mM KCl, 50 mM Tris-HCl, 1 mM KH\(_2\)PO\(_4\), 5 mM MgCl\(_2\), 1 mM EGTA, pH 7.4). Muscles were minced with scissors and trimmed clean of visible fat and connective tissues. Muscle pieces were transferred to 30 ml of isolation medium supplemented with 0.2% bovine serum albumin and 0.2 mg·m\(^{-1}\)·N\(^{-1\}\) Nagarse (Fluka, Buchs). After 1 min, muscles were homogenized using a motor-driven Plexiglas/Plexiglas potter, transferred to 120 ml of isolation medium supplemented with 0.2% bovine serum albumin, and centrifuged at 700 × g for 10 min. The

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\(1\) The abbreviations used are: PTP, permeability transition pore; CaA, cyclosporin A; ΔΔ\(_f\), transmembrane electric potential difference; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; PN, pyridine nucleotide(s); Ub0, ubiquinone 0; TMPD, \(N,N,N',N'\)-tetramethyl-1,4-phenylenediamine dihydrochloride; TPMP\(^*\), triphenylmethylphosphonium; BCECF, 2,7’-bis(carboxyethyl)-5(6)-carboxyfluorescein; Mops, 4-morpholinepropanesulfonic acid.
Regulation of Permeability Transition Pore at Complex I

RESULTS

Effect of Respiratory Substrates on PTP Opening Induced by Ca\textsuperscript{2+} Loading—Since no specific characterization of the PTP in skeletal muscle mitochondria was available, the first aim of this work was to assess the basic features of the permeability transition in these mitochondria. In the experiments of Fig. 1, we assessed the occurrence of PTP opening induced by Ca\textsuperscript{2+} addition as measured from the change of membrane potential of mitochondria incubated in an isotonic sucrose medium supplemented with 10 mM Pi.\textsuperscript{2} Addition of 100 nmol of Ca\textsuperscript{2+}·mg protein\textsuperscript{-1} to mitochondria energized with glutamate, malate, and pyruvate induced a full depolarization (trace a). Identical results were obtained using different associations of complex I substrates such as glutamate plus malate, malate plus pyruvate, or 2-oxoglutarate (not shown). When the experiment was performed in the presence of 1 mM CsA, the same Ca\textsuperscript{2+} addition induced only a moderate and transient depolarization linked to Ca\textsuperscript{2+} uptake (trace b, see also Fig. 2), indicating that the full depolarization of trace a was dependent on PTP opening. Surprisingly, when mitochondria were energized with succinate in the presence of rotenone, the same amount of Ca\textsuperscript{2+} was not able to induce a permeability transition, and only the moderate and transient depolarization accompanying Ca\textsuperscript{2+} uptake could be observed (trace c, see also Fig. 2).

The experiments of Fig. 2 were performed in order to directly measure the Ca\textsuperscript{2+} retention capacity of skeletal muscle mitochondria with different energy sources. When energized with succinate plus rotenone (trace c), skeletal muscle mitochondria displayed a Ca\textsuperscript{2+} retention capacity which was 3--4-fold higher than that observed when they were energized with glutamate, malate, and pyruvate (trace a). It should be noted that the initial kinetics of Ca\textsuperscript{2+} accumulation was the same irrespective of the respiratory substrates used. As expected, addition of CsA increased the Ca\textsuperscript{2+} retention capacity with both complex I and II substrates (traces b and d). However, even in the presence of CsA, Ca\textsuperscript{2+} retention was higher when mitochondria were energized with succinate plus rotenone rather than with glutamate, malate, and pyruvate (compare traces b and d). These data represent a first indication that PTP opening can be induced in skeletal muscle mitochondria, and that the probability of pore opening is higher when substrates are provided to respiratory complex I rather than to respiratory complex II.

