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Regulation of the Permeability Transition Pore in Skeletal Muscle Mitochondria

MODULATION BY ELECTRON FLOW THROUGH THE RESPIRATORY CHAIN COMPLEX I*

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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_i. 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_2O_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 opening is modulated by the transmembrane electrical potential, by matrix pH, by redox potential, by adenine 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_0F_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 PN, or production of H_2O_2, but rather on the rate of electron flux through complex I. Consistently, we find that PTP opening is inhibited by Ub_0. 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 Madsen *et al.* (13) with slight modifications. Albino Wistars 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_2PO_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·ml^{-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; CsA, cyclosporin A; $\Delta\psi$, transmembrane electric potential difference; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; PN, pyridine nucleotide(s); Ub_0, 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.

supernatant was decanted and centrifuged at 10,000 × *g* for 10 min. The resulting pellet was resuspended in a medium containing 250 mM sucrose, 0.1 mM EGTA-Tris, 10 mM Tris-HCl, pH 7.4, and centrifuged at 7,000 × *g* for 6 min. The final mitochondrial pellet was resuspended in 0.5 ml of the same medium at a final protein concentration of about 20 mg·ml^{-1}. All procedures were carried out at 0–4 °C. Rat liver mitochondria were prepared according to standard procedures.

Mitochondrial oxygen consumption was measured polarographically at 25 °C using a Clark-type electrode. Measurements of membrane potential, extramitochondrial Ca^{2+}, matrix pH, and PN oxidation-reduction status were carried out fluorimetrically with a Perkin-Elmer 650–40 spectrofluorimeter equipped with magnetic stirring and thermostatic control. Membrane potential was measured in the presence of 0.2 μM rhodamine 123 as described in Emaus *et al.* (14) (excitation-emission, 503–525 nm). The resting potential was determined through parallel determinations with a $TPMP^+$-selective electrode as described in Zoratti and Petronilli (15). The residual Donnan potential after full depolarization with FCCP was estimated to be −80 mV (in this $\Delta\psi$ range the electrode sensitivity is too low for a precise determination). Extramitochondrial Ca^{2+} concentration was measured in the presence of 1 μM Calcium Green-5N as described in Ichas *et al.* (5) (excitation-emission, 505–535 nm). Calibration of the signal was achieved by the addition of known amounts of Ca^{2+}. Mitochondrial loading with BCECF and pH_i determinations were carried out exactly as described in Bernardi *et al.* (16). The PN oxidation-reduction status was evaluated based on endogenous NAD(P)H fluorescence (excitation-emission, 340–460 nm). Calibration of the signal was achieved by the addition of glutamate, pyruvate, and malate (levels taken as 100% reduction) or of 0.8 μM FCCP (level taken as 100% oxidation). All chemicals were of the highest purity commercially available, and cyclosporin A was a gift of Novartis (Basel, Switzerland).

RESULTS

Effect of Respiratory Substrates on PTP Opening Induced by Ca^{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^{2+} addition as measured from the change of membrane potential of mitochondria incubated in an isotonic sucrose medium supplemented with 10 mM P_i.[2] Addition of 100 nmol of Ca^{2+}·mg protein^{-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 μM CsA, the same Ca^{2+} addition induced only a moderate and transient depolarization linked to Ca^{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^{2+} was not able to induce a permeability transition, and only the moderate and transient depolarization accompanying Ca^{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^{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^{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^{2+} accumulation was the same irrespective of the respiratory substrates used. As expected, addition of CsA increased the Ca^{2+} retention capacity with both complex I and II substrates (*traces b* and *d*). However, even in the presence of CsA, Ca^{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.

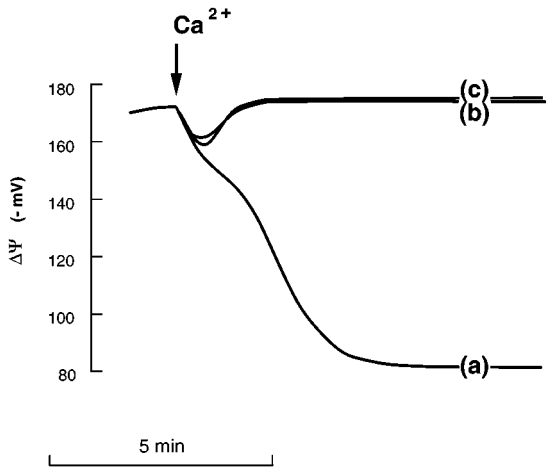

FIG. 1. **Effect of respiratory substrates on the cyclosporin A-sensitive, Ca^{2+}-induced depolarization of skeletal muscle mitochondria.** The incubation medium contained 250 mM sucrose, 10 mM P_i-Tris, 5 μM EGTA-Tris, 10 mM Tris-MOPS, 0.2 μM rhodamine 123. Final volume 2 ml, pH 7.3, 25 °C. Respiratory substrates were 5 mM glutamate-Tris, 5 mM pyruvate-Tris, and 2.5 mM malate-Tris (*traces a* and *b*), or 5 mM succinate-Tris plus 2 μM rotenone (*trace c*). Experiments were started by the addition of 0.3 mg of skeletal muscle mitochondria (not shown). Where indicated, 15 μM Ca^{2+} was added. In the experiment of *trace b*, 1 μM CsA was present.

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^{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^{2+} addition was made in the presence of CsA, stimulation of respiration was transient, mitochondria recovered the state 4 respiratory rate preceding Ca^{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^{2+} addition stimulated respiration only transiently, while higher concentrations of Ca^{2+} were required for uncoupling (*trace d*), which was largely prevented by CsA (*trace e*). These experiments indicate that Ca^{2+}-induced PTP opening is accompanied by loss of matrix PN. This, in turn, causes respiratory inhibition with NAD^+-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^{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 P_i concentration used in our experiments, no variations of matrix pH could be recorded during the phase of Ca^{2+} uptake (data not shown).

To assess whether the effect of respiratory substrates de-

[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.

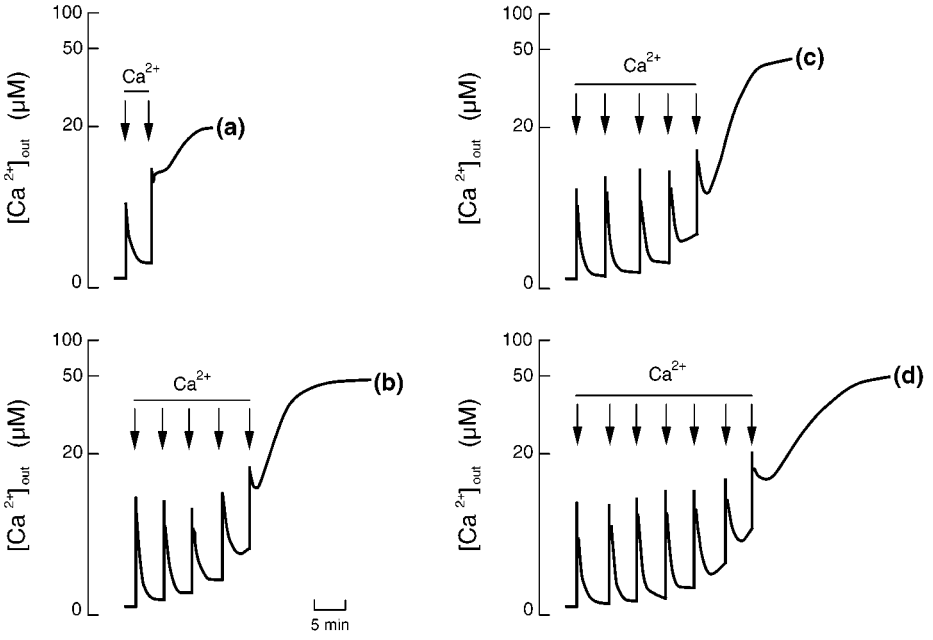

FIG. 2. **Ca^{2+} retention capacity of skeletal muscle mitochondria; effect of respiratory substrates and CsA.** Experimental conditions were as in Fig. 1, except that the incubation medium was supplemented with 1 μM Calcium Green-5N while rhodamine 123 was omitted. Respiratory substrates were 5 mM glutamate-Tris, 5 mM pyruvate-Tris, and 2.5 mM malate-Tris (*traces a* and *b*), or 5 mM succinate-Tris plus 2 μM rotenone (*traces c* and *d*). In *traces b* and *d* the medium was supplemented with 1 μM CsA. Experiments were started by the addition of 0.3 mg of mitochondria (not shown). Where indicated, 10 μM Ca^{2+} pulses were added (*arrows*).