To further characterize these effects, we measured the rate of oxygen consumption with different substrates. The experiments depicted in Fig. 3, panel A, show that, in the presence of glutamate, malate, and pyruvate, Ca\textsuperscript{2+} addition induced a transient stimulation followed by a progressive inhibition of respiration (trace a). Respiration could not be stimulated by FCCP addition (not shown), but it could be restored by the addition of NADH, and remained fully sensitive to rotenone (trace a). When the same Ca\textsuperscript{2+} addition was made in the presence of CsA, stimulation of respiration was transient, mitochondria recovered the state 4 respiratory rate preceding Ca\textsuperscript{2+} addition, and respiration could not be stimulated by the addition of NADH, but was still inhibited by rotenone (trace b). When succinate was the substrate (panel B), the same initial Ca\textsuperscript{2+} addition stimulated respiration only transiently, while higher concentrations of Ca\textsuperscript{2+} were required for uncoupling (trace d), which was largely prevented by CsA (trace e). These experiments indicate that Ca\textsuperscript{2+}--induced PTP opening is accompanied by loss of matrix PN. This, in turn, causes respiratory inhibition with NAD\textsuperscript{+}-dependent substrates, which can be bypassed by complex II electron donors. Note that the respiratory control ratio was about 30 with glutamate, malate, and pyruvate (trace c), and about 15 with succinate (trace f). Hence, the increased Ca\textsuperscript{2+} sensitivity with complex I substrates cannot be accounted for by a lower respiratory control.

Matrix pH is an important regulator of pore opening (16, 17). Measurements of matrix pH using mitochondria loaded with BCECF did not reveal any difference between complex I and II substrates. Furthermore, due to the relatively high Pi concentration used in our experiments, no variations of matrix pH could be recorded during the phase of Ca\textsuperscript{2+} uptake (data not shown).

To assess whether the effect of respiratory substrates de-

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\textsuperscript{2} Due to the low yield of mitochondria, this method was chosen for its higher sensitivity. Comparable results could be obtained in standard swelling assays based on permeabilization to sucrose, and from membrane potential measurements based on TPMP– distribution.

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pends on their oxidation, we added glutamate, malate, and succinate together. In this case all flux occurs through complex I, since succinate is not oxidized, due to the inhibition of succinate dehydrogenase of skeletal muscle mitochondria by oxaloacetate, produced here by malate oxidation (18). Under these conditions, pore opening could be easily induced by the addition of 200 nmol of Ca\(^{2+}\) per mg protein, indicating that the effects of complex I substrates are not inhibited by succinate as such (not shown). When rotenone is added, however, complex I is inhibited, oxaloacetate is no longer produced, and succinate oxidation can proceed. Under these conditions, the same 200 nmol/mg protein \(\text{Ca}^{2+}\) load was unable to induce pore opening despite the presence of glutamate and malate (not shown). These findings indicate that complex I substrates are not causing their inducing effects unless they are being oxidized.

**Effect of Respiratory Substrates on PTP Opening Induced by Depolarization**—It has been shown that in liver mitochondria the PTP behaves like a voltage-dependent channel, in that the open probability increases at decreasing membrane potentials (19). To study the voltage dependence of the PTP in skeletal muscle mitochondria, we compared the depolarization induced by increasing concentration of FCCP in the presence of CsA (i.e. with a closed PTP) to that obtained after uptake of a small \(\text{Ca}^{2+}\) load, which was unable to open the pore per se. As shown in Fig. 4, panel A, when mitochondria were energized with malate, glutamate, and pyruvate, the FCCP-induced depolarization was much more pronounced after \(\text{Ca}^{2+}\) loading (open circles) than in the presence of CsA (closed squares), revealing a major contribution of the PTP to depolarization under this particular condition. Fig. 4, panel B, shows that under identical condition of \(\text{Ca}^{2+}\) loading, but with succinate (in the presence of rotenone) as the substrate, contribution of the pore to depolarization was much less pronounced. To determine whether the promoting effect of complex I substrates on FCCP-induced pore opening only depended on a change of the threshold voltage, or also on the rate of electron flux through complex I we performed similar experiments where depolarization was rather achieved through inhibition of electron flow with cyanide. Fig. 3 It must be stressed that PTP contribution to depolarization with succinate (plus rotenone) as the substrate became more apparent at higher \(\text{Ca}^{2+}\) loads, consistent with earlier reports on liver mitochondria.
Fig. 4. Effect of respiratory substrates on depolarization induced by FCCP or KCN in skeletal muscle mitochondria. Mitochondria (0.3 mg) were incubated as described for Fig. 1. In panels A and C the substrates were 5 mM glutamate-Tris, 5 mM pyruvate-Tris, and 2.5 mM malate-Tris, while in panels B and D the substrate was 5 mM succinate-Tris plus 2 μM rotenone. Mitochondria were then loaded with 5 μM Ca²⁺ (open circles) or incubated in the presence of 1 μM CsA (closed squares). After reaching a steady state, membrane potential was decreased by sequential additions of FCCP (panels A and B) or KCN (panels C and D). Membrane potential values were measured two minutes after each addition of FCCP or KCN, i.e. when the fluorescence readings had stabilized.