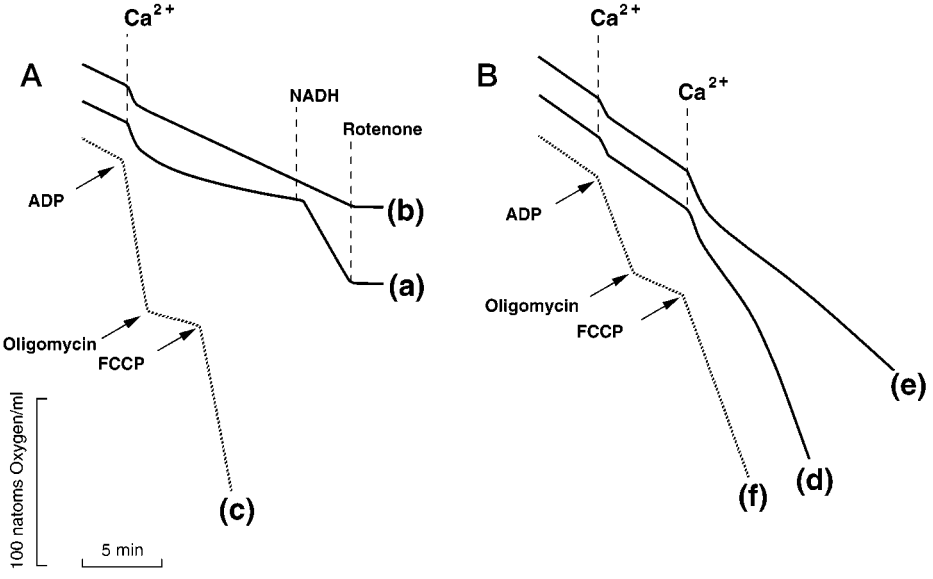

FIG. 3. **Oxygen consumption rate of skeletal muscle mitochondria with complex I or II respiratory substrates; effects of Ca^{2+}, CsA, ADP, and FCCP.** Experimental conditions were as in Fig. 1 except that rhodamine 123 was omitted. Experiments were started by the addition of 0.8 mg mitochondria (not shown). In *panel A* the substrates were 5 mM glutamate-Tris, 5 mM pyruvate-Tris, and 2.5 mM malate-Tris; where indicated 50 μM Ca^{2+}, 1 mM NADH, and 2 μM rotenone (*traces a* and *b*) or 1 mM ADP, 0.5 μM oligomycin, and 200 nM FCCP (*trace c*) were added. In *trace b* the medium was supplemented with 1 μM CsA. In *panel B* the substrate was 5 mM succinate-Tris plus 2 μM rotenone; where indicated, 50 μM (first addition) and 100 μM Ca^{2+} (second addition) (*traces d* and *e*) or 1 mM ADP, 0.5 μM oligomycin, and 200 nM FCCP (*trace f*) were added. In *trace e* the medium was supplemented with 1 μM CsA.

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+}·mg protein^{-1}, 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^{-1} 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 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 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 Ca^{2+} loading, but with succinate (in the presence of rotenone) as the substrate, contribution of the pore to depolarization was much less pronounced.[3] 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 Ca^{2+} loads, consistent with earlier reports on liver mitochondria.