4, panel C, shows that the major sensitization to PTP opening with complex I substrates disappeared in this protocol, the PTP contribution to depolarization becoming indistinguishable from that observed with succinate plus rotenone (panel D). These results underline the importance of electron flux through complex I as a major determinant of pore modulation, with an effect which can be clearly discriminated from a change in the threshold voltage for PTP opening.

Mechanism of PTP Regulation by Complex I; Inhibition by Ub⁰—We next addressed the questions of whether the inducing effect of complex I substrates on pore opening could be accounted for by an overproduction of H₂O₂, or by differences in the oxidation-reduction levels of PN. Fig. 5 shows that with complex I substrates PTP opening by Ca²⁺ was only slightly delayed in the presence of excess catalase (compare traces a and b), suggesting that overproduction of H₂O₂ is not essential for induction of PTP opening.

The question of whether the effect of complex I substrates on the PTP can be accounted for by changes of the PN oxidation-reduction status was more difficult to assess. Indeed, the addition of Ca²⁺ and/or FCCP can cause oxidation of PN only in the absence of rotenone, i.e. a condition where succinate oxidation is inhibited by oxaloacetate. We therefore chose to compare the effect of Ca²⁺ addition on the membrane potential and on the oxidation-reduction level of PN using complex I or IV substrates. As shown in Fig. 6, in the presence of glutamate, malate, and pyruvate, Ca²⁺ addition triggered pore opening...
opening. In an attempt to sort these events we tested the effects of flux and of PN oxidation-reduction level on PTP and therefore do not allow discrimination between the oxidation of PN. indicating that no endogenous substrates are available for reduction readily followed the addition of FCCP, irrespective of the substrate used (panel A, trace c) and membrane depolarization (panel B, trace c) were deeper than those observed with complex I substrates in the presence of CsA, i.e. with a closed pore (compare traces b and c in both panels). Finally, PN oxidation and mitochondrial depolarization readily followed the addition of FCCP, irrespective of the substrate used (traces b and c in both panels). It must be stressed that with complex IV substrates flux through complex I is minimal, and rereduction of PN in trace c of Fig. 6 depends only on reverse electron flow. Indeed, in the absence of added substrates no membrane potential developed (not shown), indicating that no endogenous substrates are available for reduction of PN.

Most conditions used to decrease electron flow at complex I (e.g. addition of rotenone) also lead to increased reduction of PN, and therefore do not allow discrimination between the effects of flux and of PN oxidation-reduction level on PTP opening. In an attempt to sort these events we tested the effects of the exogenous ubiquinone analogue, Ub0. Fig. 7 shows that addition of Ub0 induced a deep and sustained oxidation of PN (panel A, trace c) but did not affect the membrane potential (panel B, trace c). In the presence of Ub0 Ca2+ addition further oxidized PN (panel A, trace c) and caused a deep depolarization (panel B, trace c), but the initial levels of both PN reduction and membrane potential were regained within 5 min (panels A and B, traces c), indicating that PTP opening had been prevented. Indeed, an identical Ca2+ load in the absence of Ub0 caused full depolarization and oxidation of PN (panels A and B, traces a), which were fully sensitive to CsA (panels A and B, traces b). Note that the rate of repolarization was much slower in the presence of Ub0 than in the presence of CsA, suggesting that electron flux had been partially inhibited.

The effects of Ub0 on mitochondrial oxygen consumption with complex I substrates are presented in Fig. 8, which indeed shows that increasing Ub0 concentrations inhibited uncoupled but not state 4 respiration. Taken together, the experiments of Figs. 7 and 8 demonstrate that Ub0 decreases electron flux through complex I and inhibits the PTP despite oxidation of PN.

A major issue at this point was whether the effects of complex I substrates are specific for muscle mitochondria, or they rather underscore a general property of PTP regulation. Fig. 9 shows that rat liver mitochondria incubated in the presence of 1 mM Pi easily underwent PTP opening upon the addition of 25 μM Ca2+ irrespective of whether glutamate plus malate (panel A, trace a) or succinate (panel A, trace b) was the substrate.4 To assess whether the P2 concentration was a factor, we increased

4 Note that rotenone was omitted in the experiments with succinate, so that the degree of PN oxidation was comparable irrespective of whether complex I or II substrates were being oxidized.
Regulation of Permeability Transition Pore at Complex I

FIG. 8. Effect of Ub0 on the oxygen consumption rate of skeletal muscle mitochondria energized with complex I substrates.