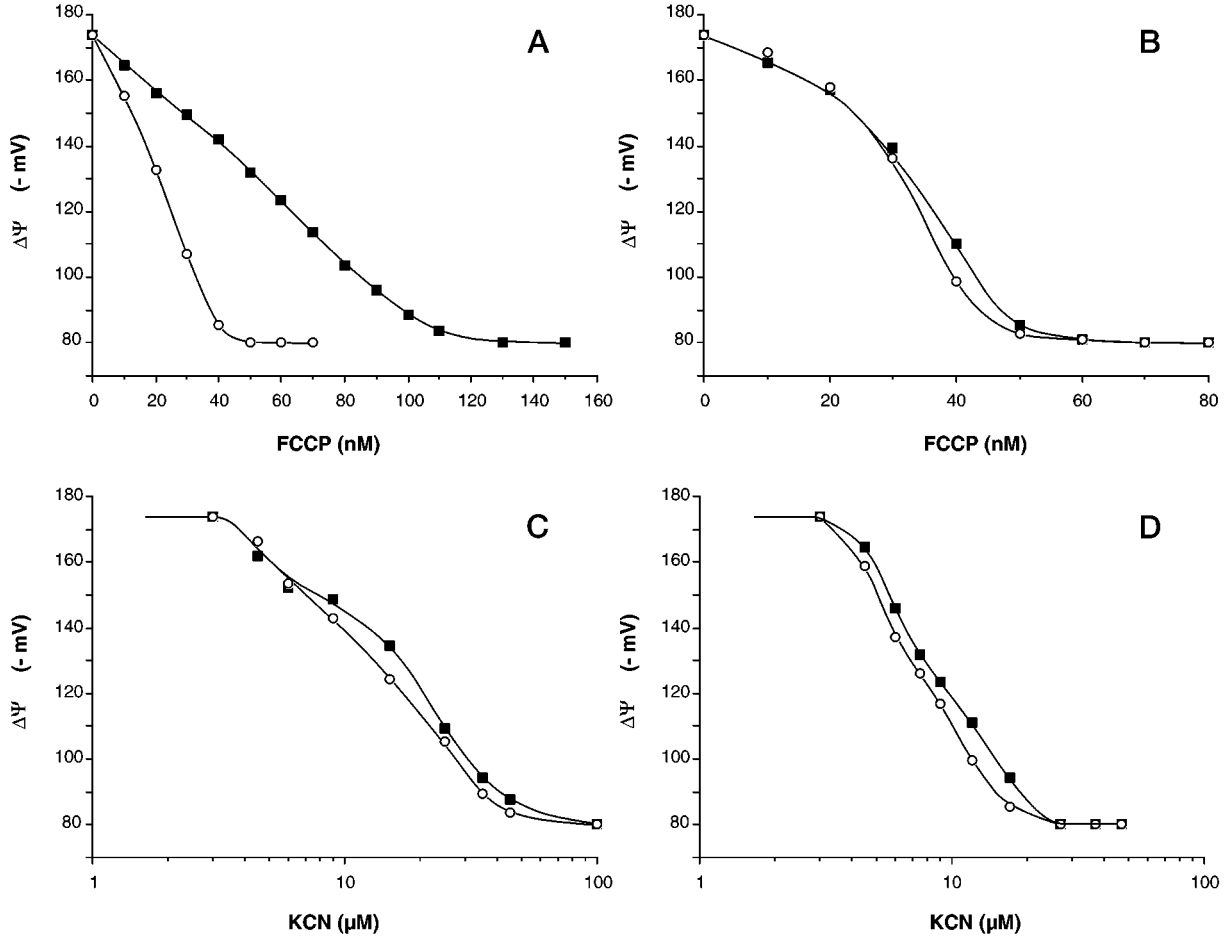

FIG. 4. **Effect of respiratory substrates on depolarization induced by FCCP or KCN in skeletal muscle mitochondria.** Mitochondria (0.3 mg) were incubated as described for Fig. 1. In *panels A* and *C* the substrates were 5 mM glutamate-Tris, 5 mM pyruvate-Tris, and 2.5 mM malate-Tris, while in *panels B* and *D* the substrate was 5 mM succinate-Tris plus 2 μM rotenone. Mitochondria were then loaded with 5 μM Ca^{2+} (*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 Ub_0—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 H_2O_2, or by differences in the oxidation-reduction levels of PN. Fig. 5 shows that with complex I substrates PTP opening by Ca^{2+} was only slightly delayed in the presence of excess catalase (compare *traces a* and *b*), suggesting that overproduction of H_2O_2 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 Ca^{2+} 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 Ca^{2+} 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, Ca^{2+} addition triggered pore opening