Skeletal muscle mitochondria (0.4 mg) were incubated as in Fig. 3 with 5 mM glutamate-Tris, 2.5 mM malate-Tris, 5 mM pyruvate-Tris, and the indicated concentrations of Ub0 in the presence of either 200 nM FCCP (closed circles) or of 0.5 μM oligomycin (open circles).

FIG. 9. Cyclosporin A-sensitive Ca2+-induced depolarization of rat liver mitochondria with complex I or II substrates; effect of Mg2+ and P1.

The incubation medium contained 250 mM sucrose, 5 μM EGTA-Tris, 10 mM Tris-MOPS, 0.2 μM rhodamine 123. Final volume 2 ml, pH 7.3, 25°C. Respiratory substrates were 5 mM glutamate-Tris and 2.5 mM malate-Tris (traces a and c), or 5 mM succinate-Tris (trace b). The medium was supplemented with 1 mM P1 (panel A) or 5 mM P1 and 2 mM MgCl2 (panel B). Experiments were started by the addition of 0.7 mg of liver mitochondria (not shown). Where indicated, 25 μM Ca2+ (panel A) or 35 μM Ca2+ (panel B) was added. In the experiments of traces a, 1 μM CaA was present (both panels).

not with succinate (panel B, trace b) as the substrate. Consistent with these results Ub0 inhibited PTP opening, decreased electron flux at complex I, and oxidized PN also in rat liver mitochondria (not shown, but results were identical to those depicted in Figs. 7 and 8).

DISCUSSION

In this study we have shown that isolated rat skeletal muscle mitochondria can undergo a CsA-sensitive permeability transition as a result of Ca2+ accumulation. Of more general interest is the observation that the probability of PTP opening is modulated by electron flow through complex I of the respiratory chain, with an effect that can be dissociated from the oxidation-reduction state of the mitochondrial PN pool.

Modulation by Complex I Substrates Is a General Feature of the Permeability Transition—A first question raised by our findings on the effects of complex I substrates and Ub0 in skeletal muscle mitochondria was whether these defined a tissue-specific modulation of the pore, or rather depended on specific incubation conditions. Most studies on the permeability transition have been performed in mitochondria isolated from liver, which are therefore the standard for the mechanistic aspects of modulation (see Refs. 1–4 for reviews). Relative to the conditions commonly employed with liver mitochondria, in the present study we used higher P1 concentrations (10 mM) because this allowed attainment of the highest respiratory control ratios (i.e. the maximum efficiency of energy coupling), and minimized matrix pH changes following Ca2+ uptake. When the P1 concentration was 5 mM we could easily observe PTP regulation by electron flux within complex I in liver mitochondria as well, provided that Mg2+ was added (Fig. 9) in agreement with a previous report (21). Furthermore, PTP inhibition by Ub0 was also detected in liver mitochondria even at lower P1 concentrations (not shown). On the other hand, we would like to stress that modulation of PTP opening by complex I substrates could still be observed in skeletal muscle at 1 mM P1 (not shown), i.e. a condition where the effect was undetectable in liver mitochondria (Fig. 9).

Taken together, these data suggest that (i) inhibition by Ub0 and regulation by electron transfer within complex I are general properties of the permeability transition and (ii) that quantitative differences exist reflecting the variable control strength exerted by this process on the overall probability of pore opening in different tissues and/or under different conditions.

Mechanism of PTP Modulation by Electron Flux at Complex I—The role of the oxidation-reduction status of PN in regulation of the PTP has been recognized for many years (22, 23). The novel contribution of the present work is the demonstration that electron transfer can independently modulate the probability of pore opening. Our results show that the relevant regulatory event(s) occur within complex I, that they are related to the rate of electron flow, and that they are independent of changes of the PN oxidation-reduction levels as such. Indeed, first, upon addition of Ca2+ (in the absence of rotenone) the PTP opens when complex I, but not complex IV substrates are being oxidized, yet PN undergo oxidation in both cases (Fig. 6). These observations demonstrate that the inducing effects of increased electron flow are site-specific and suggest regulation by a sensing element located within complex I.