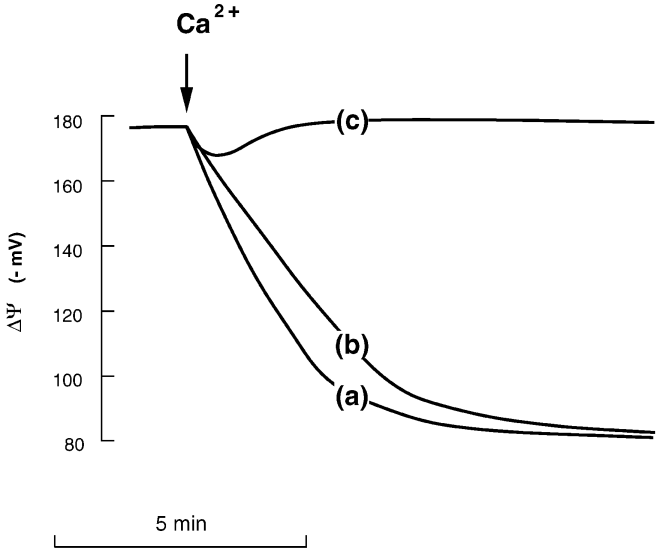

FIG. 5. **Effect of catalase on the Ca^{2+}-dependent depolarization with complex I respiratory substrates.** Experimental conditions were as in Fig. 1. Respiratory substrates were 5 mM glutamate-Tris, 5 mM pyruvate-Tris, and 2.5 mM malate-Tris (*traces a* and *b*), or 5 mM succinate-Tris plus 2 μM rotenone (*trace c*). In the experiment of trace b, 10,000 units of catalase were present. Experiments were started by the addition of 0.2 mg of mitochondria (not shown). Where indicated, 10 μM Ca^{2+} was added.

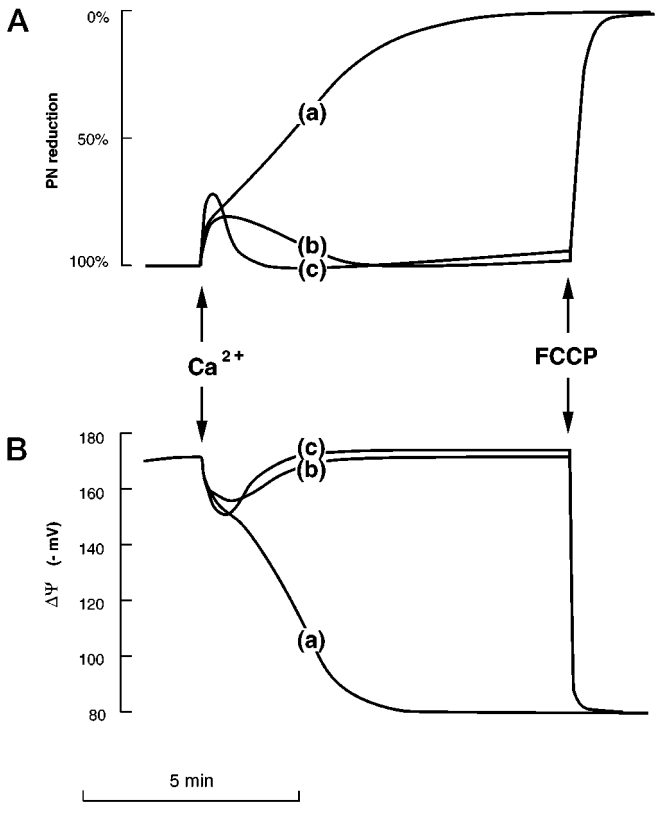

FIG. 6. **Ca^{2+}-dependent changes of membrane potential and PN oxidation-reduction state of skeletal muscle mitochondria with complex I and IV substrates.** Experimental conditions were as in Fig. 1 except that rhodamine 123 was omitted in *panel A*. Respiratory substrates were 5 mM glutamate-Tris, 5 mM pyruvate-Tris, and 2.5 mM malate-Tris (*traces a* and *b*), or 5 mM ascorbate-Tris plus 0.3 mM TMPD (*trace c*). In the experiment of *trace b* the medium was supplemented with 1 μM CsA. Experiments were started by the addition of 0.5 mg of mitochondria (not shown). Where indicated, 25 μM Ca^{2+} and 300 nM FCCP were added. *Panel A*, PN oxidation-reduction levels; *panel B*, membrane potential. Measurements were performed in parallel incubations of the same mitochondrial preparation.

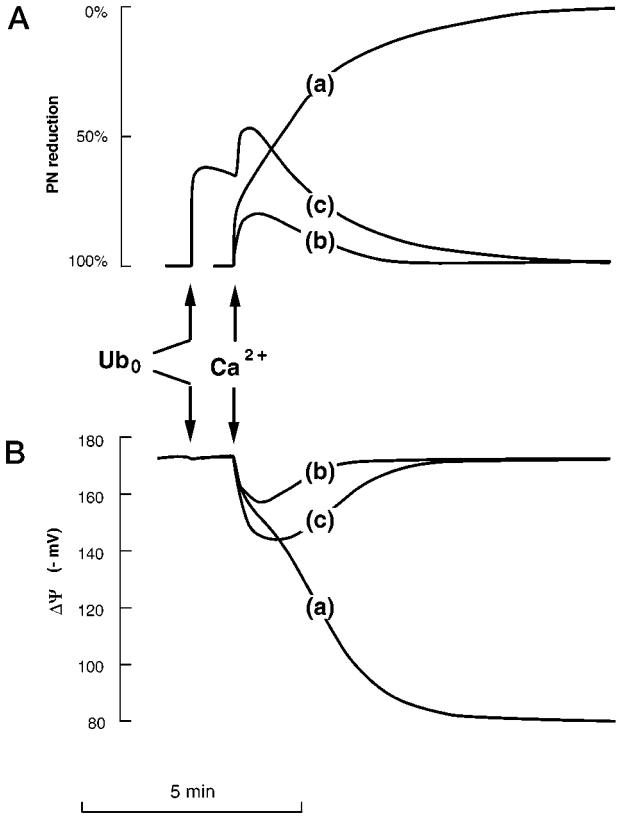

FIG. 7. **Inhibition of the Ca^{2+}-induced depolarization of skeletal muscle mitochondria with complex I substrates by Ub_0.** Experimental conditions were as in Fig. 1, except that rhodamine 123 was omitted in *panel A*. Respiratory substrates were 5 mM glutamate-Tris, 5 mM pyruvate-Tris, and 2.5 mM malate-Tris. The experiments were started by the addition of 0.4 mg of mitochondria (not shown). Where indicated, 50 μM Ub_0 (*traces c* only) and 20 μM Ca^{2+} were added. *Panel A*, PN oxidation-reduction levels; *panel B*, membrane potential. In the experiment of *trace b* the medium was supplemented with 1 μM CsA.

(*panel B*, *trace a*) which was paralleled by the oxidation of PN (*panel A*, *trace a*), which invariably follows pore opening (20). Both depolarization and PN oxidation were indeed inhibited by CsA (*panels A* and *B*, *traces b*). When ascorbate and TMPD were used as the substrates, the same Ca^{2+} load did not open the pore despite the fact that both PN oxidation (*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, Ub_0. Fig. 7 shows that addition of Ub_0 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 Ub_0 Ca^{2+} 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 Ca^{2+} load in the absence of Ub_0 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 Ub_0 than in the presence of CsA, suggesting that electron flux had been partially inhibited.

The effects of Ub_0 on mitochondrial oxygen consumption with complex I substrates are presented in Fig. 8, which indeed shows that increasing Ub_0 concentrations inhibited uncoupled but not state 4 respiration. Taken together, the experiments of Figs. 7 and 8 demonstrate that Ub_0 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 P_i easily underwent PTP opening upon the addition of 25 μM Ca^{2+} irrespective of whether glutamate plus malate (*panel A*, *trace a*) or succinate (*panel A*, *trace b*) was the substrate.[4] To assess whether the P_i 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.

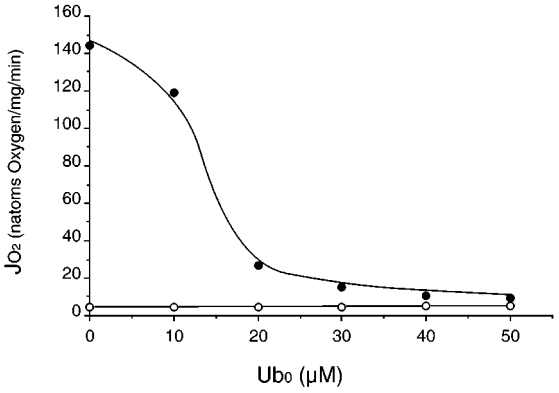

FIG. 8. **Effect of Ub_0 on the oxygen consumption rate of skeletal muscle mitochondria energized with complex I substrates.** Skeletal muscle mitochondria (0.4 mg) were incubated as in Fig. 3 with 5 mM glutamate-Tris, 2.5 mM malate-Tris, 5 mM pyruvate-Tris, and the indicated concentrations of Ub_0 in the presence of either 200 nM FCCP (*closed circles*) or of 0.5 μM oligomycin (*open circles*).