Second, PTP opening with complex I substrates is observed after the addition of FCCP but not of cyanide, despite identical levels of depolarization (Fig. 4). This regulatory effect is therefore distinct from that exerted by the transmembrane voltage since the latter can be observed irrespective of the respiratory substrate, of whether depolarization is induced with a H+ or a K+ current, and of the presence of respiratory inhibitors (19,
24, 25). Thus, the PTP is directly modulated by both electron flux (the primary event of energy transduction, which leads to charge separation through H+ extrusion at the energy-conserving sites), and by the transmembrane proton electrochemical difference (the output force of substrate oxidation).

Third, Ub0 inhibits Ca2+-dependent pore opening at concentrations that both cause oxidation of PN per se, and maximize Ca2+-dependent depolarization and PN oxidation during Ca2+ uptake (Fig. 7). Ub0 oxidizes NADH via two different electron pathways within complex I (26, 27). The first pathway goes from NADH to the physiological hydrophobic ubiquinone-binding site, and couples electron flux to proton extrusion in a rotenone-sensitive manner. The second pathway goes from NADH to a hydrophilic binding site (28), is rotenone-insensitive, and is not coupled to proton translocation (26–28). Thus, Ub0 may be able to oxidize NADH through the rotenone-insensitive site without increasing electron flux through the rotenone-sensitive pathway, consistent with the results of Fig. 7. The mechanism of inhibition of complex I by Ub0 remains unclear, but we note that a similar effect has been reported for Ub1 (29). Based on the triggering effect of electron flux through the rotenone-sensitive pathway, and on the inhibitory effect of Ub0 on PTP opening we suggest that endogenous ubiquinone may bind to a regulatory site (possibly the hydrophilic one) in complex I stabilizing the pore in the closed conformation. Increasing electron flux at complex I might displace ubiquinone from this binding site, increasing in turn the pore open probability. Conversely, addition of Ub0 might stabilize the pore in the closed conformation either through direct binding to this site, or through a decrease of electron flux through complex I. Irrespective of the detailed mechanism, the demonstration that the PTP can be inhibited by Ub0 is an original observation which may have far reaching implications for the pharmacological control of the pore.

The idea that the oxidation-reduction status of ubiquinone indirectly modulates the PTP through changes in the rate of production of H2O2 has been put forward in several studies (30, 31). It must be stressed that this mechanism is unlikely to play a role under our experimental conditions since pore opening by complex I substrates was only marginally affected by the addition of catalase (Fig. 5).

Consequences of PTP Opening on Respiration—An early consequence of PTP opening in vitro is loss of PN (21, 32, 33). Although it is almost invariably assumed that PTP opening is accompanied by uncoupling, the most likely result of a permeability transition is in fact respiratory inhibition due to loss of PN. This is clearly shown in the experiments of Fig. 3, which document that uncoupling can be observed exclusively with electron donors of complex II like succinate, while the permeability transition results in a complete inhibition of respiration with complex I electron donors. The concentration of PN is significantly higher in mitochondria than in the cytosol, and the gradient of free PN between mitochondria and the cytosol in the liver of fed rats is above 14 (34). If a permeability transition ever occurs in vivo, we predict that it will result in respiratory inhibition because of mitochondrial PN and substrate depletion, blocking in turn the Krebs cycle and oxidative metabolism.

It should be noted that respiratory inhibition following pore opening cannot be accounted for entirely by PN depletion. The maximal rate of respiration indeed remained about 50% inhibited even in the presence of millimolar concentrations of added NADH (Fig. 3). It should be mentioned that under these conditions the pore remained open, since addition of succinate plus rotenone did not repolarize mitochondria unless EGTA was also added to close the pore (not shown). This result suggests that PTP opening might also have direct consequences on electron transfer within complex I, since the inhibitory effect is not observed with succinate (Fig. 3). We cannot exclude, however, that under these particular conditions the rate-limiting step in respiration is regulated by diffusion of NADH through the pore.

Nature of the PTP—Despite recent progress in our understanding of PTP modulation, its molecular nature has remained elusive. The findings of the present study offer a hitherto unrecognized possibility, i.e. that the pore may comprise, or be in close contact with, proteins of the mitochondrial complex I. It is striking that Saccharomyces cerevisiae mitochondria, which lack a classical Ca2+ and Ca2+-sensitive permeability transition (35) also lack complex I and possess two NADH dehydrogenases, which are insensitive to rotenone and, most importantly, do not pump protons (36). Expression of mammalian complex I proteins in yeast may provide an answer to this unresolved question of mitochondrial pathophysiology.

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