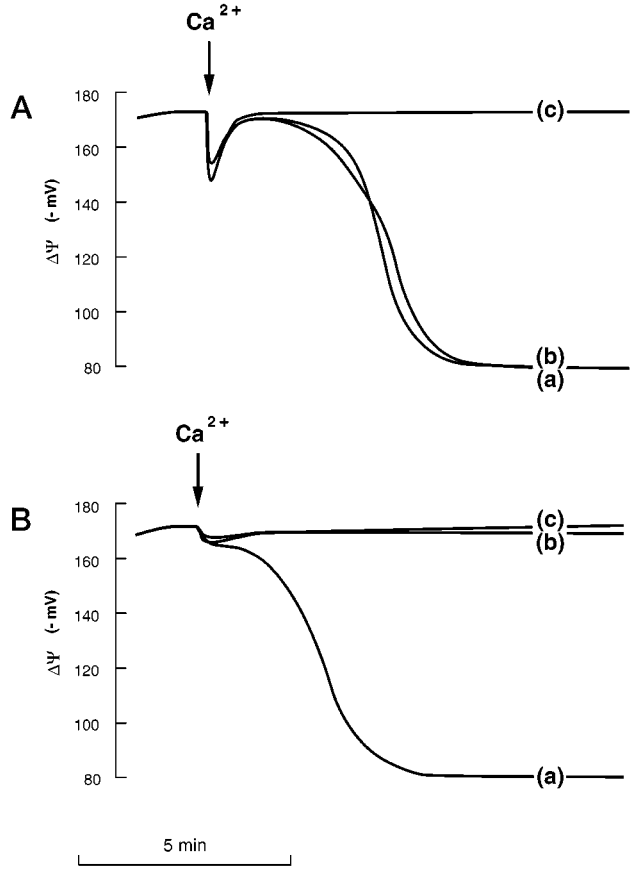

FIG. 9. **Cyclosporin A-sensitive Ca^{2+}-induced depolarization of rat liver mitochondria with complex I or II substrates; effect of Mg^{2+} and P_i.** The incubation medium contained 250 mM sucrose, 5 μM EGTA-Tris, 10 mM Tris-MOPS, 0.2 μM rhodamine 123. Final volume 2 ml, pH 7.3, 25 °C. Respiratory 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 P_i (*panel A*) or 5 mM P_i and 2 mM $MgCl_2$ (*panel B*). Experiments were started by the addition of 0.7 mg of liver mitochondria (not shown). Where indicated, 25 μM Ca^{2+} (*panel A*) or 35 μM Ca^{2+} (*panel B*) was added. In the experiments of *traces c*, 1 μM CsA was present (*both panels*).

it to 5 mM, and found PTP opening at negligible Ca^{2+} loads. To circumvent this problem we added 2 mM Mg^{2+} to our incubations. Under these conditions a clear effect of substrates could be detected, and the PTP opening readily followed the addition of 35 μM Ca^{2+} with glutamate plus malate (*panel B*, *trace a*) but not with succinate (*panel B*, *trace b*) as the substrate. Consistent with these results Ub_0 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 Ca^{2+} 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 Ub_0 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 P_i 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 Ca^{2+} uptake. When the P_i concentration was 5 mM we could easily observe PTP regulation by electron flux within complex I in liver mitochondria as well, provided that Mg^{2+} was added (Fig. 9) in agreement with a previous report (21). Furthermore, PTP inhibition by Ub_0 was also detected in liver mitochondria even at lower P_i 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 P_i (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 Ub_0 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 Ca^{2+} (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, Ub_0 inhibits Ca^{2+}-dependent pore opening at concentrations that both cause oxidation of PN *per se*, and maximize Ca^{2+}-dependent depolarization and PN oxidation during Ca^{2+} uptake (Fig. 7). Ub_0 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, Ub_0 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 Ub_0 remains unclear, but we note that a similar effect has been reported for Ub_3 (29). Based on the triggering effect of electron flux through the rotenone-sensitive pathway, and on the inhibitory effect of Ub_0 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 Ub_0 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 Ub_0 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 H_2O_2 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 represented 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 Ca^{2+}- and CsA-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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Regulation of the Permeability Transition Pore in Skeletal Muscle Mitochondria: MODULATION BY ELECTRON FLOW THROUGH THE RESPIRATORY CHAIN COMPLEX I

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